AMINO ACID PERMEASE INVOLVEMENT IN THE VOLATILE ANESTHETIC RESPONSE OF SACCHAROMYCES CEREVISIAE

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Despite the clinical importance of volatile anesthetics, their mechanisms of action remain unknown. Understanding how anesthetics produce their numerous cellular effects will facilitate the design of safer anesthetics with fewer undesirable side effects. The yeast Saccharomyces cerevisiae is being used as a model to elucidate anesthetic actions. Studies in yeast show that availability of specific amino acids plays a key role in the response of this organism to anesthetics. The volatile anesthetic isoflurane inhibits the uptake of leucine and tryptophan, inducing a starvation response and growth arrest in appropriately auxotrophic strains. Overexpression of $TAT1$, $TAT2$, or $BAP2$ genes encoding permeases that import these amino acids, renders such strains resistant to volatile anesthetics. Conversely, deletion of these genes leads to increased sensitivity to anesthetics. These findings implicate specific amino acid permeases as candidate targets of anesthetics.

The high-affinity tryptophan transporter Tat2p, which is encoded by $TAT2$, was inhibited in a time- and dose-dependent manner during isoflurane exposure. This inhibition was rapid and reversible. Amino acid uptake studies revealed that the inhibition of Tat2p by isoflurane was not competitive, suggesting that anesthetics do not affect the ability of Tat2p to bind tryptophan. Inhibition of transport was not due to degradation or this permease or relocalization away from the plasma membrane. These findings are consistent with a model where isoflurane decreases Tat2p transporter activity, either through a direct interaction or a membrane-mediated effect.
Mutations within *TAT2* that render cells resistant to isoflurane were isolated. Characterization of these mutants revealed that anesthetic resistance can result from an overall increase in tryptophan uptake in the presence and absence of drug, a decrease in the inhibitory effects of anesthetic on uptake, or a combination of these two factors.

*Tat2p* provides a model of anesthetic effects on a candidate drug target in a single-celled organism. A better understanding of the effects of volatile anesthetics on amino acid permeases will provide insight into the mechanisms of action of these clinically essential drugs.
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LIST OF ABBREVIATIONS AND SYMBOLS

α    alpha
β    beta
γ    gamma
μ    micro
Δ    gene deletion
Ω    Ohm
ALP  alkaline phosphatase
DMSO dimethyl sulfoxide
DNA  deoxyribonucleic acid
dNTP 2’-deoxynucleoside 5’-triphosphate
EDTA ethylenediaminetetraacetic acid
FM® 4-64 N- (3-triethylammoniumpropyl) -4- (6- (4- (diethylamino) phenyl) hexatrienyl) pyridinium dibromide
g    gravity
GAAC general amino acid control
GABA γ-aminobutyric acid
GDW glass-distilled water
GFP  green fluorescent protein
HA  hemagglutinin A
Iso  isoflurane
Kb  kilobase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>LiOAc</td>
<td>lithium acetate dihydrate</td>
</tr>
<tr>
<td>MAC</td>
<td>minimum alveolar concentration</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>Min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SC</td>
<td>synthetic complete</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TAP</td>
<td>tandem affinity purification</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween-20</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>V</td>
<td>volt</td>
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"Suffering so great as I underwent cannot be expressed in words . . . The particular pangs are now forgotten; but the blank whirlwind of emotion, the horror of great darkness, and the sense of desertion by God and man, bordering close upon despair, which swept through my mind, and overwhelmed my heart, I can never forget."

-George Wilson on surgery before anesthesia

Ashhurst J. Surgery before the days of anesthesia. Boston Medical and Surgical Journal. October 1896; 135(16):378-380
CHAPTER ONE
GENERAL INTRODUCTION

Theories of volatile anesthetic action

The discovery of volatile anesthetics 160 years ago was a turning point in medical practice and a major American contribution to the field of medicine. The mechanism of action of volatile anesthetics remains a major unsolved scientific mystery (Kennedy and Norman 2005) despite ceaseless use and intense research. Advances in understanding how volatile anesthetics produce their numerous effects on various cells, tissues and organisms have been made, yet no current model adequately explains how volatile anesthetics work. Characterization of drug actions involves identification of drug targets, characterization of drug/target interactions, and elucidation of downstream pathways that lead to the known observable outcomes of the drug. A critical step in understanding the mechanism of drug action involves the characterization of direct targets of the drug that are physiologically important and this step will be the focus of this work.

General anesthesia is defined as immobility, amnesia, hypnosis, and insensitivity to pain (Eckenhoff 2001). While many classes of drugs can impact these parameters, only general anesthetics accomplish all four outcomes. All general anesthetics are inhaled compounds, including both gases and volatile liquids. The potency of a general anesthetic is defined as the minimum alveolar concentration (MAC) of anesthetic required to prevent response to a painful stimulus in 50% of subjects. A variety of
chemically divergent molecules act as general anesthetics (Figure 1). These molecules vary in structural complexity, ranging from elemental xenon to branched halogenated hydrocarbons.

Figure 1. Chemically divergent compounds act as anesthetics. Volatile as well as gaseous compounds of various sizes and from different chemical classes act as general anesthetics. Modified from: (Campagna, Miller et al. 2003).

The pivotal findings in the late 19th century by Meyer and Overton that the potency of a volatile anesthetic increases with its lipophilicity as measured by solubility in olive oil (Figure 2) led to the hypothesis that anesthetics have a hydrophobic site of action (Campagna, Miller et al. 2003). When it was later discovered that the cellular
membrane is made up largely of lipids, and therefore shares key properties with olive oil, the Meyer-Overton correlation formed the basis for lipid-based hypotheses of anesthetic action. The correlation between potency and solubility improves when membrane lipids are the solvent as opposed to olive oil, providing further support for membrane models (Koblin 2005). A variety of membrane-based hypotheses propose that volatile anesthetics act by inserting into the hydrophobic cellular membrane, causing an alteration of some physical property of the lipid bilayer such as ion permeability, fluidity, volume, or thickness (Koblin 2005). Additional support for the cellular membrane as the site of

**Figure 2.** The Meyer-Overton rule: lipophilicity correlates with the potency of volatile anesthetics. There is a strong correlation between hydrophobicity as measured by olive oil-gas partition coefficient and anesthetic potency. This correlation led to the theory that anesthetics have a hydrophobic site of action. ● Anesthetic potency measured by the concentration required to prevent movement in response to incision in humans (MAC-immobility); ■ MAC-immobility in animals. Source: (Campagna, Miller et al. 2003).
anesthetic action comes from the finding that volatile anesthetics affect every cell type and organism tested, including bacteria and plants. It was reasoned that the hydrophobic cellular membrane is more similar among these diverse cell types than any protein target would likely be (Eckenhoff 2001).

Several lines of evidence challenge the hypothesis that the cellular membrane is the direct target of volatile anesthetics. Certain pairs of stereoisomers of volatile anesthetics possess different potencies despite having the same lipophilicity (Dickinson, White et al. 2000). This finding is difficult to explain under lipid-based hypotheses as it suggests that the site of anesthetic action has more ability to distinguish between stereoisomers than is expected for the cellular membrane (Dickinson, Franks et al. 1994). Additionally, there are compounds that are structurally similar to volatile anesthetics and are lipophilic, but do not have the immobilizing effects of anesthetics (Fang, Sonner et al. 1996). These compounds, called nonimmobilizers, seemingly defy lipid-based hypotheses. Their lipophilicity suggests they would partition into and alter the membrane in the same way as anesthetics, yet they lack anesthetic action. Finally, while volatile anesthetics do cause measurable alterations in physical properties of cellular membranes, most alterations are small and none has been causally related to anesthesia. For example, volatile anesthetics measurably increase membrane fluidity (Eckenhoff 2001). However, other means of attaining the same alteration in fluidity, such as the small temperature increase associated with a low-grade fever, neither produce anesthesia nor increase anesthetic potency (Eckenhoff 2001).
In the face of such challenges to lipid-based models of anesthetic action, proteins were suggested as the direct targets of anesthetics. Protein-based models of volatile anesthetic action hypothesize that anesthetics bind directly to protein(s) and alter their activity. While the Meyer-Overton rule formed the basis of membrane models of anesthetic action, any hydrophobic site is consistent with the correlation between potency and lipophilicity. Because proteins possess hydrophobic cavities, protein-based theories can account for the Meyer-Overton rule (Eckenhoff 2001). The idea of proteins as direct targets of anesthetics gained momentum when it was shown that purified firefly luciferase, a soluble enzyme, is inhibited by anesthetics (Franks 1984). This lipid-free system obeys the Meyer-Overton rule over a 100,000-fold range of anesthetic potencies (Franks 1984), defying the assertion that the correlation between lipophilicity and anesthetic potency requires a membrane site of anesthetic action. Protein-based hypotheses can account for some aspects of anesthetics not well explained by lipid-based theories. The different anesthetic potencies of stereoisomers can be explained by chiral anesthetic-binding sites within a protein (Yamakura, Bertaccini et al. 2001). Nonimmobilizing compounds can also be explained by protein theories. Just as anesthetic-binding sites in proteins distinguish between pairs of stereoisomers, they can also distinguish between anesthetics and similar compounds that do not act as anesthetics. Anesthetics, but not nonimmobilizers, have been shown to affect the activity of many proteins including the neurotransmitter receptors for GABA, glycine, serotonin, and glutamate (Koblin 2005).
However, protein-based models of anesthetic action face their own set of challenges. The GABA$_A$ receptor, which mediates the action of the major inhibitory neurotransmitter in the brain, has been implicated as a potential anesthetic target (Yamakura, Bertaccini et al. 2001) and will be used to exemplify issues faced when attempting to prove a protein is involved in anesthesia. Studies of effects of anesthetics on neurotransmission give variable results depending on the biological preparation examined (Koblin 2005). For example, halothane and isoflurane cause little or no change to the depolarizing effects of GABA application to neocortical slices, yet they enhance the currents evoked by GABA application to rat hippocampal neurons (Koblin 2005). Means of evoking neurotransmission can also affect results. In rat synaptosomes, anesthetics have no effect on potassium-induced GABA release (Salord, Keita et al. 1997), but they inhibit 4-aminopyridine-evoked GABA release (Westphalen and Hemmings 2006). The effects of anesthetics on GABA$_A$ receptors are agent-specific. Xenon, nitrous oxide, cyclopropane and butane have little or no ability to potentiate (enhance the activity of) GABA$_A$ receptors, in contrast with halothane and isoflurane (Campagna, Miller et al. 2003). This is logical, as it is difficult to envision an anesthetic binding site within a protein that can accommodate small anesthetics such as elemental xenon as well as larger halogenated hydrocarbon molecules such as sevoflurane (Franks 1984). Another complication in linking a specific protein to anesthetic mechanisms is that anesthetics have endpoint-specific effects. For instance, while the knockout of the $\beta$3 subunit of the GABA$_A$ receptor affects the concentration of anesthetic required to affect the response to a painful stimulus in mice, it has no effect on loss-of-righting reflex
(Tanner 2000). It is apparent from studies of the GABA<sub>A</sub> receptor that volatile anesthetic actions are complex. The GABA<sub>A</sub> receptor may play a role in some physiological responses to certain anesthetics, but it seems likely that the action of volatile anesthetics does not involve a simple interaction with a single protein receptor.

A third model of anesthetic action incorporates both proteins and lipids by asserting that anesthetics act by inserting into the membrane and altering the activity of integral membrane protein(s). In this model, alterations in membrane protein activity may occur by insertion of the anesthetic at the lipid/protein interface or in bulk membrane. Support for the binding of anesthetics at lipid/protein interfaces comes from finding that the amino acid residues in some ligand-gated ion channels conferring sensitivity to anesthetics are found in transmembrane domains (Siegwart, Jurd et al. 2002). This suggests protein as well as lipid components may make up the anesthetic binding site. Additional support for lipid/protein theories is found in evidence of the strong influence of membrane composition on embedded protein conformation and activity (Trudell and Bertaccini 2002). However, it is difficult to distinguish protein and lipid/protein theories because integral membrane proteins are difficult to purify from the plasma membrane. Once purified, these proteins must be reincorporated into artificial membranes to make functional assays possible. Thus, while these models provide an appealing combination of lipid and protein involvement in anesthetic action, they are likely to be difficult to rigorously test.
Identification of drug targets

One approach to identify the target of a drug involves the search for a high-affinity drug receptor. A common method involves the creation of a radiolabeled photoactivatable drug analogue that is used to probe cells or tissues in photoaffinity experiments. Alternatively, drugs can be immobilized in an affinity column and used to purify potential targets from a cell extract. These techniques are useful in identification of drug targets provided that the interaction between the target and drug is sufficiently strong and there is a low background of off-target binding. However, volatile anesthetics, which are effective at a plasma concentration in the low millimolar range, are likely to bind protein targets with much lower affinity than many drugs (Eckenhoff 2001). The therapeutic level of halothane in the plasma is 20,000 times greater than typical concentrations used for opiate drugs (Tanner 2000). An additional difficulty posed by anesthetics is their promiscuous binding to many proteins. A rat brain section photoaffinity labeled with a halothane analogue shows widespread halothane binding with little preference for any given region of the brain (Eckenhoff and Eckenhoff 1998). To test if this binding pattern results from binding to a small number of widespread targets or binding to many proteins, extracts were prepared from halothane-labeled brain samples. Many proteins from both soluble- and membrane-protein preparations were found to be labeled, leading to the conclusion that the anesthetic-binding motif is sufficiently degenerate to be found in many proteins (Eckenhoff, Chan et al. 2002). It is unlikely that all of the anesthetic-bound proteins are affected by anesthetics in a way that is relevant to the state of anesthesia.
Due to such complications, affinity-based approaches have not led to experimental identification of physiologically relevant anesthetic targets. An alternative approach has been to study proteins that may logically be thought to be involved in anesthesia. The choice of a plausible target is based on a limited understanding of consciousness, which has been described as, “arguably the least understood aspect of biology” (Eckenhoff 2001). Plausible anesthetic targets include voltage or ligand-gated ion channels due to their direct involvement in neurotransmission. The activity of many ion channels is affected by anesthetics. In addition to the GABA_A receptor, which was previously discussed, anesthetics affect nicotinic acetylcholine receptors (Rada, Tharakan et al. 2003), glycine receptors (Tassonyi, Charpantier et al.), serotonin receptors (Martin, Adams et al. 1990), N-methyl-D-aspartate receptors (Dickinson 2007), kainate receptors (Trudell and Bertaccini 2002), glutamate transporters (Huang, Feng et al. 2006) and potassium, sodium and calcium channels (Koblin 2005). Due to the direct involvement of these proteins in neurotransmission, finding that the activities of these proteins are affected by anesthetics can lead to the formation of models of anesthetic action. For example, glutamate transporters remove the excitatory neurotransmitter glutamate from the extracellular space and prevent over stimulation of glutamate receptors (Huang, Feng et al. 2006). The activity of one such transporter is enhanced by isoflurane, leading to a model in which anesthesia results from decreased excitatory neurotransmission (Huang, Feng et al. 2006).

However, in addition to plausible targets, anesthetics also affect the activity of proteins whose potential role in anesthesia is not readily obvious. Examples include
carrier proteins hemoglobin, myoglobin, and serum albumin and structural proteins such as tubulin (Tanner 2000, Franks, 1984 #348). Even the activity of proteins with no potential role in anesthesia, such as firefly luciferase, can be inhibited by anesthetics in a manner that obeys key expectations of anesthetic targets, such as the Meyer-Overton rule (Franks 1984). The study of these proteins as models can provide valuable information.

Bovine serum albumin was used to characterize binding energetics of anesthetic/protein interactions (Eckenhoff and Johansson 1997), firefly luciferase has provided a model for the thermodynamics of anesthetic binding (Dickinson, Franks et al. 1993), and cholesterol oxidase and others have been crystallized with anesthetics to allow structural characterization of anesthetic binding sites (Bertaccini, Trudell et al. 2007). However, because anesthetics interact with and affect the activity of so many proteins, finding that the activity of a plausible target is affected by anesthetics is not sufficient. The problem arises in linking an alteration in protein activity to a biologically relevant effect.

Genetic methods provide another alternative for identification of drug targets. Genetic analysis in a variety of organisms has been used to study activities of volatile anesthetics. Organisms used include various mammals, the nematode Caenorhabditis elegens, the fruit fly Drosophila melanogaster, and the yeast Saccharomyces cerevisiae (Humphrey, Sedensky et al. 2002) (Tanner 2000). Mammals such as mice are of value because their response to volatile anesthetics is similar to that of humans. However, genetic manipulation of mice for drug target identification is challenging. Because the GABA\textsubscript{A} receptor is a logical and popular candidate anesthetic target, knockout mice lacking various subunits of the GABA\textsubscript{A} receptor have been made and tested for altered
anesthetic sensitivity. However, results regarding anesthetic phenotype have not provided strong support for these receptors as mediators of anesthetic sensitivity (Nash 2002). This may be due to compensatory gene expression or functional redundancy, problems that plague such experiments.

