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# STRUCTURAL CHARACTERIZATION OF PEPTIDE FRAGMENTS DERIVED FROM THE SEQUENCE OF CYTOCHROME *b*<sub>5</sub>

A Thesis in

Chemistry

by

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#### ABSTRACT

Rat microsomal cytochrome  $b_5$  is a membrane-bound hemoprotein consisting of two domains: an N-terminal heme binding region and C-terminal hydrophobic membrane anchor. Removal of the hydrophobic region results in an excised heme-binding domain of approximately 100 residues (referred to in this document as "cyt  $b_5$ "), the solubility properties of which make it suitable for spectroscopic study in aqueous solutions. Cyt  $b_5$ can be further sub-divided into residues responsible for the formation of two hydrophobic cores. The first of these cores (core 1) forms the contacts between the protein and bound heme and loses most of its secondary structure in the apoprotein state. The second hydrophobic core (core 2) is responsible for stabilization of a scaffold that retains its secondary and tertiary structure in the apoprotein state.

The secondary structure of the holoprotein is described sequentially by  $\beta$ 1-H1- $\beta$ 4- $\beta$ 3-H2-H3- $\beta$ 5-H4-H5- $\beta$ 2-H6. This is the  $\alpha$ - $\beta$  topology that defines the cyt  $b_5$  fold. In this study, the role of local interactions in the behavior of apo- and holocyt  $b_5$  was investigated using peptide fragments. Each fragment was designed to preserve the ability to sample the secondary structure of the native holoprotein state, while eliminating conformational restraints arising from higher order structure in the protein. In this way, the effect of sequence on structural propensity in both the presence and absence of heme coud be examined.

One of the elements of secondary structure of interest was the C-terminal  $3_{10}$  helix, H6. The sequence of this helix starts with HPDD and, with respect to the *cis-trans* isomerization of the Xxx-Pro bond, contains a potentially catalytic, positively-charged imidazolium side chain (His80). In the apoprotein, H6 fluctuates between two states.

The conformational exchange is slow on the NMR chemical shift time scale, The peptidyl-prolyl bond of the model peptide for helix H6 (H6P-1) was found by NMR spectroscopy to isomerize at rates inconsistent with the fluctuations observed in this region of the intact apoprotein,. This led to the conclusion that a *cis-trans* isomerization event did not account for the minor conformer observed in apocyt  $b_5$ . NMR data demonstrated that the peptide sampled a native-like conformation including an N-