Besides the targeted disruption of genes encoding suspected drug targets, other genetic approaches in mammals are based on comparisons between strains. One such approach with mice arises from finding that the MAC values for different mice strains commonly used in the laboratory can vary by as much as 55% (Sonner, Gong et al. 2000). Sorting out the genetic differences responsible for this MAC variation between strains is yet to be accomplished but may hold potential to provide insight into anesthetic actions. MAC comparisons between strains that are more closely related may also be informative. Recombinant inbred strains with increased sensitivity to ethanol are also more sensitive to some anesthetics including isoflurane and enflurane (Humphrey, Sedensky et al. 2002). Candidate genes can be tested to determine if they differ between sensitive and resistant strains. The GABA_A receptor differs between these strains, but a complication is that there are estimated to be at least nine loci contributing to the strain differences (Humphrey, Sedensky et al. 2002). Another complication to these types of genetic analyses is the inability of strain comparison studies to distinguish between drug targets, upstream or downstream effects, or unrelated genes that segregate between the two lines (Harris 1991).

Multi-celled organisms with fewer cell types such as fruit flies and nematodes have also been used in the genetic studies of anesthetics. Shorter generation times, well-
mapped genomes, and relatively simpler molecular genetic manipulation facilitate more rapid analysis. Chemical mutagenesis studies with *D. melanogaster* were followed by a clever column-chromatography-like scheme that selects for flies able to remain standing on a series of platforms in the presence of anesthetics (Krishnan and Nash 1990). These studies implicate a role for calcium channels in the anesthetic response of this organism (Nash, Scott et al. 2002). Resistance of these mutants varies depending on the anesthetic tested, suggesting that different anesthetics have different targets in this organism (Krishnan and Nash 1990). Other studies implicate a role for potassium channels in the anesthetic response of this organism. The *Shaker* gene, named for mutations leading to leg twitch in response to ether, encodes a potassium channel involved in neuronal function. Mutations in this gene lead to halothane resistance (Walcourt, Scott et al. 2001). The finding that human potassium channels are partially inhibited by volatile anesthetics at clinically relevant concentrations may provide a potential link between the findings in fruit flies and anesthetic effects in humans (Koblin 2005). This suggests that work in relatively simple organisms can provide useful and relevant information regarding anesthetic mechanism of action.

Two endpoints have been used during studies with *C. elegans*, complete immobility for ten seconds and absence of movement towards food (Humphrey, Sedensky et al. 2002). Each endpoint is readily measurable and obeys the Meyer-Overton rule as well as other expectations of anesthetic response. Screening of mutants in this organism for ability to disperse towards food in the presence of isoflurane and halothane uncovered a potential role for neuronal syntaxin or syntaxin-binding proteins.
These proteins are involved in vesicular fusion events necessary for neurotransmission. Another screen of existing mutants using complete immobility as an anesthetic endpoint has implicated stomatin proteins (Nash 2002). These proteins are associated with lipid rafts, and are proposed to aid in the transfer of raft-associated proteins from endocytic vesicles and the cell surface (Sedensky, Siefker et al. 2001). Because lipid rafts contain high concentrations of ion channels, and ion channels may be anesthetic targets, these proteins may be accessories to actual drug targets. A screen of *de novo* mutants for hypersensitivity to anesthetic-induced immobility indicates a role for the mitochondrial Complex I, involved in electron transport and proton pumping (Morgan and Sedensky 1994; Nash 2002). Finding that humans with specific mitochondrial defects have greatly increased sensitivity to sevoflurane (Humphrey, Sedensky et al. 2002) provides a potential link between mutants in nematodes and mammals.

**Yeast as a model for studying effects of volatile anesthetics in animals**

Yeast offers numerous advantages in the study of the mechanisms of action of drugs. The evolutionary conservation of many cellular pathways and the powerful molecular and genetic tools available for this eukaryotic organism have been utilized to gain insights into activities of a variety of clinically relevant drugs including chemotherapeutics, immunosuppressants and antimalarials [for examples, see (Emerson, Nau et al. 2002; Welsch, Hagiwara et al. 2003; Liu, Brusilow et al. 2004)]. The yeast genome was the first eukaryotic genome to be fully sequenced, and studies with yeast
benefit from extensive previous genetic, molecular and biochemical characterization (Hughes 2002).

Using inhibition of yeast growth as an endpoint, there are many similarities in anesthetic response with mammals (Figure 3). The potency of anesthetics as inhibitors of yeast growth is predicted by the Meyer-Overton rule (Figure 3A and (Keil, Wolfe et al. 1996; Koblin 2000; Koblin 2005)). As the oil/gas partition coefficient of anesthetics increases, the concentration of anesthetic required to inhibit visible yeast growth on an agar plate decreases (Figure 3A). This concentration is defined as the minimum inhibitory concentration (MIC). The effect of different anesthetics on yeast growth is additive (Figure 3B and (Wolfe, Hester et al. 1998; Ropcke, Wirz et al. 2001)). That is, the combined concentrations of different anesthetics provide the same growth-inhibitory effects as the full dose of a single anesthetic (Wolfe, Hester et al. 1998).

Nonimmobilizers, compounds that are chemically similar to volatile anesthetics yet do not induce anesthesia in animals (Fang, Sonner et al. 1996), do not inhibit yeast growth alone, or in combination with near-inhibitory concentrations of anesthetics (Figure 3C and (Wolfe, Hester et al. 1998)). Yeast display a sharp dose response curve, meaning that near the effective anesthetic dose, a small change in concentration has a large effect on response, as measured by yeast cell division or mammalian behavioral response (Figure 3D and (Keil, Wolfe et al. 1996) (Koblin 2005)). Yeast growth is rapidly (in less than 15 minutes) arrested by anesthetics (Figure 3E and (Palmer, Wolfe et al. 2002)). Anesthetic-induced growth arrest is reversible (Figure 3E), an important characteristic of anesthesia in
**Figure 3.** Growth inhibition of yeast obeys the expectations of model anesthetic response.  

A. Yeast growth inhibition obeys the Meyer-Overton rule. Yeast MIC (circles) is the Minimum Inhibitory Concentration of an anesthetic sufficient to inhibit growth. Human MAC is also shown (squares).  

B. Volatile anesthetics have additive inhibitory effects on yeast growth. C. Nonimmobilizers (NA1 and NA2 refer to 1,2-dichlorohexafluorocyclobutane and 2,3-dichlorooctafluorobutane, respectively) do not inhibit yeast growth alone (not shown) or in combination with the anesthetic methoxyflurane. MIC$_p$, predicted MIC of nonimmobilizers based on lipophilicity.  

D. Yeast growth inhibition displays a sharp dose response curve, as small increases in isoflurane concentration have a large effect on visible growth. E. Cells were plated (first panel), incubated in the presence of the isoflurane MIC for 13 hours (second panel), removed from anesthetic and grown for 3 hours (third panel) and 9 hours (fourth panel). Growth inhibition is rapid as evidenced by inhibition of bud formation in unbudded cells (2$^{nd}$ panel) and reversible, as evidenced by colony formation upon anesthetic removal (3$^{rd}$ and 4$^{th}$ panel). Modified from: (Keil, Wolfe et al. 1996; Wolfe, Hester et al. 1998)
mammals. Thus, there are extensive similarities in the response of mammals and yeast to volatile anesthetics. This suggests that yeast, like more complicated organisms, is a good model for anesthetic studies and that growth inhibition is an appropriate endpoint for measuring the volatile anesthetic response of this organism.

**Availability of critical amino acids during anesthetic exposure**

Alteration of gene dosage forms a basis for genetic studies in yeast to identify direct drug targets. The rationale for such gene-dosage-based approaches is that decreased expression of a gene encoding a drug target may result in increased sensitivity (super-sensitivity) to the drug while increased expression of the gene may decrease drug sensitivity (resistance) (Hughes 2002) (Rine, Hansen et al. 1983). These approaches are based on the assumption that gene dosage affects the level of the encoded proteins. Screens or selections based on this concept can be employed for drug target identification. Genes encoding certain amino acid transporters, including those that transport leucine or tryptophan, obey the gene dosage expectations of direct drug targets (Hughes 2002; Palmer, Wolfe et al. 2002). These genes render yeast strains resistant to the growth inhibitory effects of anesthetics when present on multicopy plasmids, provided that the strains are auxotrophic for the corresponding amino acid (Palmer, Wolfe et al. 2002). Consistent with the expectation that decreased expression of a drug target should lead to increased drug sensitivity, deletion of genes encoding these transporters renders cells hypersensitive to isoflurane (Palmer, Wolfe et al. 2002). These findings suggest that availability of amino acids plays a key role in anesthetic response.
The amino acid substrates of these transporters are defined as critical to anesthetic response. Genes identified include TAT1 and TAT2, which encode low- and high-affinity tryptophan transporters, respectively (Schmidt, Hall et al. 1994). Not all permease genes alter anesthetic sensitivity when deleted or overexpressed, indicating some specificity in the role of nutrient transporters during anesthetic response (Palmer, Wolfe et al. 2002). Also, not all plasma-membrane-localized transporters are inhibited by anesthetics. Specifically, uracil uptake by Fur4p is not inhibited by isoflurane, indicating anesthetics do not disrupt the proton gradient that drives transport across the plasma membrane (Palmer 2002).

Other lines of evidence also demonstrate the importance of the availability of critical amino acids to the action of anesthetics in yeast (Palmer, Wolfe et al. 2002). Increased levels of these amino acids in the medium enhance anesthetic resistance as measured by growth of yeast (Palmer, Wolfe et al. 2002). Yeast cells capable of synthesizing all amino acids are resistant to volatile anesthetics, presumably because their growth is not dependent on import of amino acids from the medium (Palmer, Wolfe et al. 2002). Consistent with the idea that specific amino acid transporters may be direct targets of volatile anesthetics, isoflurane inhibits the uptake of tryptophan (Palmer, Wolfe et al. 2002).

**Yeast amino acid transporters**

Yeast cells import amino acids for protein synthesis and to be used as nitrogen sources. Yeast cells have 25 transporters in the amino acid permease family (Roth, Wan
et al. 2006). The permeases are active transporters and can import amino acids against large concentration gradients by using proton symport (Andre 1995). The amino acid permeases can be divided into 2 groups: general, low-affinity transporters and more specific, high-affinity transporters (Beck, Schmidt et al. 1999). The general amino acid permease Gap1p is one well-characterized member of the first group, and is transcriptionally and post-translationally up-regulated in response to limited nitrogen availability (Beck, Schmidt et al. 1999). Gap1p transports all amino acids. In contrast, the members of the high-affinity transporter group are expressed when cells are not starved for nitrogen (Beck, Schmidt et al. 1999). That is, they are inversely regulated to members of the general low affinity permeases. High-affinity transporters are specific for a few, or even a single, amino acid and they transport amino acids primarily destined for use in protein synthesis (Beck, Schmidt et al. 1999). Tat2p is a relatively well-characterized member of this group of transporters and it has been suggested to import phenylalanine and tyrosine as well as tryptophan (Regenberg, During-Olsen et al. 1999).

Along with all plasma membrane proteins, Tat2p is synthesized in the endoplasmic reticulum and travels via vesicular transport to the Golgi (Chang 2002). Tat2p is then either deposited at its active location on the plasma membrane when extracellular tryptophan is abundant or routed directly to the vacuole for degradation when extracellular tryptophan is not sensed (Beck, Schmidt et al. 1999). Transcriptional induction of \textit{TAT2} and other high-affinity permease genes is dependent on the amino acid sensor Ssy1p (Kodama, Omura et al. 2002). Ssy1p is a transporter-like protein that signals the availability of extracellular nutrients but has no known transporter activity.
(Iraqui, Vissers et al. 1999). In addition to transcriptional regulation, amino acid permeases are regulated post-translationally. Nutrient-based sorting of amino acid permeases is dependent on the ubiquitination components Rsp5/Bul1/Bul2 (Pizzirusso and Chang 2004). Once Tat2p has reached the plasma membrane, the nutritional status of the cell continues to affect its stability. Treatment with the immunosuppressant rapamycin, a drug whose mechanism of action has been characterized in yeast, has been shown to induce the internalization and degradation of Tat2p (Beck, Schmidt et al. 1999). Starvation has the same effect on Tat2p (Beck, Schmidt et al. 1999). In fact, the degradation of high-affinity nutrient transporters including Tat2p has been asserted to be a general characteristic of starvation-induced growth arrest (Schmidt, Beck et al. 1998). The studies reported here assay the stability and localization of Tat2p in the presence of growth-inhibitory concentrations of isoflurane. These studies allow refinement of the model of the effects of isoflurane on Tat2p (Figure 4) as well as a more complete understanding of the effects of starvation on high-affinity amino acid permeases.
Figure 4. Models for the involvement of Tat2p in anesthetic response. A. Direct inhibition of Tat2p activity by isoflurane by competitive binding at the tryptophan binding site (ii) or binding elsewhere (iii). B. Indirect inhibition of Tat2p activity by isoflurane due to induction of post-translational modification alone (ii) or in combination with internalization (iii), possibly followed by degradation (not shown). C. Tat2p as an exporter of isoflurane. Open circles, tryptophan; barrel, Tat2p; curved double line, plasma membrane; solid straight arrow, transport of tryptophan; open straight arrow, export of isoflurane; triangle, isoflurane; X, transport inhibition; lollipop, post-translational modification; curved arrow, internalization. Modified from (Palmer, Wolfe et al. 2002)
Models for the involvement of amino acid permeases in anesthetic response

Several models of direct or indirect involvement of amino acid permeases in anesthetic response exist (Figure 4). To directly test the ability of anesthetics to inhibit the transport of amino acids, the uptake of radiolabeled amino acid was assayed in the presence and absence of a growth-inhibitory concentration of the anesthetic isoflurane. Anesthetics were found to significantly decrease the uptake of tryptophan (Palmer, Wolfe et al. 2002). There are many ways anesthetics could affect the ability of permeases to transport amino acids. Models for the involvement of amino acid permeases in volatile anesthetic response proposed in Figure 4, such as inhibition of Tat2p by a competitive (Figure 4A ii) or not competitive (Figure 4A iii) mechanism were tested and will be discussed in this dissertation. In previous work, anesthetics were not found to induce Tat2p degradation within the first hour of exposure [Figure 4B iii and (Palmer, Wolfe et al. 2002)]. This model was tested further in this work.

One potential role of amino acid transporters could be the transport of anesthetic molecules out of the cell (Figure 4C). While this possibility does not necessarily involve inhibition of tryptophan import by Tat2p, the export of anesthetic would decrease the effective concentration of anesthetic within the cells, and would be consistent with the anesthetic phenotypes of cells overexpressing or deleted for amino acid permeases. However, this model does not explain the finding that deletion of a tryptophan permease leads to hypersensitivity (more anesthetic sensitivity than the wild-type) only in cells that are appropriately auxotrophic (Leu⁺Trp⁻, but not Leu⁺Trp⁺ or Leu⁻Trp⁻) (Palmer, Wolfe et al. 2002). Likewise, overexpression of a tryptophan transporter in a Leu⁺Trp⁺ strain has
no effect on anesthetic phenotype (Palmer, Wolfe et al. 2002). These data indicate that the amino acid transport function is relevant to anesthetic phenotype rather than a drug efflux effect.

**The study of amino acid transporters by mutagenesis**

Nutrient transport plays an important role in cellular metabolism, yet much remains unknown about the proteins responsible for this critical process. The regulation and function of nutrient transporters have ramifications on normal growth and development, cancer, obesity, immune system function, viral infection, and neurotransmission (Edinger 2007). *Saccharomyces cerevisiae* provides an ideal system for studying fundamental processes such as nutrient transport due to a high degree of conservation among eukaryotes. Nutrient transport is accomplished by members of the amino acid/polyamine/organocation (APC) superfamily, which includes transporters from bacteria to animals (Jack, Paulsen et al. 2000). As mentioned previously, amino acid transport in yeast is accomplished by a group of proteins called the amino acid permease family. The amino acid permease family is a sub-family of the APC superfamily (Andre 1995). Tat2p, the high-affinity tryptophan transporter (Schmidt, Hall et al. 1994), is a relatively well-studied member of the amino acid permease family, yet a detailed understanding of the structure-function relationship of this protein does not exist.

Study of the effects of mutations within a gene of interest allows characterization of important functional regions of the encoded protein. Mutations within *TAT2* that have been characterized include N-terminal truncations and substitution of lysines in the N-
terminus to arginine (Beck, Schmidt et al. 1999). Analysis of these mutants revealed that Tat2p is recognized for ubiquitination and degraded in response to starvation or rapamycin treatment (Beck, Schmidt et al. 1999). This degradation is disrupted if all five lysines in the N-terminal 31 amino acids are mutated or deleted (Beck, Schmidt et al. 1999). Besides these directed mutations within \textit{TAT2}, studies of mutations in other yeast and bacterial transporter proteins have provided some insight into functional elements within these proteins. Conserved residues in the third and fifth transmembrane domains of bacterial aromatic amino acid transporter AroP have been implicated in the proton relay necessary for active solute transport (Cosgriff and Pittard 1997). Residues in helix three and extracellular loop four are proposed to be important for substrate specificity as determined by random mutagenesis studies of the arginine permease Can1p and glutamine-asparagine transporter Gnp1p (Regenberg and Kielland-Brandt 2001). Due to a high degree of similarity between amino acid permeases, these findings have potential implications for Tat2p. Randomly-generated mutations within \textit{TAT2} that were selected using anesthetics as a tool will have potential implications for other members of the amino acid permease family.

While no member of the amino acid permease family has been crystallized, the structure of a functionally related protein, LeuT\textsubscript{Aa}, has recently been solved (Yamashita, Singh et al. 2005). LeuT\textsubscript{Aa} is a bacterial leucine transporter with similar activity and topology to that of amino acid permease family members. Specifically, both LeuT\textsubscript{Aa} and Tat2p have 12 transmembrane-spanning helices and transport an amino acid substrate against a concentration gradient into the cell. The structure of LeuT\textsubscript{Aa} has served as the
basis for homology modeling of the human serotonin transporter SERT (Ravna, Jaronczyk et al. 2006), human GABA transporter GAT1 (Pallo, Bencsura et al. 2007), and for Tat2p [homology model constructed by Dan DeCotiis (Keasey, De Cotiis et al. 2007)]. This homology model provides a framework for understanding randomly generated mutations, and a basis for site-directed mutagenesis.

**Research objectives**

The focus of this thesis is to examine the effects of anesthetics on specific amino acid permeases and the role of these permeases during the anesthetic response. This work investigated the nature of the nutrient transport inhibition by volatile anesthetics on the tryptophan transporter encoded by *TAT2* as well as the localization and levels of this permease during anesthetic exposure. Isolation and characterization of anesthetic-resistant mutants implicated regions within this permease important for anesthetic response as well as for tryptophan transport under normal growth conditions. A more detailed understanding of the role of amino acid permeases during anesthetic response will allow further refinement of the model of volatile anesthetic mechanism(s) of action.
CHAPTER TWO

EXPERIMENTAL PROCEDURES

Strains and media

Yeast strains were derived from RLK88-3C (Table 1 lists all yeast strains used in this work). P2495 was constructed from RLK88-3C to study the role of the tryptophan transporter encoded by $TAT2$ in anesthetic response. The relevant genotype of this strain is $tat1\Delta tat2\Delta YCpTAT1$. Following transformation with a plasmid containing $TAT2$ and selection for loss of pTAT1, the only tryptophan transporter capable of supporting growth is encoded by the plasmid-borne $TAT2$. P2495 was transformed with pL5296 (YCp $TAT2$), pL5330 (YCp $TAT2$-TAP), pL5334 (YCp $TAT2$-GFP), or pL5605 (YEp $TAT2$-TAP) (Table 2). Loss of the $URA3$-marked pTAT1 plasmid was selected on medium containing 5-fluoroorotic acid to produce P2683, P2684, P2688, or P3144, respectively.

Unless otherwise noted, liquid yeast media was SC with 50 $\mu$M trp (Palmer, 2002 #38) and agar-solidified SC media containing 250 $\mu$M tryptophan and lacking appropriate nutrients to select for maintenance of plasmids.

*Escherichia coli* strains DH5$\alpha$, DH10$\beta$, and NM522, XL1-Blue (Stratagene), XL2-Blue (Stratagene), or XL10-Gold (Stratagene) were used for standard plasmid propagation. XL1-Red (Stratagene) was used for random mutagenesis. Bacteria were grown and handled according to standard protocols (Sambrook, 1989 #296) or the manufacturer’s instructions.
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</tr>
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<td>MATa his3 leu2 met15 ura3</td>
<td>(Brachmann, Davies et al. 1998)</td>
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<td>BY4741 TAT2-GFP</td>
<td>Invitrogen</td>
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Plasmid constructions

The PstI to EcoRI fragment containing TAT2 and surrounding genomic DNA was excised from pTAT2 [a gift from A. Schmidt (Schmidt, Hall et al. 1994)] and ligated into YCplac111 (LEU2) digested with the same enzymes to produce pL5296. To C-terminally tag the TAT2 gene in pL5296, a TAT2-specific TAP-tagging cassette (Ghaemmaghami, Huh et al. 2003) containing S. kluyveri HIS3 was amplified by PCR from the Tat2p-TAP strain (Open Biosystems) using primers O-657 and O-658 (Table 3), which anneal approximately 500 bases before the beginning and after the end of the TAP tag. The PCR product was transformed into BY4741 (relevant genotype his3 leu2) containing pL5296. Cells containing a TAT2-TAP fusion as a result of in vivo homologous recombination (Oldenburg, Vo et al. 1997) were selected on SC-leu-his medium. To identify colonies containing the TAP fusion on the plasmid-borne TAT2 rather than the chromosomal TAT2, transformants were grown in complete medium to permit plasmid loss, plated on SC and replica-plated to SC-leu and SC-his. Co-loss of prototrophy for leucine and histidine indicated the TAT2-TAP fusion was on the plasmid while inability to lose the histidine marker indicated the TAT2-TAP fusion was on the chromosome. The entire sequence of the TAT2-TAP region of the resulting plasmid (pL5330) was verified. To create YEp TAT2-TAP, TAT2-TAP was excised from pL5330 with SacI and HindIII and ligated into the YEp vector pRS425 (Christianson, Sikorski et al. 1992) cut with the same enzymes to produce pL5605. Sequencing verified the appropriate insertion and lack of mutations in TAT2-TAP on this vector.
<table>
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</tr>
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<td>YEp <em>TAT2-TAP LEU2</em></td>
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*Source* This work
TAT2 was also C-terminally tagged with GFP. A TAT2-specific GFP-tagging cassette was amplified from the Tat2p-GFP strain (Invitrogen) (Huh, Falvo et al. 2003) using O-657 and O-658 and a plasmid with the appropriate fusion, pL5334, was derived as described above.

Plasmid pL5330 was modified by site-directed mutagenesis to create appropriately located unique restriction sites to permit the excision of mutagenized TAT2-TAP for ligation into a naïve vector. Briefly, the EcoRI site in the TAP tag of the TAT2-TAP plasmid pL5330 was removed without affecting the encoded amino acids (GAA TTC→GAG TTC; Glu Phe→Glu Phe) to produce pL5342. The Ndel site in the S. kluyveri HIS3 selectable marker of the TAP insertion cassette was removed without affecting the encoded amino acids (CAT ATG→CAC ATG His Met→His Met) to produce pL5352. This plasmid contains TAT2-TAP on a 3.9 kB EcoRI-Ndel fragment. Sequencing verified no additional mutations were introduced.

Plasmid pL5352 was used as a template for site-directed mutagenesis to create mutants F101T (GAA→CAA) or E286Q (TTT→ACT), producing pL5603 or pL5601, respectively. DNA sequencing confirmed that only these mutations were present.

Transformation

E. coli transformations were performed using the heat-shock method (Sambrook, 1989 #296) or by electroporation with the Gene Pulser system (Bio-Rad) set to 25 µF, 2.5 kV, 200 Ω. When using commercially available ultracompetent or supercompetent cells (Stratagene XL1-Blue, XL2-Blue or XL10-Gold), the manufacturer’s instructions were
followed with the following exceptions; LB medium was used in place of NZY\(^+\) broth and standard microfuge tubes were used in place of Falcon 2059 polypropylene tubes. Yeast transformations were performed according to a standard LiOAc method (Schiestl and Gietz 1989).

**Yeast genomic DNA preparations**

A saturated culture of yeast was grown in appropriate selective medium. The cells from 1.5 mL of culture were collected by centrifugation at 2100 g for 1 minute. The pelleted cells were resuspended in 150 µL Solution A (14 mM β-mercaptoethanol, 0.1 M EDTA pH 7.5, 0.9 M sorbitol, 150 µg/mL zymolyase). To form spheroplasts, the cells were incubated for 1 hour at 37\(^\circ\) with occasional inversion. Spheroplasts were collected by centrifugation at 4600 g for 3 minute. The pellet was resuspended in 150 µL Solution B (1% SDS in TE) and 50 µL of Solution C (5 M ammonium acetate pH 7) was added. The suspension was vortexed and then centrifuged for 3 minutes at 13,000 g. The supernatant was carefully removed and added to 150 µL of isopropanol. This mixture was frozen at -80\(^\circ\) and the DNA was pelleted by centrifugation at 13,000 g. The pellet was dried in a Savant Integrated SpeedVac ISS 100 System. For most applications, DNA was resuspended in 100 µL TE. However, DNA used for bacterial electroporation was rinsed in 200 µL 70% ethanol, frozen at -80\(^\circ\), collected by centrifugation at 13,000 g and resuspended in 50 µL GDW. One µL was used for electroporation.
PCR

PCR reactions for yeast genomic DNA preparations contained 1 µL of a 1:10 dilution of yeast genomic DNA, 2 µL 10x PfuTurbo reaction buffer (Stratagene), 0.8 µL dNTP mix (Stratagene), 0.2 µL of a 20 µM solution of each primer, 15.7 µL GDW, and 0.25 units PfuTurbo DNA polymerase (Stratagene). Primers were synthesized by the Penn State Hershey Macromolecular Core Facility. Alternatively, a Fail-Safe PCR PreMix Kit (Epicentre Biotechnologies) was used according to manufacturer’s instructions.

For PCR amplification from bacterial plasmids, plasmid DNA was prepared using Qiagen QIAprep Spin Miniprep Kit. Reactions contained 0.25 µL of a 1:10 dilution of plasmid DNA, 5 µL 10x PfuTurbo reaction buffer, 2.5 µL dNTP mix, 1 µL of a 20 µM solution of each primer, 40 µL GDW, and 0.6 units PfuTurbo DNA polymerase.

PCR reactions were incubated in a Hybaid OmniGene Labnet PCR machine. The program parameters were: pre-warm lid for 15 minutes at 50°C; ‘hot start’ for 5 minutes at 94°C; denature for 1 minute at 94°C, anneal for 1 minute at 54°C, and extend for 2 minutes at 72°C. Denature/anneal/extend steps were repeated a total of 35 times, and followed by a 10-minute incubation at 72°C. For products greater than 2 kb in length, the ‘extend’ portion of the cycle was increased to 4 or 6 minutes as needed.

PCR for site-directed mutagenesis was carried out according to manufacturer’s instructions. Fifty µL reactions contained 125 ng of each primer, 1 µL dNTP mix (Stratagene), 2.5 units PfuTurbo DNA polymerase (Stratagene), 5 µL 10x reaction buffer
(Stratagene), and 5, 10 or 20 ng of template DNA. Cycling parameters were: pre-warm lid for 15 minutes at 50°C; ‘hot start’ for 5 minutes at 95°C; denature for 30 seconds at 95°C, anneal for 1 minute at 55°C, and extend for ten minutes at 68°C. Denature/anneal/extend steps were cycled 12 times for single-base changes, 16 times for 2- or 3-base changes. Success of the PCR reaction was verified by running 2 µL of the reaction on an agarose gel. As necessary for reactions that failed, the number of cycles was increased (up to 18 cycles), elongation time was extended (up to 20 minutes) or DMSO was added (2.5 µL). Ten units of DpnI were then added and the reaction was incubated at 37°C for 60 minutes to digest template DNA. One µL of this reaction was transformed into Supercompetent XL1-Blue. DNA sequencing confirmed that only the desired mutation was present within TAT2.

Table 3

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' -&gt; 3')</th>
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<tbody>
<tr>
<td>O-214</td>
<td>GAAGAACATAAGCTAAGCAATAATTACGCAACACACTCTCATCAGGTGACAAACCTTAAT</td>
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<td>O-215</td>
<td>AAATATTCTACAAAAATAAATTGAAACTTGGTTTGTTCGATGATTGATCTGATATCACCTA</td>
</tr>
<tr>
<td>O-198</td>
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<td>O-723</td>
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</tr>
<tr>
<td>O-724</td>
<td>CAGTATAGGTTACTGTTTGGNNNGTCTGCTAGTGTAAGGC</td>
</tr>
</tbody>
</table>
Gene deletions

Gene deletions were carried out by amplification of the G418<sup>R</sup> gene flanked by lox<em>P</em> sites from the pUG6 vector (Guldener, Heck et al. 1996) with primers containing pUG6 sequence as well as yeast genomic sequence flanking the gene of interest. One primer contained 39 nucleotides of genomic sequence immediately upstream of the start codon of the gene to be deleted followed by the sequence 5’- GTG GAT CTG ATA TCA CCT A -3’ from pUG6. The second primer contained 39 nucleotides of sequence immediately after the stop codon of the gene to be deleted, followed by the sequence: 5’-CAG GTC GAC AAC CCT TAA T -3’ from pUG6. The resulting PCR product was transformed into the appropriate yeast strain. Gene deletion was confirmed by PCR using a primer internal to the G418<sup>R</sup> marker and one downstream of the deleted gene. PCR using a primer internal to the gene that was deleted and one downstream of the gene was used to ensure that no wild-type copy of the gene remained.

Tryptophan uptake assay

Yeast cells were grown overnight to early log phase and 30 mL of culture were introduced into 250-mL evacuated bottles (Baxter Healthcare Corp) containing no drug, 195 µL volatilized isoflurane (11.5%), or 100 µg/mL cycloheximide. The yeast culture was introduced using a 60 mL syringe (Beckton Dickson) with a sterile 18-guage needle (Beckton-Dickson). For preincubations of 15 minutes, the OD<sub>600</sub> at the time of culture injection was approximately 0.15-0.2, while for 120-minute preincubations, the starting OD<sub>600</sub> was approximately 0.1. Following the injection, the pressure inside and outside
the bottle was equalized by piercing the septum with a sterile 18- or 21-gauge needle. Cultures remained in bottles in the presence or absence of anesthetic for the duration of the experiment. Following a 15- or 120-minute preincubation with isoflurane, 0.5 µCi L-[5-³H] tryptophan (Amersham Biosciences) per mL of culture were introduced into each bottle. Using a 5-mL syringe and an 18-gauge needle, 4-mL samples were collected immediately. One mL was stored on ice to determine the OD₆₀₀ and total radioactivity as described below. The remaining 3 mL were added to approximately 5 mL of ice-cold wash media (SC or GDW containing 9.8 mM tryptophan) on top of 25 mm glass-fiber filters (Whatman GF/C) loaded in a 12-port vacuum manifold (Millipore). GF/C filters were rinsed three times with approximately 5 mL of ice-cold wash medium from a squirt bottle. Following the rinse, the ports were plugged with stoppers. The samples were collected at three-minute intervals for 15 minutes. Upon completion of sampling, stoppers were gently removed by twisting to avoid tearing the filter. Filters were transferred with tweezers to scintillation vials and treated with 350 µL 1 M sodium hydroxide. To read the total radioactivity in each culture, 20 µL of the initial culture was added in duplicate to vials containing a glass-fiber filter pre-wetted with 350 µL 1 M sodium hydroxide. All vials were incubated overnight. Following neutralization with 400 µL of 1 M acetic acid, 5 mL of Scintiverse BD (Fisher) scintillant were added. The vials were vortexed prior to determination of radioactive counts on a Beckman LS 6000 IC Liquid Scintillation System that read each vial for 5 minutes. Tryptophan uptake is defined as the slope of the line for µM tryptophan imported per OD₆₀₀ unit of cells (OD₆₀₀ = product of optical density and culture volume) versus time. Uptake inhibition is
defined as the percent difference between the slope of the lines for the no-drug control and the drug-treated sample.

The dose response of amino acid uptake to isoflurane was performed as described above except that 0, 2.5%, 5.5%, 8.5%, 11.5%, 12.5% or 14.5% isoflurane was used.

**Tryptophan export assay**

Yeast cells were grown overnight to early log phase. Tracer amounts of tryptophan were added at the beginning of a 15-minute preincubation in the presence or absence of 11.5% isoflurane. Following preincubation, cells were centrifuged at 500 g and medium containing $^3$H tryptophan was removed. Fresh medium containing 0% or 11.5% isoflurane was added and counts were followed as listed in tryptophan uptake assay above. Time zero counts were set to 100%.

**Assay for competitive inhibition of tryptophan uptake by isoflurane**

To determine whether the inhibition of tryptophan transport is competitive, cultures were grown overnight in SC medium containing 50 μM tryptophan. Following a 15-minute preincubation in the presence or absence of isoflurane, tryptophan was added to a final concentration of 50, 250, 750 or 1500 μM. The additional tryptophan was added as a mixture of 44 mM tryptophan stock containing tracer amounts of $^3$H tryptophan, so that the radiolabeled tryptophan was increased proportional to the total tryptophan concentration to maintain equal specific radioactivity among conditions. Alternatively, supplemental tryptophan was added to the yeast cultures either 18 hours
before the preincubation or at the beginning of the 15-minute preincubation, separately from radioactive tryptophan. Uptake was assayed by the procedure described above.

Phenylalanine was also assayed for the ability to competitively inhibit tryptophan uptake. Cultures were grown overnight in SC medium containing 50 µM tryptophan and 300 µM phenylalanine (standard concentration in SC). Following a 15-minute preincubation in 300 µM or 3 mM phenylalanine, tryptophan was added to a final concentration of 50, 250, or 750 µM and uptake was assayed as described above except that duplicate samples were analyzed at 0, 15, and 30 minutes.

**Tryptophan incorporation into protein during anesthetic exposure**

For determination of protein synthesis, duplicate 3-mL samples were collected at each time point. One sample was assayed for tryptophan uptake as described above and the other was treated for 5 minutes with ice-cold 10% TCA on the GF/C filter. Additional 10 percent TCA was added throughout the 5-minute incubation to assure cells on the filter were continually submerged. Filters were then rinsed three times with 5 mL ice-cold 5 percent TCA.

**Recovery of tryptophan uptake following anesthetic exposure**

To examine the recovery of cells from the isoflurane-induced inhibition of amino acid uptake, cultures were incubated with isoflurane for 120 minutes, then 60 mL cultures were split and half the culture was subjected to cavitation to remove dissolved isoflurane. Briefly, the culture was drawn into a 60 mL syringe (Beckton-Dickson), the needle was
removed and the end of the syringe was capped with a gloved finger. The plunger was pulled back and gases released by cavitation were expelled. The process was repeated four times. These cultures were then introduced into fresh evacuated containers, the vacuum was released to achieve atmospheric pressure, and tryptophan uptake was measured as described above. The cavitation procedure and introduction into fresh bottles took approximately 4 minutes. The remaining 30 mL of culture was left in its original bottle. Samples were collected immediately and at 10-minute intervals for 30 minutes. Tryptophan uptake was measured as described above.

**Cell extract preparation**

Yeast cells were grown to early log phase (OD<sub>600</sub> approximately 0.1-0.15) and 50 mL were introduced into evacuated bottles containing no drug, 11.5% isoflurane, or 20 ng/mL rapamycin as described for studies of tryptophan uptake. Following incubation for 0, 15 or 120 minutes, sodium azide and sodium fluoride were added to a final concentration of 10 mM from 1 M stock solutions. Cultures were quickly cooled by mixing with approximately 15 g of ice in a 50-mL polypropylene tube. Cells were collected by centrifugation and washed once with 10 mL ice-cold GDW. The washed cell pellet was frozen at -80° until all pellets were ready for extraction. The pellets were resuspended in 20 µL cracking buffer per OD<sub>600</sub> unit of cells. Cracking buffer was pre-warmed to 70° and contained 5% SDS, 8 M urea, 40 mM Tris-HCl pH 6.8, 0.1 mM EDTA, 58 mM N-ethylmaleimide and Complete Minitab Protease Inhibitor (Roche Applied Science; one protease inhibitor tablet was used per 7 mL buffer). Cell
suspensions were incubated in a 70°C-water bath for 10 minutes, and then transferred to microfuge tubes. Glass beads (425 to 600 µm) were added approximately 2/3 of the way to the meniscus and the mixture was vortexed 4 times for 4 minutes at 4°C, with four minutes of incubation on ice between each cycle of vortexing. To maximize extract recovery, a flamed 21-gauge needle was used to pierce the bottom of the microfuge tube. The tube was then placed in a glass test tube and centrifuged for 1 minute at 120 g. Extracts were transferred to a fresh microfuge tube and clarified by spinning at 12,900 g for 5 minutes. The supernatant was removed and put on ice. To enhance protein extraction, pellets were resuspended in fresh cracking buffer, boiled for 5 minutes, briefly vortexed and clarified a second time. Supernatants from both clarification steps were combined and assayed for total protein concentration (Bio-Rad DC Protein Assay). Cell extracts containing 80 µg total protein were run on SDS-PAGE.

**Western blot analysis**

SDS-PAGE gels were made with 10% acrylamide for the resolving gel and 5% acrylamide for the stacking gel. The Mini-Protean II or III system (Bio Rad) was used for pouring the gel and for the subsequent electrophoresis. The electrophoresis was set to 65 V until the dye front reached the stacking/resolving interface and then the setting was increased to 120 V. Following electrophoresis, the proteins were transferred from gels to PVDF membranes activated with methanol using the semi-dry transfer technique at 15 V for 60 minutes. Membranes were blocked in 5% w/v milk solution in TBST (20 mM Tris pH 7.6, 137 mM NaCl, 0.1% Tween-20) for at least 60-minutes at RT or overnight at 4°C.
Blocking solution was removed by rinsing with TBST. Primary antibodies were diluted in TBST without milk (primary antibody solutions were stored at 4°C between uses).

Dilutions of primary antibodies were rabbit anti-TAP 1:5000 (Open Biosystems), rabbit anti-eIF2α 1:5000 [gift from R. Wek (Narasimhan, Staschke et al. 2004)], mouse anti-Pma1p 1:5000 (Abcam), and mouse anti-ALP 1:100 (Molecular Probes). Membranes were incubated with primary antibody solution for at least 60 minutes at RT or overnight at 4°C. Primary antibody solutions were removed by rinsing with TBST. Secondary antibodies (from Pierce chemiluminescent kit) were diluted 1:5000 in TBST and incubated for 60 minutes at RT. Membranes were rinsed with TBST to remove secondary antibody solution. Chemiluminescent detection was carried out with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) detected in the GeneGnome. Quantitation analysis was performed using the densitometric analysis program GeneTools, version 3.02 (Syngene).

**Fractionation of cellular membranes**

The distribution of Tat2p among various membranes was determined using renografin density gradient separation (Chang 2002). Cultures of P2684 were grown and harvested as described above. Cell pellets were washed once with ice-cold TE buffer (50 mM Tris, pH 7.5, 1 mM EDTA) and then resuspended in TE buffer containing one tablet Complete Minitab Protease Inhibitor per 7 mL (Roche Diagnostics). Glass beads were added 2/3 of the way to the meniscus and cells were vortexed seven times for 30 seconds at 4°C with 30 seconds between each cycle. Unbroken cells were removed by
centrifugation for 5 minute at 500 g and the supernatant was loaded in the bottom of an ultracentrifuge tube (Beckman Ultra-Clear 14 x 89mm) in 38% Renografin in TE buffer (Reno-60, Bracco Diagnostics). Gradients were made by carefully layering 2.2 mL aliquots of ice-cold 34, 30, 26 and 22% renografin in TE buffer on top of the extract using a one mL syringe and 21-gauge needle. Gradients were spun for 16-18 hours at 150,000 g in an SW 41 Ti rotor in a Beckman Optima LE-80K Ultracentrifuge. Fourteen 800-µL fractions were removed from the top of the gradient, diluted 1:6.5 in TE buffer, and spun for 1 hour at 100,000 g in micro ultracentrifuge tubes (Beckman part #357448) in a TLA 100.3 rotor in a Beckman Coulter Optima MAX Ultracentrifuge. The supernatant was removed carefully and the pellet was resuspended in reducing Laemmli sample buffer (UK), denatured for 10 minutes at 37°, and run on SDS-PAGE as above. The resulting blots were probed with rabbit anti-TAP (Open Biosystems), mouse anti-Pma1 (Abcam), or mouse anti-ALP (Molecular Probes).

**Localization of Tat2p-GFP in live cells**

For live-cell microscopy, 25 mL cultures of P2688 were grown to early log phase (OD<sub>600</sub> approximately 0.1) and the cells were collected by centrifugation at 6,300 g for three minutes. Cells were protected from light throughout the experiment to prevent photo bleaching of GFP. The cells were resuspended in 1 mL of 40 µM solution of the vacuolar-membrane-specific vital dye FM 4-64 in PBS and incubated with agitation for 10 minutes at 30°. The cells were collected by centrifugation as above, washed once with
15 mL SC, and resuspended in 1 mL SC. The cells were visualized on a Nikon Eclipse E1000 with a 100x oil-immersion objective.

**Localization of Tat2p-GFP in fixed cells**

For visualization of Tat2-GFP in untreated or isoflurane-treated cells, 25 mL of early log-phase cultures (OD$_{600}$ of approximately 0.1) of P2688 were introduced into bottles with or without 11.5% isoflurane as described. Following incubation with shaking at 30° for 15, 60, or 120 minutes, cells were collected by centrifugation, and fixed by resuspending in 1 mL of freshly dissolved 3% w/v paraformaldehyde and incubating for 10 minutes at RT. To dissolve paraformaldehyde, the mixture was gently heated with stirring and sodium hydroxide was added from a 5 M stock solution to an approximate final concentration of 10 mM. The cells were washed in 15 mL of PBS and resuspended in 1 mL of PBS for visualization as described above.

**TAT2 mutagenesis**

To isolate mutations in TAT2 that affect anesthetic response, plasmid pL5352 was transformed into XL1-Red (Stratagene) mutagenic bacteria according to the manufacturer’s instructions and plated on LB + AMP. Following 24 hours of growth, cells were resuspended in 5 mL of LB + AMP and grown for 18 hours prior to isolation of plasmid DNA. Plasmid DNA was transformed into XL1-Blue (Stratagene) cells. The transformants were plated on LB + AMP and grown overnight. The colonies were resuspended in 5 mL LB + AMP and plasmid DNA was prepared without additional
growth. To ensure independent mutations, multiple transformations into XL1-Red were performed and the resulting plasmid pools were maintained separately.

To ensure that mutations responsible for altered anesthetic response were located in the sequences encoding Tat2p rather than transcription regulatory regions or vector sequences, the TAT2-TAP cassette was excised from this mutagenized plasmid library with Ndel and EcoRI. The Ndel site includes the start codon of TAT2 and EcoRI cuts after the HIS3 marker of the TAP insertion cassette. The TAT2-TAP fragment was ligated into pL5247, a TAT2 plasmid in which the Ndel to EcoRI TAT2 fragment was replaced with an Ndel-EcoRI oligonucleotide linker.

Degenerate nucleotide PCR was used to introduce mutations at amino acid 101 of Tat2p. O-724 and O-725 oligonucleotides were used to amplify TAT2 from pL5334. Reactions were transformed into XL1-Blue, and resulting DNA from pooled transformants was used to transform P2495. Forty-eight colonies were screened for plasmid loss and isoflurane phenotype. Plasmid loss analysis was performed by diluting saturated cultures 1:1000 in uracil-containing medium, growing 3 days, repeating dilution, and plating 100 µL of a 1:10 dilution on 5-FOA-containing medium. While the cultures were allowed to grow 3 days to saturation, differences in viable cell count remain a possibility.

**Isolation of Tat2p mutants resistant to isoflurane**

Plasmids containing randomly mutagenized TAT2 in the unmutagenized vector were introduced into the yeast strain P1848. Transformants were incubated in the
presence of 12 or 13% isoflurane, concentrations that are inhibitory to growth of cells containing the plasmid with a wild-type TAT2-TAP. Plasmids containing mutations that potentially conferred resistance to the growth-inhibitory effects of isoflurane were recovered into E. coli and transformed into P2495 to assess whether the phenotype was due to a plasmid-borne mutation. Strain P2495 permitted the selection of derivatives that lost the URA3-marked pTAT1 on medium containing 5-FOA, leaving the candidate Tat2p mutant as the only tryptophan transporter capable of supporting growth.
CHAPTER THREE

CHARACTERIZATION OF Tat2p, A CANDIDATE TARGET OF INHALED ANESTHETICS

Isoflurane inhibition of tryptophan uptake by Tat2p is time- and dose-dependent

To simplify the study of isoflurane effects on Tat2p, a tat1Δ strain was used. When yeast cells that are auxotrophic for tryptophan are grown on a good nitrogen source, deletion of TAT1 and TAT2 is synthetically lethal, that is, lethal when deleted in combination (Schmidt, Hall et al. 1994). To confirm that either TAT1 or TAT2 is required for yeast growth in standard yeast growth medium, which contains a rich nitrogen source, a new strain was constructed. The strain was auxotrophic for tryptophan, had TAT1 and TAT2 deleted from the chromosome, and contained TAT1 on a plasmid, YCpTAT1. A plasmid-loss strategy was used. When YCpTAT2 was transformed into this strain, cells that lost YCpTAT1 were able to grow. However, when an empty vector was transformed into this strain rather than YCpTAT2, no colonies grew on medium that selected for the loss of YCpTAT1. These results indicate that the transporter encoded by TAT2 is necessary and sufficient to import the level of tryptophan required to support growth in this strain. Therefore, growth of strain P2684 (relevant genotype trp1-III tat1Δ tat2Δ YCpTAT2) used for these studies depends on uptake of tryptophan from the medium by plasmid-encoded Tat2p. The results of the plasmid-loss assay were identical when a
TAP-tagged or GFP-tagged TAT2 gene was present on the plasmid (pL5330 or pL5334, respectively; data not shown), indicating that these C-terminal tagged versions of Tat2p are functional.

Figure 5. Isoflurane inhibits tryptophan uptake in a time- and dose-dependent manner. After cells were incubated in the presence or absence of isoflurane for 15 or 120 minutes, $^3$H tryptophan was added, and samples were collected immediately and at 3-minute intervals for 15 minutes. Samples were analyzed for tryptophan uptake as described in Experimental Procedures. A. 15-minute preincubation with 11.5% isoflurane (long-dashed line) and 120-minute preincubation (short-dashed line). Solid line with squares, 15 minute no-isoflurane control; solid line with triangles, 120 minute no isoflurane control. Data are representative from five experiments. B. Inhibition of tryptophan import following 15-minute preincubation with 0%, 2.5%, 5.5%, 8.5%, 11.5%, 12.5%, and 14.5% isoflurane. Data from two experiments are plotted against percent isoflurane. Lines were fitted to data from 0-5.5% isoflurane and 8.5-14.5% isoflurane, and connected.

It was previously reported that isoflurane inhibits the uptake of tryptophan by the strain RLK88-3C following a short (15-minute) exposure to 12% isoflurane (Palmer, Wolfe et al. 2002). For the studies reported here, the procedure was modified to detect the pattern of uptake over time by assaying single samples every three minutes. This approach revealed linearity of tryptophan uptake over the time course of the assay.
(Figure 5A) and a 58 +/- 5% (n=5) inhibition of uptake following a 15-minute preincubation with 11.5% isoflurane (Figure 5A). Following a 120-minute exposure to isoflurane, uptake inhibition increased to 77 +/- 4% (Figure 5A) indicating it takes more than 15 minutes for maximum inhibition to occur. Labeled tryptophan imported by cells in the presence or absence of anesthetics is not released from the cells to an appreciable degree during the time frame of the experiment (Figure 6), indicating the inhibition is due to uptake, and not a combination of changes in uptake and release. Inhibition of uptake is also dose-dependent (Figure 5B). Strikingly, inhibition showed a sharp increase between 5.5% isoflurane, where there was only slight inhibition, to 8.5%, where inhibition was much more dramatic.

![Figure 6](image)

**Figure 6.** Loss of $^3$H tryptophan from cells is small. Following a 15-minute preincubation in the presence or absence of isoflurane in medium containing $^3$H tryptophan, medium was removed and fresh medium without $^3$H tryptophan was added. Radioactivity remaining in the cells was measured for 15 minutes. The time zero measurement was set to 100%. Solid line, export no drug; dashed line, export isoflurane.
Overexpression of Tat2p affects tryptophan uptake, but not isoflurane-induced inhibition of tryptophan uptake

Previous work showed that introduction of the \textit{TAT2} gene on a multicopy vector (YE\textit{p}) into an appropriately auxotrophic strain led to anesthetic resistance (Palmer, Wolfe et al. 2002). Western blot analysis has now confirmed that expression from the YE\textit{p}TAT2 vector increased Tat2p levels when compared to expression from a single-copy vector (YC\textit{p}TAT2) (\textit{Figure 7A}). Specifically, YE\textit{p}TAT2 produced a 2.5-fold (+/- 0.2, \(p<0.001\)) increase in the level of Tat2p compared to YC\textit{p}TAT2.

The increase in Tat2p levels led to an increase in tryptophan uptake of about 40% in either the presence or absence of drug (\textit{Figure 7B}). Thus, the percent inhibition of uptake by isoflurane was not altered by increased levels of Tat2p (\textit{Figure 7B}). The percent inhibition was also unaffected following a 120-minute preincubation with isoflurane (data not shown), indicating that inhibition of tryptophan uptake appears to be independent of Tat2p concentration or the rate of tryptophan uptake.
Figure 7. Tat2p expression from a YEp vector increases Tat2p levels and tryptophan import. A. Triplicate extracts from cells containing YCp TAT2-TAP (strain P2684) or YEp TAT2-TAP (strain P3144) were processed for Western blot analysis as described in Experimental Procedures and blots were probed for the TAP tag and eIF2α as a loading control (not shown). B. Following a 15-minute preincubation in the presence or absence of isoflurane, tryptophan import was assayed as previously described. Bold solid line, YEp no drug; bold dashed line, YEp with isoflurane; light solid line, YCp no drug; light dashed line, YCp with isoflurane. Representative data from three replicates.

Extended exposure to isoflurane decreases protein synthesis

The incorporation of imported tryptophan into protein was measured in the presence or absence of isoflurane. The lower level of tryptophan uptake after a 15-minute preincubation and 30-minute experiment in the presence of isoflurane only slightly decreased the rate of protein synthesis, as measured by tryptophan label incorporation into acid-precipitable material (Table 4A), indicating that a longer exposure is required to halt translation. Thus, a higher percentage of imported tryptophan was incorporated into protein following this length of exposure to isoflurane (Table 4A).
A 120-minute preincubation with isoflurane induced a greater inhibition of tryptophan uptake and a dramatic inhibition of protein synthesis (Table 4B).

As a control, incorporation of tryptophan into protein in cells exposed to cycloheximide, an inhibitor of translation elongation, was measured. Although cells imported substantial amounts of tryptophan following a 15-minute exposure to this drug, little was incorporated into proteins (Table 4A) due to blockage of translation elongation.

**TABLE 4**

**Extended exposure to isoflurane affects protein synthesis.**

<table>
<thead>
<tr>
<th>Treatment:</th>
<th>Tryptophan import</th>
<th>TCA</th>
<th>incorporation into protein (%)</th>
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<tr>
<td>A 15 min.</td>
<td>no drug</td>
<td>1</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>isoflurane</td>
<td>0.38</td>
<td>0.31</td>
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<tr>
<td></td>
<td>cycloheximide</td>
<td>0.63</td>
<td>0.04</td>
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<tr>
<td>B 120 min.</td>
<td>no drug</td>
<td>1</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>isoflurane</td>
<td>0.12</td>
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</table>

*Cells were exposed to isoflurane, cycloheximide or no drug for 15 minutes (A) or 120 minutes (B) prior to addition of $^3$H tryptophan. Samples were collected immediately following addition of radiolabeled tryptophan and after 30 minutes. Samples were analyzed for either total or TCA-precipitable counts as described in Experimental Procedures. Total tryptophan import and TCA-precipitable counts were calculated as the difference between the 0 and 30 minute measurements. The tryptophan import in the absence of drug was set to 1 independently for part A and B, and was used to normalize tryptophan import and TCA data. Data are the average from 3 experiments. Incorporation into protein (%) is calculated as (TCA precipitable counts ÷ tryptophan import)*100.*
Tryptophan import recovers rapidly after isoflurane removal

Earlier studies showed isoflurane-induced growth inhibition is reversible (Keil, Wolfe et al. 1996). This suggests that the inhibition of tryptophan import is also reversible. To assess the rapidity of the recovery of tryptophan uptake, cells were incubated in the presence or absence of isoflurane for 120 minutes. Half of each culture was then subjected to a partial vacuum to remove dissolved gases including isoflurane, a process called cavitation. The other half of each culture continued to be incubated without cavitation. Removal of dissolved gases and introduction into fresh bottles took approximately 4 minutes. Samples were taken at 10-minute intervals thereafter, and tryptophan uptake was measured.

Following cavitation, recovery of uptake was apparent at the earliest 10-minute interval (Figure 8). Additional evidence of the rapidity of the recovery of uptake comes from the finding that even 4 minutes after cavitation was begun, cultures from which isoflurane was removed accumulated significantly higher amounts of tryptophan (30.1 +/- 8.6 µM) than the cultures that still contain isoflurane (22.3 +/- 3.7 µM, p<0.05, n=8). The increased uptake was not induced by the cavitation procedure since cavitation of cultures not exposed to anesthetic did not show increased uptake. The slight increase in uptake observed in the isoflurane-treated culture not subjected to cavitation may be due to a decrease in isoflurane concentration due to half of the culture being removed (Figure 8).
Figure 8. Uptake of tryptophan recovers rapidly following anesthetic removal. Cells were incubated in the presence or absence of isoflurane for 120 minutes. Half of each culture was subjected to cavitation to remove dissolved gases including isoflurane and then analyzed for uptake of $^3$H tryptophan. Solid line with filled squares: no drug, no cavitation; dotted line with open squares: no drug, with cavitation; solid line with filled triangles: isoflurane, no cavitation; dotted line with open triangles: isoflurane removed by cavitation. Numbers represent uptake of tryptophan as measured by slope of the line (see Experimental Procedures) for each 10-minute time period (isoflurane with and without cavitation) or for the best-fit line over the 30-minute incubation (no drug with and without cavitation).

I Isoflurane does not competitively inhibit tryptophan uptake

To further characterize the nature of the inhibition of tryptophan uptake by isoflurane, inhibition was measured at various tryptophan concentrations. Cells were initially grown in medium containing 50 µM tryptophan. At time zero, tryptophan stock containing tracer amounts of $^3$H-tryptophan was added to give the indicated concentration
(Figure 9). Increasing the concentration of tryptophan as much as thirty-fold did not decrease the isoflurane-induced inhibition of tryptophan uptake (Figure 9B) although the total amount of tryptophan imported in the presence or absence of isoflurane increased (Figure 9A). Alternative experimental designs were used to assure that cells had adequate time to adapt to medium containing additional tryptophan. Specifically, the concentration of tryptophan in the medium was increased at times prior to the addition of tracer amounts of $^3$H tryptophan. Adding the extra tryptophan at the start of the 15-minute isoflurane preincubation or 18 hours before assaying uptake had no effect on the observed inhibition (data not shown). From these experiments, isoflurane does not appear to be a competitive inhibitor of tryptophan uptake by Tat2p. This appears to exclude the possibility that isoflurane affects tryptophan binding by Tat2p. Rather, isoflurane may affect the tryptophan translocation activity of Tat2p or may cause a tryptophan-independent decrease in Tat2p on the plasma membrane, either by affecting Tat2p synthesis or degradation or by affecting the localization of Tat2p in the cell.
Figure 9. Inhibition of amino acid uptake by isoflurane is not competitive. Cultures were grown in varying concentrations of tryptophan in the presence or absence of isoflurane and assayed for tryptophan uptake as described in Experimental Procedures. A. Uptake in the absence (filled squares) or presence (open squares) of isoflurane at various concentrations of tryptophan. Cultures were initially grown in medium with 50 μM tryptophan and additional tryptophan to achieve the desired concentration was added at time zero when \(^{3}\text{H}\) tryptophan was added. Representative data from three experiments are shown. B. Percent inhibition of uptake, defined as percent difference between uptake by isoflurane-treated samples and no-drug control, at various tryptophan concentrations. Calculations use data from A.
Uptake of phenylalanine increases when *TAT2* is present on a multicopy plasmid, suggesting that Tat2p has the capacity to import phenylalanine (Regenberg, During-Olsen et al. 1999). If a single transporter takes up more than one amino acid, one would expect competitive inhibition between the amino acids. To verify that the methods used would reveal competitive inhibition if it occurred, the uptake of tryptophan was measured in the presence of the standard SC medium concentration of phenylalanine as well as a 10-fold increased concentration. Phenylalanine did inhibit tryptophan uptake (Figure 10). Tryptophan concentration was varied to determine whether increased tryptophan levels were able to overcome phenylalanine-induced tryptophan uptake inhibition. While there was some variability between experiments, the overall trend was a decrease in tryptophan uptake inhibition as tryptophan concentration increases. This trend is consistent with competitive inhibition by phenylalanine, as predicted.
**Figure 10.** Phenylalanine competitively inhibits tryptophan uptake. Cultures were grown in varying concentrations of tryptophan in the standard SC concentration of phenylalanine, or a 10-fold increased (10x) concentration and assayed for tryptophan uptake as described in *Experimental Procedures*.  

A. Uptake in standard phenylalanine medium (filled diamonds) or 10x phenylalanine (open diamonds). Representative data from two experiments are shown.  

B. Percent inhibition of uptake, defined as percent difference between uptake in standard phenylalanine and 10x phenylalanine, at various tryptophan concentrations. Inhibition data are shown from two independent experiments.

**Tat2p is not degraded during isoflurane exposure**

Rapamycin, a drug that inhibits tryptophan uptake by Tat2p, induces ubiquitination and degradation of plasma-membrane-localized Tat2p and a re-routing of newly-synthesized Tat2p from the secretory pathway directly to the vacuole leading to decreased cellular levels of Tat2p (Beck, Schmidt et al. 1999). To determine whether volatile anesthetic exposure induces similar effects, levels of Tat2p in the cell were assayed. Unlike rapamycin treatment, Tat2p levels remained unchanged following 15 minutes of exposure to isoflurane (*Figure 11*) and actually increased slightly after 120 minutes of exposure (*Figure 11*, p<0.001). This increased level of Tat2p occurred at a
time when tryptophan uptake was inhibited by about 80%, demonstrating that loss of Tat2p was not responsible for the decreased import. As a control, rapamycin-treated cells showed a dramatic decrease in Tat2p levels after a 120-minute exposure (Figure 11).

**Figure 11.** Isoflurane does not induce Tat2p degradation. A. Cells were exposed to isoflurane (iso), rapamycin (rapa) or no drug for the indicated periods of time. Cells were processed for Western blot analysis as described in *Experimental Procedures* and blots were probed for the TAP tag or eIF2α as a loading control. TAP antibody is specific for the TAP tag on Tat2p, as shown by the absence of signal in P2683, the untagged Tat2p control (lane 1). B. Quantitation was performed by densitometric analysis and statistics were calculated from ten replicates. *** p<0.001
Tat2p does not relocalize during isoflurane exposure

While the total amount of Tat2p present in cells did not decrease during isoflurane exposure, a change in the proportion of plasma-membrane-localized Tat2p would decrease the proportion of Tat2p capable of transporting tryptophan into the cell. To analyze Tat2p localization qualitatively, a Tat2p-GFP fusion was used. Microscopic analysis showed that in the absence of anesthetic, most Tat2p was present in the plasma membrane and the vacuole (Figure 12A), whose identity was confirmed using the vacuolar-membrane-specific dye FM 4-64 (Vida and Emr 1995). This localization is consistent with previous reports (Huh, Falvo et al. 2003; Umebayashi and Nakano 2003). Some cells had faint cytoplasmic foci of GFP signal, which always colocalized with FM 4-64 foci (data not shown). FM 4-64 is used to trace endocytosis (Vida and Emr 1995) suggesting these GFP foci contained endocytosed Tat2p destined for the vacuole. Exposure to isoflurane for 15, 60, or 120 minutes did not induce a detectable decrease in plasma-membrane-localized Tat2p (Figure 12B and data not shown).

Analysis with the GFP-tagged Tat2p did not reveal a change in Tat2p localization, and it seems unlikely that a relocalization that was sufficient to account for the 60 to 80% inhibition of uptake would not be obvious even in this qualitative assay. However, to further test this possibility and to potentially detect a slight change in distribution, we quantitated Tat2p distribution in the cells by using renografin gradients to separate internal-membrane-localized proteins from plasma-membrane-localized proteins (Chang 2002). Extracts from cells incubated in the presence or absence of isoflurane for 15 or
**Figure 12.** Qualitative Tat2p localization is not altered by isoflurane. 
A. Live cells containing Tat2p-GFP (strain P2688) were stained with vital vacuolar dye FM 4-64 and visualized by microscopy. Left panel: Tat2-GFP is visible in plasma membrane and the vacuole; center panel: FM 4-64 stains the vacuolar membrane; right panel: merge of GFP and FM 4-64 demonstrates that internal Tat2p-GFP signal localizes to the vacuole. B. Cells grown in the absence (left panel) or presence (right panel) of isoflurane for 60 minutes were fixed in formaldehyde and visualized for GFP fluorescence.
120 minutes were examined for the membrane localization of Tat2p. To determine the location of different cellular membranes within the density gradient, fractions were probed for control proteins known to reside mainly in a single type of membrane. Localization of Pma1p, which resides in the plasma membrane (Bagnat, Chang et al. 2001; Navarre, Degand et al. 2002), peaks in fractions 8 to 9 from renografin density gradients [(Chang 2002) and Figure 13A and C] while alkaline phosphatase (ALP), which resides in the vacuolar membrane (Klionsky and Emr 1989), was present largely in fractions 1 to 3 [(Klionsky and Emr) and Figure 13C]. Tat2p-TAP was present in both low-density fractions with ALP and higher density fractions with Pma1p (Figure 13) as expected from the results with GFP-tagged Tat2p. In addition, a third peak of intermediate density was observed (Figure 13B and C). Previous work demonstrated that membrane density decreases as it changes from plasma membrane to membranes of early, then late endosomes (Singer-Kruger, Frank et al. 1993). Because Tat2p is removed from the plasma membrane via endocytosis (Beck, Schmidt et al. 1999), the intermediate peak likely contains Tat2p present in cytoplasmic vesicles destined for the vacuole. This is consistent with the cytoplasmic foci containing FM 4-64 and Tat2p discussed in the GFP microscopy section. When FM 4-64 is added to cells, it first stains plasma membranes, then is endocytosed and stains cytoplasmic foci, and finally arrives at the vacuolar membrane (Vida and Emr 1995). Once a vacuolar membrane staining pattern has been established, this pattern is stable (Vida and Emr 1995). Because FM 4-64 does not stain cytoplasmic foci once it has reached the vacuolar membrane (Vida and Emr 1995), these Tat2p foci must be in endocytic vesicles destined for the vacuole.
Quantitative analysis of fractionated density gradients revealed that approximately 50% of membrane-localized Tat2p was located in the vacuolar membrane (fractions 1-3), approximately 20% was in the intermediate peak (fractions 4-6), and approximately 30% was in the plasma membrane (fractions 7-10). These proportions did not change for cells exposed to isoflurane for 15 or 120 minutes (Figure 13B). This finding is consistent with the qualitative GFP analysis and suggests that Tat2p membrane localization is not changed during exposure to isoflurane.

Figure 13. Quantitative Tat2p localization does not change during isoflurane incubation. Cells containing Tat2p-TAP were grown in the presence or absence of isoflurane for 15 or 120 minutes. Extracts were loaded in the bottom of renografin density gradients, spun overnight, fractionated and run on SDS PAGE. Blots were probed with antibodies against TAP, Pma1p (a control plasma membrane protein) or ALP (a control vacuolar membrane protein). A. Representative Western blots of renografin gradient fractions probed for TAP and Pma1. B. Densitometric analysis of Tat2-TAP bands from A. Numbers represent the percent of Tat2p present in lanes 1 through 3 (vacuole), lanes 4 through 6 (intermediate peak) and lanes 7 through 10 (plasma membrane). Tick marks under 15-minute no isoflurane trace delineate peak valleys. C. Overlay of Tat2p-TAP (solid line), Pma1p (long-dash line), and ALP (short-dash line) densitometric analysis from representative Western blots of cells not exposed to isoflurane.
CHAPTER FOUR

ANESTHETIC-RESISTANT MUTANTS OF Tat2p HAVE ALTERED TRYPTOPHAN UPTAKE CHARACTERISTICS

Specific mutations in TAT2 lead to volatile anesthetic resistance

To generate Tat2p mutants that alter the anesthetic response of yeast, random mutagenesis of the TAT2 gene was followed by selection for isoflurane-resistant mutants. Random mutagenesis of the entire TAT2 gene allows identification of regions throughout Tat2p that affect sensitivity of yeast growth to isoflurane. Random mutagenesis was performed using XL1-Red Mutagenic bacteria (Stratagene). Due to deletions of DNA repair enzymes, this strain introduces random mutations throughout the introduced plasmid. This creates a library of plasmids that must be recovered into a non-mutator strain, XL1-Blue (Stratagene), for amplification of DNA. Table 5 summarizes the results of TAT2 mutagenesis at various steps.

From this selection, ten different TAT2 mutations that render yeast isoflurane-resistant were obtained (Figure 14A). Mutations were identified by DNA sequence. Mutations S95G, W171C and T295A were independently isolated two, two, and six times, respectively, suggesting either hypermutability of these sites or a selection bias for these mutant alleles. The Tat2p mutants were characterized in a strain auxotrophic for tryptophan whose sole tryptophan transporter capable of supporting growth is encoded by
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<sup>a</sup>Each mutagenesis is an independent pool of mutants generated in XL 1 Red

<sup>b</sup>Bacteria column refers to the approximate number of XL 1 Blue bacterial colonies resuspended for DNA pool preparation

<sup>c</sup>Total yeast refers to the approximate total number of yeast transformants that were subjected to selection in isoflurane

<sup>d</sup>Original refers to the number of yeast colonies that grew at the selective isoflurane concentration

<sup>e</sup>Naïve retest is the number of isoflurane-resistant isolates after transfer of the candidate mutant plasmid into a naïve strain of yeast

<sup>f</sup>Mutants that tested positive in the naïve retest but are not in the "new mutants" column were either repeats of previously identified mutants, false positives, or were not sequenced

<sup>g</sup>Too numerous to count
the plasmid-borne, mutant TAT2 gene. Isoflurane resistance ranged from slight (E27F) to strong (S95N, V368A) based on the amount of visible growth in the presence of increasing concentrations of isoflurane (Figure 14). All mutants were tested for cross-resistance to halothane, another clinically relevant volatile anesthetic. With the exception of the weakly resistant mutant E27F, all mutants exhibit approximately the same relative level of resistance to both anesthetics. The E27F showed no growth resistance to halothane at the concentrations tested (Figure 14B). Eight of the ten mutations selected based on anesthetic resistance phenotype are located in predicted transmembrane helices (Figure 15). Helices one and six each contain three of the mutations, while helices three and eight contain one mutation each. The E27F mutation is located in the N-terminal cytoplasmic tail while N330D is predicted to be in the fourth extracellular loop (Figure 15).

**Anesthetic-resistant permease mutants alter tryptophan uptake**

Isoflurane inhibits tryptophan uptake by wild-type Tat2p in a time-dependent manner as previously reported and confirmed here [Chapter 3 and (Palmer, Wolfe et al. 2002)]. Analysis of tryptophan uptake in the Tat2p mutants revealed two different means of producing the isoflurane-resistant phenotype. In some mutants, tryptophan transport activity was refractory to the inhibitory effects of isoflurane. For example, inhibition of tryptophan uptake in the V368A mutant was markedly less than in wild-type Tat2p following a 15- or 120-minute preincubation with isoflurane (Figure 16). Mutants with decreased isoflurane-induced inhibition of tryptophan uptake are termed isoflurane-
Figure 14. Specific mutations within TAT2 lead to volatile anesthetic resistance. Wild-type (P2684) and Tat2p mutants incubated in the absence or presence of varying concentrations of isoflurane (A) or halothane (B) for 5 days as described in Experimental Procedures.
Figure 15. Isoflurane-resistant mutations cluster in helices surrounding the ligand binding site. Proposed secondary structure is shown, according to the structural homology model of Tat2p based on LeuT<sub>Aa</sub>. Mutations noted at their approximate location in Tat2p. Mutations marked in green were selected for isoflurane-resistance from a library of random mutants. Mutations noted in red indicate site-directed mutants. N- and C-termini are cytoplasmic. T in triangle, tryptophan cargo; black circle, proton; numbered rectangles, transmembrane helices; EL, extracellular loops; IL, intracellular loops. Modified from (Kanner 2005)

refractory. Amino acid uptake analysis of all Tat2p mutants is summarized in Figures 17 and 18. Uptake inhibition of each mutant was compared to the wild-type control. Six mutants were significantly refractory to the effects of isoflurane (Figure 18). The majority of these mutants showed an increased inhibition of tryptophan uptake by isoflurane following 120-minute preincubation as compared to a 15-minute preincubation. However, I285T and N330D did not behave in this manner.
Another means to achieve the isoflurane resistance involves increased tryptophan uptake by the mutant compared to wild type, regardless of the presence of isoflurane. For example, mutant N330D led to an increase in the rate of tryptophan uptake in the absence and presence of anesthetic (Figure 16). This is termed a hyperactive mutant. Because the uptake rate increased proportionately in both conditions, the percent inhibition in this hyperactive mutant did not differ significantly from wild type (Figure 18). Six of the ten mutants were hyperactive (Figure 17). Three of the mutants, S95G, S95N and M287I, were both hyperactive and isoflurane-refractory, suggesting that both factors contribute to
the anesthetic resistance in these mutants. Mutants W171C and V368A decreased uptake in the absence of anesthetic (Figure 17). These are termed hypoactive mutants. Interestingly, these two hypoactive mutants had the strongest isoflurane-refractory phenotype (Figure 18), meaning that they are resistant to isoflurane despite a decreased ability to import tryptophan in the absence of isoflurane. In mutants E27F and I285T, uptake was not significantly changed. Mutants with normal or hypoactive uptake are isoflurane-refractory, meaning that the isoflurane-resistant phenotype is attributable to decreased inhibition of uptake by isoflurane. The E27F mutant was the one exception, as it was neither hyperactive nor isoflurane-refractory. E27F had the weakest isoflurane-resistant phenotype of the ten mutants (Figure 14), and was consistently slightly hyperactive. Although this increased rate of uptake was not statistically significant, the slight hyperactivity appears to be the most likely explanation for the slight isoflurane resistance of this mutant.
**Figure 17.** *TAT2* mutants can alter tryptophan uptake in the absence of anesthetic. The tryptophan uptake of each mutant is shown relative to the wild-type control, which was set to 1. Data are averaged from at least three independent experiments per mutant. Dashed line represents tryptophan uptake by wild type. *p<0.05, **p<0.01, ***p<0.001.

**Figure 18.** Effect of *TAT2* mutants on tryptophan uptake inhibition varies. Inhibition of tryptophan uptake for the various *TAT2* mutants following a 15 minute (gray bars) or 120 minute (dark bars) preincubation with isoflurane. Inhibition of uptake is calculated as the percent difference between tryptophan uptake in the presence versus absence of isoflurane. Inhibition data are averaged from two or more independent experiments per mutant. Dashed line indicates wild-type uptake inhibition following 15 minutes preincubation. Dot-dash line indicates wild-type uptake inhibition following 120 minutes preincubation. * p<0.05, ** p<0.01, *** p<0.001.
Some hyperactive mutants have increased Tat2p levels

One potential explanation for mutants that increase uptake in the absence of isoflurane is that the mutation increases the level of Tat2p. Western blot analysis revealed that some, but not all, hyperactive mutants had increased levels of Tat2p (Figure 19C). Hyperactive mutants A91T, M287I, and T295A had increased Tat2p levels. The increased Tat2p may account for the hyperactivity, provided the localization patterns in these mutants remain unchanged. This has not been tested. However, not all hyperactive mutants had increased Tat2p levels. Mutant N330D was present at the same level as wild-type Tat2p (Figure 19A), as were S95G and S95N. This indicates that increased Tat2p levels do not always account for the hyperactive tryptophan uptake phenotype. However, the localization of Tat2p in each mutant is yet to be characterized.

Interestingly, the hypoactive W171C and V368A mutants had the most marked increase in Tat2p levels (Figure 19B and C). The increase in Tat2p for these mutants was greater than 2-fold compared to wild type (Figure 19C). Finally, mutant I285T, which had normal uptake in the absence of anesthetic, had increased Tat2p levels. In general, the Tat2p levels of the mutants in the absence of anesthetic did not correlate with tryptophan import under the same condition. However, a more informed correlation would take mutant Tat2p localization into account, as only Tat2p localized to the plasma membrane is available to transport tryptophan.
Figure 19. Some mutants had increased Tat2p levels. Cells containing the indicated mutant Tat2p were processed for Western blot analysis as described in Experimental Procedures and blots were probed for the TAP tag or eIF2α as a loading control. Bands were quantitated by densitometry and TAP signal was normalized using the loading control. The average normalized TAP signal for the wild-type control lanes from each blot was set to 1 arbitrary unit (a.u.). Representative blots are shown for N330D (A) and V368A (B). C. Tat2p levels for the various mutants. Error bars represent standard deviation. Data are averaged from at least five independent cell extracts. **p<0.01, ***p<0.001

Hypoactive mutants have a slow growth phenotype

Altered uptake of an essential amino acid is likely to affect the growth rate of yeast. The effect of the TAT2 mutations on growth rate in the absence of anesthetic was determined. Mutations that decreased tryptophan uptake decreased growth rate (Figure
This suggests that when Tat2p is hypoactive, tryptophan uptake becomes limiting, leading to a significant decrease in growth rate. Hyperactive mutants did not affect the growth rate (**Figure 20**). Also, mutants with unaltered uptake in the absence of drug, E27F and M287I, had no effect on growth rate (**Figure 20**). This indicates that tryptophan uptake is not limiting to growth of cells containing wild-type Tat2p in the absence of drug.

**Figure 20.** Generation time is increased in hypoactive Tat2p mutants. Generation times were determined from logarithmically growing cultures. The average generation time for each mutant was determined from at least four independent experiments.

**Hypoactive Tat2p mutants affect growth on media containing low tryptophan**

The effects of the Tat2p mutants on tryptophan uptake suggested that these mutants might affect the ability of strains to grow on medium containing decreased tryptophan concentrations. Strains were grown on plates containing various concentrations of tryptophan and scored for growth daily. Following one day of
incubation, hypoactive mutant W171C showed dramatically decreased growth at all concentrations of tryptophan compared to wild-type Tat2p (Figure 21). After six days growth of this strain on 250 µM tryptophan was indistinguishable from wild type while growth on 12.5 µM tryptophan remained affected. This suggests the result was caused by low tryptophan and not by a general slow growth phenotype (Figure 20). The other hypoactive Tat2p mutant, V368A, also displayed a growth defect on low tryptophan medium, although the effect was less striking. The hyperactive mutants did not show an improvement in ability to grow on medium containing low tryptophan (Figure 21).

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**Figure 21.** Hypoactive Tat2p mutants grow poorly on low-tryptophan media. Cells were grown to saturation, diluted 1:50, and spotted on media containing the indicated concentrations of tryptophan. The standard concentration of tryptophan in our media is 250 µM. A. Mutants of Tat2p were tested in a tat1Δ tat2Δ strain background (P2495 derivatives). B. Mutants were tested in a TAT1 tat2Δ background (P1848).
Site-directed mutagenesis supports the Tat2p structural homology model.

A structural homology model of Tat2p was constructed by Dan DeCotiis (Keasey, De Cotiis et al. 2007) based on the crystal structure of LeuT_{Aa} (Figure 22) (Yamashita, Singh et al. 2005; Keasey, De Cotiis et al. 2007). To experimentally validate this model, predictive mutations were designed in regions of Tat2p suspected to be important for tryptophan transport. The phenylalanine at position 101 is predicted by the structural model to point into the tryptophan transport channel (Yamashita, Singh et al. 2005; Keasey, De Cotiis et al. 2007). The position of this residue with respect to the amino acid binding site suggests the potential for aromatic stacking interactions with the tryptophan cargo (Keasey, De Cotiis et al. 2007). Mutation to threonine would remove potential stacking interactions and was predicted to decrease Tat2p activity (Keasey, De Cotiis et al. 2007). Analysis of this and other mutants is summarized in Table 6. The F101T mutant was constructed by site-directed mutagenesis and amino acid uptake analysis revealed that transport was hypoactive as was predicted (Figure 17; mutations produced by site-directed mutagenesis are shown in red italics). There was no effect on inhibition of uptake by isoflurane (Figure 18). As with other hypoactive Tat2p mutants, F101T leads to an increase in Tat2p levels (Figure 19). Also consistent with other hypoactive mutants, this mutant significantly decreased growth rate (Figure 20), likely a result of tryptophan becoming limiting for growth. To address this assertion, growth of this mutant on low tryptophan media is shown in both TAT1 tat2Δ and tat1Δ tat2Δ strain backgrounds. This allows comparisons to be made among F101T and the random
**Figure 22.** A structural homology model of Tat2p was constructed based on the crystal structure of LeuT<sub>Ac</sub>. This picture is generated as though the cytoplasmic face is at the superior aspect of each model. Coloring for Tat2p: helices 1, 3, 6, and 8 are magenta. Remaining residues are tan. Coloring for LeuT<sub>Ac</sub>: helices 1, 3, 6, and 8 are blue. These helices contain amino acids that are directly responsible for substrate trafficking. Remaining orange residues do not directly participate in substrate trafficking. Source: Dan De Cotiis
mutants (Figure 21 A) as well as to the other site-directed mutant (Figure 21 B), which cannot be analyzed in a tat1Δ tat2Δ background, as will be discussed below. In each case, results indicate a diminished ability of this strain to grow at low tryptophan compared to wild-type controls (Figure 21A and B). This mutant does grow better than a strain carrying no Tat2p (Figure 21B), in agreement with its tryptophan uptake characterization, which indicates partial Tat2p activity. In addition to the specifically constructed F101T mutation, random mutations at F101 were also generated. Forty-eight yeast transformants were screened for 1) the ability to lose YCpTAT1, an indication of Tat2p activity and 2) growth in the presence of isoflurane. For strains containing 11 of the 48 plasmids, loss of the YCpTAT1 plasmid was decreased and growth was super-sensitive (more sensitive than wild-type control) to isoflurane. Sequencing of select mutants revealed that mutation of phenylalanine at position 101 to isoleucine prevented plasmid loss comparable to an empty vector and mutation to methionine or tyrosine decreased plasmid loss and rendered cells super-sensitive to isoflurane. In one case loss of YCpTAT1 was increased and growth was resistant to isoflurane. Sequencing revealed

![Figure 23](image)

**Figure 23.** Mutations at F101 alter plasmid loss and isoflurane phenotype. Cells were grown in the absence of selection for YCpURA3 TAT1 and tested for plasmid loss as described in Experimental Procedures. For strains capable of losing TAT1 plasmid, isoflurane phenotype was tested. Growth in 9% isoflurane is shown.
mutation to tryptophan in this plasmid (Figure 23). The remaining plasmids were not remarkably different than the wild type or gave inconclusive results.

Based on the structural model of Tat2p, the E286 residue is also predicted to point into the tryptophan transport channel, and is postulated to contribute to hydrogen bonding and charge stabilization of the cargo tryptophan (Yamashita, Singh et al. 2005; Keasey, De Cotiis et al. 2007). Mutation to glutamine preserves hydrogen-bonding capacity, but abolishes charge stabilization. Therefore, E286Q was predicted to be detrimental to Tat2p activity (Keasey, De Cotiis et al. 2007). The plasmid containing the E286Q mutation in Tat2p was transformed into P2495, relevant genotype $tat1^{\Delta} tat2^{\Delta} trp1-{\text{HIII}}$ YCp$URA3$ TAT1. No colonies grew on medium containing 5-fluoroorotic acid, which selects for cells that are $ura3^{-}$. This indicates that the E286Q mutant was unable to import sufficient tryptophan to support growth. The limited growth of a strain containing a chromosomal copy of TAT1 and a plasmid containing the E286Q Tat2p mutant on low-tryptophan media supports this conclusion. Specifically, the E286Q mutation showed markedly less growth than wild type on 100 $\mu$M tryptophan (Figure 21B). Growth on this medium was even less than that observed for the hypoactive F101T Tat2p. The E286Q mutant had approximately the same growth on low tryptophan media as a strain containing the empty vector (no Tat2p) despite being present at levels greater than wild-type Tat2p (Figure 21B and data from Dan De Cotiis, not shown).
Table 6. Summary of mutant Tat2p phenotypes

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Apparent Location</th>
<th>No drug</th>
<th>Inhibition by isoﬂurane</th>
<th>Tat2p levels</th>
<th>Growth rate</th>
<th>Low tryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td>E27F</td>
<td>N-terminal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>A91T</td>
<td>helix 1a</td>
<td>hyperactive</td>
<td>normal</td>
<td>↑(^a)</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>S95G</td>
<td>helix 1a</td>
<td>hyperactive</td>
<td>less inhibited</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>S95N</td>
<td>helix 1a</td>
<td>hyperactive</td>
<td>less inhibited</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>W171C</td>
<td>helix 3</td>
<td>hypoactive</td>
<td>less inhibited</td>
<td>↑↑(^b)</td>
<td>slow</td>
<td>weak</td>
</tr>
<tr>
<td>I285T</td>
<td>helix 6 unwound</td>
<td>normal</td>
<td>less inhibited(^c)</td>
<td>↑</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>M287I</td>
<td>helix 6b</td>
<td>hyperactive</td>
<td>less inhibited</td>
<td>↑</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>T295A</td>
<td>helix 6b</td>
<td>hyperactive</td>
<td>normal</td>
<td>↑</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>N330D</td>
<td>EC 4</td>
<td>hyperactive</td>
<td>normal</td>
<td>↑</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>V368A</td>
<td>helix 8</td>
<td>hypoactive</td>
<td>less inhibited</td>
<td>↑↑</td>
<td>slow</td>
<td>weak</td>
</tr>
<tr>
<td><strong>F101T</strong></td>
<td>helix 1 unwound</td>
<td>hypoactive</td>
<td>normal</td>
<td>↑↑</td>
<td>slow</td>
<td>weak</td>
</tr>
<tr>
<td><strong>E286Q</strong></td>
<td>helix 6b</td>
<td>severely hypoactive(^d)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>very weak</td>
</tr>
</tbody>
</table>

\(^a\) Tat2p levels are increased less than 2-fold over wild type

\(^b\) Tat2p levels are increased greater than 2-fold over wild type

\(^c\) less inhibited than wild-type only after extended isoﬂurane exposure

\(^d\) severe hypoactivity not assayed by tryptophan uptake as this mutant was unable to be separated from TAT1; see Results section for details

\(^e\) equivalent to empty vector control
CHAPTER FIVE
GENERAL DISCUSSION AND FUTURE DIRECTIONS

General anesthetics are administered 20 million times per year in the United States alone (Eckenhoff 2001). Due to the widespread and increasing use of anesthetics, and the risks inherent in the use of these drugs, elucidation of the molecular basis of their effects is critical. However, understanding of anesthetic actions remains incomplete. A key first step in understanding the mechanisms of action of a drug is the identification and characterization of its targets. Previous findings implicated specific amino acid permeases as candidate targets of anesthetics in yeast (Palmer, Wolfe et al. 2002). Work presented here involves the characterization of amino acid permeases, and specifically Tat2p. The study of Tat2p during anesthetic exposure of yeast provides a readily manipulatable model to study effects of anesthetics on transporter proteins. Anesthetics inhibit amino acid transport by Tat2p in a relatively rapid and reversible manner, and by a mechanism that is not competitive. This inhibition occurs without associated internalization or degradation of the transporter. Additionally, isoflurane provides a tool to select mutations within TAT2 that alter tryptophan transport.

Involvement of Tat2p, a candidate drug target, in volatile anesthetic response

Genetic evidence involving overexpression or deletion of amino acid permease genes, including TAT2, suggests that permeases are targets of anesthetics. Specifically, deletion of TAT2 renders cells more sensitive to isoflurane while expression from a multi-
copy plasmid renders cells more resistant. Several previously proposed models of direct and indirect involvement of amino acid permeases in anesthetic response have been tested in this work (Figure 24). Volatile anesthetics have been shown to competitively inhibit the activity of many proteins (Franks 1984; Martin, Adams et al. 1990; Rada, Tharakan et al. 2003; Dickinson 2007), so competitive inhibition of Tat2p seemed a likely possibility. However, increasing the concentration of tryptophan does not decrease isoflurane-induced inhibition of uptake (Figure 24 A ii), indicating that tryptophan and isoflurane are not interacting competitively. Possibilities for indirect inhibition have been also been explored. Tat2p is not internalized or degraded in response to isoflurane treatment (Figure 24 B iii), further narrowing the possible effects of anesthetics on Tat2p. Models of anesthetic action involving less Tat2p on the plasma membrane, or a decreased ability of Tat2p to bind tryptophan have been excluded by these studies.

Several possibilities for the effects of anesthetics on Tat2p remain. Isoflurane exposure may induce a post-translational modification of Tat2p that affects its ability to transport tryptophan. Tat2p has been suggested to be phosphorylated by the nutrient-responsive kinase Npr1 in response to starvation (Schmidt, Beck et al. 1998). However, this proposed phosphorylation leads to Tat2p degradation and, therefore, does not appear to be consistent with the effects of isoflurane on Tat2p. Phosphorylation of other sites on Tat2p or other post-translational modifications remain a possibility. For example, other yeast amino acid permeases undergo reversible palmitoylation and Tat2p has a candidate recognition sequence for palmitoylation enzymes (Roth, Wan et al. 2006). A test for
Figure 24. Models for the involvement of Tat2p in anesthetic response. A. Direct inhibition of Tat2p activity by isoflurane by competitive binding at the tryptophan binding site (ii) or binding elsewhere (iii). B. Indirect inhibition of Tat2p activity by isoflurane due to induction of post-translational modification in combination with internalization (ii), followed by degradation (iii) or post-translational modification alone (iv). Indirect inhibition of Tat2p by disruption of lipid rafts (v). C. Tat2p as an exporter of isoflurane (ii). Modified from (Palmer, Wolfe et al. 2002)
altered post-translational modification was attempted using isoelectric focusing, a method that allows detection of any charged modification. These preliminary attempts were inconclusive as only a smeared Tat2p signal was observed following deglycosylation (data not shown). Future attempts at this method could include alteration of protein extraction conditions or ampholyte reagents. Alternatively, mass spectometry of purified Tat2p from cultures differentially labeled with isotopes would likely provide a more sensitive and quantitative method of detecting alterations in post-translational modification, including those that do not affect charge (Goshe and Smith 2003).

Alternatively, isoflurane may inhibit Tat2p either by binding directly to the protein or inserting at the lipid-protein interface or in the plasma membrane. Anesthetics are used clinically at plasma concentrations in the low millimolar range (Eckenhoff 2001). The high active plasma concentration of anesthetics suggests that their net binding energies are small (Trudell and Bertaccini 2002). Many proteins have pre-existing cavities adequately sized for binding anesthetics (Eckenhoff 2001). Membrane proteins tend to have larger cavities, often found between transmembrane helices. Anesthetic binding to these hydrophobic cavities are proposed to be low energy events (Eckenhoff 2001). One model of anesthetic action involves the anesthetic-induced stabilization of cavity-containing conformations of protein targets (Eckenhoff 2001). If the cavity-containing conformation has a different activity than other conformations, anesthetics may alter the equilibrium and therefore the activity by binding. This model remains a possibility for the interaction of isoflurane and Tat2p.
As mentioned in Chapter One, it is difficult to distinguish between the alteration of a membrane protein activity by direct binding to the protein or by inserting into the surrounding membrane. One membrane-based hypothesis that can be tested is whether anesthetics inhibit Tat2p by disrupting the organization of lipid rafts. Stomatin, a protein that is located in lipid rafts (Sedensky, Siefker et al. 2004), has been suggested to be important for lipid raft organization and was shown to be important in determining anesthetic sensitivity in C. elegans (Sedensky, Siefker et al. 2001). Tat2p also has been shown to associate with lipid rafts (Grossmann, Opekarova et al. 2007). Lipid rafts in yeast are areas of the membrane that are detergent-resistant and rich in ergosterol, the yeast equivalent of cholesterol (Umebayashi and Nakano 2003). The activity of membrane proteins depends upon properties of the surrounding lipids. More specifically, lipid-raft-associated proteins can be inhibited by drugs that disrupt the rafts (Morrow and Parton 2005). The association of Tat2p with lipid rafts could be tested based on the buoyancy and detergent-insolubility of these membrane regions (Kubler, Dohlman et al. 1996). While the relative level of Tat2p in various cellular membranes has already been analyzed, the protocol used was not designed to separate detergent-resistant membranes from the surrounding plasma membrane, as the buffer contained no detergent (Chang 2002). A change in the proportion of Tat2p localized in lipid rafts in the presence of anesthetics would provide a potential explanation for the anesthetic-induced inhibition of the activity of Tat2p.
Potential relationship of isoflurane-induced inhibition of tryptophan uptake and cell division

Several lines of evidence link the isoflurane-induced inhibition of nutrient uptake to the inhibition of yeast cell division. Previous work from our laboratory showed that for critical amino acids, increased amino acid permease levels, increased amino acid levels, and amino acid prototrophy all increase anesthetic resistance using growth as an endpoint (Palmer, Wolfe et al. 2002). Evidence from the results reported here, combined with previous findings, provides additional links between nutrient availability and growth during anesthetic exposure. Within a narrow range of anesthetic concentration, yeast growth goes from being unaffected to completely inhibited (Figure 3). Here we show that the dose dependence of amino acid uptake inhibition has a similar sharp response. Between 5.5% and 8.5% isoflurane, the inhibition of amino acid uptake increases markedly (Figure 5B). Inhibition of both amino acid import and of growth has the appearance of a threshold effect. Additionally, the onset of cell division arrest (Figure 3) and tryptophan uptake inhibition (Figure 5) both occur within 15 minutes of isoflurane exposure. Finally, both growth arrest and tryptophan uptake inhibition are readily reversible upon removal of cells from the anesthetics (Figure 3E and 8). Because the inhibition of amino acid import leads to amino acid starvation, and starvation leads to cell-cycle arrest, the isoflurane-induced growth inhibition appears to be due to amino acid permease inhibition.

It was previously shown that increased tryptophan in growth medium increases anesthetic resistance as measured by cell growth. Here we show that increased
tryptophan does not alleviate isoflurane-induced inhibition of tryptophan import. At first glance this may seem contradictory, but it is not. This is because it does not appear to be the magnitude of the inhibition of tryptophan import that dictates the ability of a yeast cell to grow in anesthetic, but instead the total amount of tryptophan imported during anesthetic exposure. Because the amount of this amino acid that is imported increases as tryptophan concentration increases, despite the presence of isoflurane, cells are able to grow in concentrations of isoflurane that would otherwise be inhibitory to growth. These data provide a more detailed understanding of the previous finding that increased tryptophan concentrations increase anesthetic resistance. Because Tat2p is the only tryptophan transporter in the strain used, these findings provide a correlation between anesthetic resistance in high-tryptophan media and an effect of isoflurane on Tat2p. Other evidence linking this permease to the ability of yeast cells to grow in anesthetic comes from finding that mutations in TAT2 that lead to increased tryptophan uptake during isoflurane exposure also lead to isoflurane resistance (see Chapter 4).

**Tat2p as a model of anesthetic effects on transport**

Inhibition of yeast growth was the anesthetic endpoint during the characterization of yeast as a model for the study of anesthetics (Figure 3). There are several studies involving yeast and anesthetics that have been done using growth inhibition as an anesthetic endpoint, but have not been done using tryptophan transport inhibition as the endpoint. Further characterization of Tat2p as a model of anesthetic effects is merited. One set of experiments that will broaden the understanding of Tat2p involves examining
the import of tryptophan in the presence of a variety of anesthetics. Several anesthetics have been shown to inhibit yeast growth [Figure 3A and (Keil, Wolfe et al. 1996)], but have not been tested for the ability to inhibit tryptophan import. Additivity of multiple anesthetics as inhibitors of yeast growth has also been demonstrated [Figure 3B and (Wolfe, Hester et al. 1998)]. Similar studies using tryptophan uptake inhibition rather than growth inhibition would provide further validation of Tat2p as a model. Another area that remains to be explored is the effect of nonimmobilizers on Tat2p activity. Early characterization of yeast as a model for the study of anesthetics involved the determination of the effects of nonimmobilizers on yeast growth (Wolfe, Hester et al. 1998). Nonimmobilizers were found not to inhibit yeast growth, either alone or in combination with near-inhibitory concentrations of anesthetics [Figure 3C and (Wolfe, Hester et al. 1998)], as previously mentioned. Nonimmobilizers have also been found not to affect the activity of several anesthetic-sensitive neurotransmitter receptors (Koblin 2005). Because nonimmobilizers do not inhibit yeast growth, it is expected that they would not inhibit tryptophan transport. Finding that nonimmobilizers do not inhibit tryptophan transport would mean the effect of anesthetics on Tat2p is specific among volatile lipophilic compounds. These studies would also provide additional experimental support for the link between the two anesthetic endpoints in yeast, inhibition of growth and tryptophan uptake.

The mutations within Tat2p that render cells resistant to anesthetics hold the potential for further studies as well. Work in other organisms shows that some mutations affect sensitivity to all anesthetics tested, while others affect sensitivity to only a subset
(Campbell and Nash 1994; Morgan and Sedensky 1994). All isoflurane-resistant mutations within TAT2 appear to be resistant to halothane as well (Figure 14). It would be of interest to further the test the cross-resistance to a more chemically diverse set of anesthetics such as xenon, cyclopropane, and sevoflurane. If isoflurane-resistant TAT2 mutants were found not to be cross resistant to every volatile anesthetic, the findings from other organisms would be extended into yeast and would support the idea that different anesthetics can have different targets.

**Isoflurane treatment differs from other Tat2p-mediated stresses**

Tryptophan uptake has been called the “Achilles’s heel” in yeast due to its prominent role in the response to a wide array of stress conditions (Abe and Iida 2003). Specifically, the tryptophan transporter Tat2p plays a role in the response to a number of drugs and stresses. Exposure to rapamycin (Schmidt, Beck et al. 1998) 4-phenylbutyrate (Liu, Brusilow et al. 2004), zaragozic acid (Daicho, Maruyama et al. 2007), starvation (Beck, Schmidt et al. 1999), or elevated pressure (Abe and Horikoshi 2000; Abe and Iida 2003) induces ubiquitin-dependent internalization and vacuolar degradation. In contrast, isoflurane induces neither internalization nor degradation of Tat2p. In fact, after 2 hours of isoflurane exposure when tryptophan uptake is inhibited almost 80% levels of Tat2p increase slightly (Figure 11) and no relocalization is evident (Figures 12 and 13). It is interesting that while amino acid starvation and treatment with isoflurane all induce the general amino acid control (GAAC) starvation response and cell cycle arrest (Beck, Schmidt et al. 1999; Hinnebusch and Natarajan 2002; Cherkasova and Hinnebusch 2003;
Palmer, Shoemaker et al. 2005), Tat2p levels increase during isoflurane exposure but
decrease when tryptophan is removed from the growth medium of tryptophan auxotrophs
(Schmidt, Beck et al. 1998; Beck, Schmidt et al. 1999). This indicates that Tat2p
degradation does not appear to be a general characteristic associated with the starvation-
induced cell cycle arrest as has been previously suggested (Schmidt, Beck et al. 1998).

Like isoflurane, the immunosuppressant FTY720 inhibits Tat2p activity in yeast
without inducing Tat2p degradation (Welsch, Hagiwara et al. 2003). However, in contrast
to isoflurane (Palmer, Wolfe et al. 2002), increased tryptophan levels in the growth
medium do not decrease sensitivity to FTY720 (Welsch, Hagiwara et al. 2003).

**Volatile anesthetics affect the functions of diverse proteins**

As mentioned previously (Chapter 1), the activities of many proteins are affected
by volatile anesthetics. In some cases, such as the GABA<sub>A</sub> (Pittson, Himmel et al. 2004)
or glycine receptor (Yamakura, Bertaccini et al. 2001), anesthetics potentiate receptor
activity leading to enhancement of protein function rather than inhibition as found with
Tat2p. In other cases volatile anesthetics have been shown to act as competitive
inhibitors. Enflurane competitively inhibits the uptake of serotonin by synaptosomes
(Martin, Adams et al. 1990). Recent studies show that certain anesthetics competitively
inhibit the binding of glycine to the N-methyl-D-aspartate receptor (Dickinson 2007).
Volatile agents isoflurane and sevoflurane reduce the affinity of nicotinic acetylcholine
receptors in the brain for agonist in a manner consistent with competitive inhibition
(Rada, Tharakan et al. 2003). A well-characterized example of a protein inhibited by
anesthetics is the soluble light-producing enzyme firefly luciferase. The inhibition of
firefly luciferase was found to be largely competitive in nature, with anesthetics
competing for the binding of the substrate luciferin (Franks 1984). This led the authors to
pose the question, “Do general anaesthetics act by competitive binding to specific
receptors?” (Franks 1984). Upon further characterization it was shown that two
anesthetic molecules bind luciferase, one of which inhibits competitively and another that
contributes to inhibition by a mechanism that is not competitive (Franks, Jenkins et al.
1998). Increased levels of tryptophan do not overcome isoflurane-induced inhibition of
tryptophan uptake, suggesting that inhibition of Tat2p is not competitive. So while the
literature is dominated by proteins found to be competitively inhibited by anesthetics,
competitive inhibition is not the only means by which anesthetics can affect the function
of a protein.

**Tat2p overexpression does not affect inhibition by isoflurane**

As mentioned previously, a classical genetic approach to drug target discovery is
based on the idea that drug resistance is induced by overexpression of a drug target while
drug sensitivity is induced by decreased expression (Hughes 2002). *TAT2* on a multi-
copy plasmid leads to isoflurane resistance while deletion leads to super-sensitivity
[increased sensitivity relative to wild-type control (Palmer, Wolfe et al. 2002)],
demonstrating that Tat2p conforms to the expectations of a drug target. A simplistic
model for this is that when the target level is increased, a higher concentration of the drug
is required to inhibit the increased number of targets and less drug is needed when less
target is present. This model is not consistent with our data. The increased levels of Tat2p caused by multi-copy plasmid expression lead to increased tryptophan uptake, but this uptake is still inhibited by isoflurane to the same degree as Tat2p expressed from a single-copy plasmid (Figure 7). Finding that Tat2p, when overexpressed, is subject to the same degree of inhibition by isoflurane suggests that changing the Tat2p levels 2.5-fold is negligible with respect to the number of isoflurane molecules. That is, isoflurane is not limiting when Tat2p is overexpressed. Based on a rough calculation, there are approximately $5 \times 10^8$ molecules of isoflurane per Tat2p (see below). Considering the vast excess of isoflurane, it is not surprising that overexpression of Tatp2 has no effect on the ability of this anesthetic to inhibit tryptophan uptake.

# Tat2p molecules per mL:

750 copies Tat2p / cell$^a$ * 2$^b$ * $5 \times 10^6$ cells / mL$^c$ = $7.5 \times 10^9$ Tat2p / mL

# Isoflurane molecules per mL:

195 µL isoflurane$^c$ / 250 mL bottle$^c$ * 1.495 g isoflurane / 1000µL * 1 mole / 184.5 g isoflurane * $6.022 \times 10^{23}$ molecules/mole = $3.8 \times 10^{18}$ molecules/mL

d Isoflurane molecules per Tat2p ≈ $5 \times 10^8$

$^a$ Quantitation data were obtained from the [www.yeastgfp.ucsf.edu](http://www.yeastgfp.ucsf.edu), a website that contains supplemental data from (Ghaemmaghami, Huh et al. 2003).

$^b$ Tat2p levels are approximately double in strain P2684, in which $TAT1$ is deleted, relative to a strain containing $TAT1$.

$^c$ Values for number of cells per mL and concentration of isoflurane represent our standard experimental conditions.

$^d$ This simplified calculation does not take into account the partition of anesthetic into the cellular membrane, which is approximately 50 – 150, depending on the lipid contents of the model bilayer (Dickinson, Franks et al. 1994).
Mutations implicate four important helices in Tat2p

Volatile anesthetics provide a novel tool for the study of amino acid permeases in yeast (Palmer, Wolfe et al. 2002). In this work, molecular genetic approaches were used to isolate and characterize mutations in the \textit{TAT2} gene that alter amino acid transport. These studies provide further understanding of the functional elements within Tat2p. Specifically, the mutations isolated cluster in transmembrane helical regions that align with the predicted cargo-binding regions in LeuT\textsubscript{Aa}, providing experimental support for the structural homology model of Tat2p. Amino acid uptake analysis reveals that isoflurane-resistant mutations can 1) increase amino acid transport by Tat2p, 2) decrease the inhibitory effect of isoflurane on Tat2p activity, or 3) affect both parameters.

Based on the Tat2p structural homology model, we find that mutations leading to volatile anesthetic resistance are located in clusters surrounding the proposed tryptophan and cation binding sites, as well as in nearby helices that buttress the binding site during conformational changes necessary for transport. The mutations obtained suggest important tryptophan transport domains of Tat2p are located in the portions of helices 1 and 6 nearest the cytoplasm while helices 3 and 8 play an important role in tryptophan transport during anesthetic exposure (data summarized in Table 6). The structure of LeuT\textsubscript{Aa}, the basis of the homology model of Tat2p, suggests helices 1 and 6 have unwound portions, breaking each helix into two segments (Yamashita, Singh et al. 2005). Together these 4 helical segments form the amino acid binding pocket (Yamashita, Singh et al. 2005). Six of the ten mutations arising from random mutagenesis cluster in these key helical regions. Helices 3 and 8 are also implicated in ion and amino acid binding in
the LeuT\textsubscript{Aa} structure (Yamashita, Singh et al. 2005). Two of the mutations isolated in the work reported here are found in these helices. Helix 3 has been previously implicated as being critical by mutagenic studies of Gnp1p, Can1p, and AroP as discussed in the introduction of this chapter (Cosgriff and Pittard 1997; Regenberg and Kielland-Brandt 2001). It is striking that the mutations leading to increased uptake of tryptophan concentrate in helices implicated by the proposed structural homology model to have key roles in transport (Kanner 2005).

**Isoflurane-refractory mutants provide insight into regions of Tat2p critical for anesthetic action**

Some of the mutations in a region necessary for a drug to affect its protein target can be expected to decrease the ability of the drug to cause the expected alteration of protein activity. The mutants that are refractory to the effects of isoflurane, that is, mutants in which uptake is less inhibited by isoflurane, provide candidate regions whose structure and function are affected by presence of isoflurane (S95G/N, W171C, I285T, M287I, V368A). Two striking isoflurane-refractory mutants are W171C (helix 3) and V368A (helix 8). In the proposed structure of Tat2p, helices 1-5 and 6-10 are related by a pseudo two-fold axis of symmetry in the plane of the membrane (Yamashita, Singh et al. 2005). Helices 3 and 8 are both elongated and tilted relative to the other helices, occupy equivalent positions in the symmetrical pair, and are proposed to participate in cargo and cation binding (Yamashita, Singh et al. 2005). It is interesting that these two
functionally relevant and symmetrically related helices contain mutations with similar characteristics (summarized in Table 6).

One possible explanation for the most isoflurane-refractory mutant V368A comes from the hypothesis that volatile anesthetics act by altering lateral pressure within the lipid bilayer (Cantor 1997). This physical alteration of the membrane is postulated to induce anesthesia by altering the equilibrium between membrane protein conformers having different levels of activity. Because β-branched amino acids are proposed to destabilize alpha helices (Deber, Khan et al. 1993), the mutation of the β-branched valine 368 to alanine, which has a high alpha helical propensity, would be expected to increase the rigidity of helix 8 (Deber, Khan et al. 1993). The hypoactivity of this mutant suggests this helix stabilization may make the conformational change necessary to transport tryptophan more difficult. However, decreased inhibition by isoflurane may result from increased resistance to the structural changes caused by lateral pressure inflicted by the presence of isoflurane.

Hypoactive mutants provide potential insight into Tat2p regulation

All mutations leading to decreased tryptophan uptake in the absence of isoflurane also lead to an increase in steady state levels of Tat2p (Figure 17 and Figure 19C). Because all hypoactive mutants have a greater than 2-fold increase in Tat2p levels (Figures 17 and 19C), it is possible there is a link between Tat2p hypoactivity and increased Tat2p levels. It is possible that a sensor recognizes the decreased intracellular tryptophan and produces a signal that leads to increased Tat2p levels.
Three lines of evidence presented here suggest a link between intracellular tryptophan levels and Tat2p levels (Figure 24). First, deletion of TAT1, which encodes the low-affinity tryptophan transporter, leads to increased steady state levels of Tat2p (data not shown). Second, mutations within TAT2 that decrease tryptophan uptake lead to increased levels of Tat2p. Third, the isoflurane-induced inhibition of tryptophan uptake leads to slightly increased levels of Tat2p. Another way to inhibit tryptophan uptake is incubation with the immunosuppressive drug FTY720, and this also leads to an increase in Tat2p levels (Welsch, Hagiwara et al. 2003). Together, these data suggest that yeast cells sense a decrease in intracellular tryptophan levels and respond by regulating Tat2p.

In experiments where yeast are incubated with tritiated tryptophan, the level of intracellular $^{3}$H label incorporated into protein has been measured in the presence or absence of anesthetics. Following a 15-minute anesthetic exposure, the amount of label not incorporated into protein in the presence of anesthetics is 0.07 units as compared to 0.65 units in the absence of anesthetic (See Table 4; subtract TCA from tryptophan import; 1 unit is defined as the tryptophan import in the absence of anesthetic as described in Table 4 legend). Following a 120-minute exposure, isoflurane-treated cells have only 0.04 units of unincorporated tryptophan. These data indicate that intracellular levels of tritium label not incorporated into protein have decreased. Other methods such as ion exchange columns would be useful to more directly measure the levels of intracellular tryptophan not incorporated into protein, as some portion of tritium label may represent a tryptophan catabolite. Additionally, the amount of free tryptophan
should be measured in cells deleted for \textit{TAT1} or containing hypoactive mutant \textit{TAT2} to confirm that these conditions decrease intracellular tryptophan as predicted.

Yeast possess a sensor of extracellular amino acids called Ssy1p. Ssy1p is a member of the amino acid permease family, yet it has no apparent ability to transport amino acids (Iraqui, Vissers et al. 1999). Similar to sugar sensors in yeast, Ssy1p has unique features relative to actual transporters in its family. Two extended extracellular loops and a long N-terminus (Iraqui, Vissers et al. 1999) are proposed to allow Ssy1p to sense amino acids by binding them as a permease would. However rather than transporting the amino acids, Ssy1p undergoes a conformational change that signals to transcription factors inside the cell. In response to extracellular amino acids, Ssy1p modulates the transcription of amino acid transporters including Tat2p. More recent work suggests that Ssy1p also detects intracellular amino acid levels (Wu, Ottow et al. 2006). This sensing is proposed to occur by intracellular amino acids trapping Ssy1p in an inward-facing, non-signaling conformation, as opposed to the outward-facing conformation that is capable of sensing and responding to the presence of extracellular amino acids (Wu, Ottow et al. 2006). This competition between intra- and extra-cellular amino acids provides a potential explanation for the apparent correlation between intracellular tryptophan levels and Tat2p levels (Figure 25).
Figure 25. Intracellular tryptophan can be decreased by three means of impairing transport. Tryptophan transport is decreased when *TAT1* is deleted (A), when isoflurane inhibits Tat2p activity (B) or when a mutation within *TAT2* leads to hypoactive Tat2p activity (C). Higher intracellular tryptophan levels compete with extracellular tryptophan by trapping Ssy1p in an inward-facing non-signaling conformation (D, left). Lower intracellular tryptophan levels are proposed to allow the equilibrium of Ssy1p to shift towards the outward-facing conformation that signals the presence of extracellular tryptophan and induces the transcription of amino acid permease genes including *TAT2* (D, right).
To test this possibility, several mutant forms of Ssy1 may be informative. Mutants Ssy1$^{382R}$ or Ssy1$^{382K}$ confer constitutive signaling (Gaber, Ottow et al. 2003). If Ssy1 signaling is involved in the increase in Tat2p associated with the conditions listed above, then two expectations follow. The constitutive signaling in these mutants should increase the amount of Tat2p, and should cause them to be refractory to the increase in Tat2p involved with isoflurane treatment, deletion of $TAT1$, or mutation of $TAT2$ to hypoactive forms. Mutants Ssy1$^{382H}$ and Ssy1$^{382L}$ are not constitutive, but display an increased sensitivity to extracellular amino acids (Gaber, Ottow et al. 2003). These forms would be expected to have a decreased response to intracellular amino acids, and therefore show less increase in Tat2p levels.

In contrast, other groups have found that Tat2p is negatively regulated in response to tryptophan starvation (Beck, Schmidt et al. 1999) and various conditions that inhibit Tat2p (Schmidt, Beck et al. 1998; Abe and Horikoshi 2000; Liu, Brusilow et al. 2004; Daicho, Maruyama et al. 2007). One would expect decreased intracellular tryptophan levels in each of these cases. By the model proposed in Figure 25, this should decrease the proportion of Ssy1p trapped in the inward-facing, non-signaling conformation, leading to increased Ssy1 signaling and increased Tat2p levels. Instead, the opposite is true. To investigate this apparent contradiction, it would be useful to directly measure the free levels of tryptophan in cells from each condition. One possibility is that the magnitude of intracellular tryptophan depletion determines the cellular response, in which case one would expect to find more marked decreases of intracellular tryptophan...
levels in the conditions that cause Tat2p depletion. In this case, another pathway that is activated by more severe nutrient depletion would be expected to override Ssy1p signaling. This possibility is consistent with previous findings that removal of all tryptophan from growth medium is required for the starvation-induced down-regulation of Tat2p while decreasing the concentration of tryptophan 10-fold does not induce degradation (Beck, Schmidt et al. 1999).

**Hyperactive mutants shed light on Tat2p structure-function relationship**

Hyperactive mutants A91T, T295A and N330D and hypoactive mutant F101T respond to isoflurane in a manner similar to wild-type Tat2p, as indicated by wild-type levels of inhibition of tryptophan uptake during isoflurane exposure. These mutants, therefore, do not appear to provide direct clues about potential interactions between isoflurane and Tat2p. They do, however, provide important clues about Tat2p activity. For example, mutant N330D lies in the fourth extracellular loop, a region implicated by mutagenesis of Can1p to be important for substrate binding (Regenberg and Kielland-Brandt 2001). Tryptophan-binding studies using purified N330D Tat2p reconstituted in an artificial membrane system would allow further exploration of the role of this extracellular loop.

The increased uptake of tryptophan by three of the hyperactive isoflurane-resistant mutants (A91T, M287I, T295A) may be explained by significantly increased Tat2p levels. In each case, a significant increase in Tat2p levels accompanies the hyperactivity of tryptophan uptake (**Figures 17 and 19C**), although it should be noted that
subcellular localization of these mutant forms has not been examined. These mutants located in helices 1 and 6 provide novel potential sites of regulation of Tat2p levels. Some mutations (S95G, S95N, N330D) cause an increase in tryptophan uptake in normal growth conditions without an associated significant increase in permease levels. Mutant N330D, which was discussed above, transports twice as much tryptophan as the wild type despite being present at normal levels. Several explanations for these findings are possible including a change in the proportion of Tat2p at its active location on the plasma membrane, alterations of channel selectivity, or changes in cargo binding, translocation or release. Further study is necessary to elucidate the reasons behind these hyperactive mutants.

**Mutations validate predictions of the Tat2p structural model**

Phenylalanine at position 101 of Tat2p is predicted to participate in stacking interactions with the tryptophan cargo based on its position in the transport channel, as predicted by the structural homology model. Mutation to methionine or isoleucine removes the potential for stacking interactions and each of these appears to decrease tryptophan transport by Tat2p based on isoflurane phenotype and the ability to lose the \(TAT1\) plasmid (Figure 23). Mutation to threonine also removes stacking potential, and tryptophan uptake analysis reveals Tat2p hypoactivity. Mutation to tyrosine also appears to decrease Tat2p function. While tyrosine is capable of stacking interactions, the addition of the hydroxyl group makes this a polar residue, which may interfere with the proposed stacking interaction with tryptophan. On the other hand, assays of a F101W
mutant indicate a potential increase in Tat2p activity. Substitution of tryptophan for phenylalanine preserves the proposed stacking capability at this position. Collectively, these mutants are consistent with the idea that the residue at position 101 participates in stacking interaction with the cargo. Transport of tryptophan in the F101 mutants, needs to be measured directly to further test this possibility.

Based on the structural homology model of Tat2p, the glutamic acid residue at position 286 is proposed to participate in hydrogen bonding and charge stabilization with the cargo tryptophan (Keasey, De Cotiis et al. 2007). Mutant E286Q still has the ability to participate in hydrogen bonding but not in charge stabilization. Since this mutant is unable to support growth of a tryptophan auxotroph in the absence of Tat1p, further analysis of this mutant would be done in a strain containing TAT1.

**Cell-free assays hold potential to further investigate Tat2p involvement in anesthetic response**

The analysis of tryptophan import by purified Tat2p reconstituted into artificial membranes would provide additional information regarding the potential interaction of Tat2p and anesthetics. Finding that purified Tat2p is inhibited by anesthetics in a cell-free assay would rule out several possibilities presented in Figure 24. For example, one model that currently remains a possibility is that anesthetics induce a post-translational modification of Tat2p that alters its activity (Figure 24). The enzyme(s) necessary to modify Tat2p would be absent in the artificial membrane experiment, meaning that if anesthetics inhibit Tat2p in this system, alterations in post-translational modification are
not necessary for the inhibition to occur. Additionally, this cell-free system would further permit the in-depth study of the kinetic parameters of anesthetic inhibition of Tat2p. Advanced techniques involving rapid mixing and flux measurements can in some cases even distinguish between lipid-mediated effects and specific protein-binding effects (Wood, Tonner et al. 1995). These studies could provide support for either a lipid- or protein-based model for the inhibition of Tat2p by anesthetics.

One potential critique of yeast as a model for the study of volatile anesthetics is the high concentration required to inhibit yeast growth compared to that which is routinely used in a clinical setting (Figure 3). There is a debate in the field of anesthetic research regarding the validity of using “clinically relevant” concentrations as a cutoff for the study of anesthetic actions (Eckenhoff and Johansson 1999; Eckenhoff and Johansson 2001; Eger, Fisher et al. 2001). Not all researchers agree that the use of concentrations in excess of those used in standard surgical settings invalidates a model. This issue could be addressed by cell-free Tat2p assays. Because yeast is a free-living organism in nature, incapable of controlling its environment, it has been suggested that yeast has evolved defenses that make it relatively resistant to many compounds (Jungwirth and Kuchler 2006). Finding that in the absence of these defenses, the activity of Tat2p can be affected at lower concentrations than is necessary to inhibit cell growth would address the “clinically relevant” concentration concern.
Conclusions

The data presented here combined with previous gene dosage findings are consistent with Tat2p being a primary target of volatile anesthetic action in yeast. During anesthetic exposure, Tat2p activity is decreased in a time- and dose-dependent manner. This inhibition occurs without degradation or relocalization of Tat2p. The timing of Tat2p inhibition obeys expectations of anesthetic targets in that it is rapid and reversible. Additionally, mutations within \textit{TAT2} that increase tryptophan uptake during anesthetic exposure lead to anesthetic resistance.

Transporters of neurotransmitters and their precursors are involved in several steps of transport, each of which is required for appropriate neurotransmission (Figure 26). Neurotransmitter precursors, which can be amino acids, must be transported into the pre-synaptic cell where they are converted into neurotransmitters. Neurotransmitters, which can also be amino acids, are then packaged into vesicles. This is another step that requires specific transporters. Upon release into the synapse, neurotransmitters activate the appropriate receptor, transducing the excitatory or inhibitory signal. Once in the synapse, transporters located either in pre- or post-synaptic neurons or glial cells are responsible for clearing neurotransmitters from the synaptic cleft. An effect of anesthetics on any step in this process has the potential to alter neurotransmission. Many neurotransmitter transporter proteins have a similar topology as Tat2p, having 12 transmembrane-spanning domains. These proteins also share functional similarities with Tat2p, as they are responsible for the cation-dependent high-affinity uptake of amino acids such as glycine or derivatives of amino acids such as serotonin against a
concentration gradient. Several neurotransmitter transporters expressed in the mammalian central nervous system have been modeled from LeuTₐₐ, the same protein that allowed structural modeling of Tat2p. This facilitates the transfer of findings from Tat2p in yeast into mammalian proteins with a potential role in anesthesia.

Figure 26. Multiple steps involving transport are necessary for appropriate neurotransmission. Precursors are transported into presynaptic cells where they are modified to become neurotransmitters, which must then be loaded into vesicles. After release into the synapse, transporters on neuronal or glial cells must remove neurotransmitters from the synaptic cleft. Modified from (Masson, Sagne et al. 1999)
REFERENCES


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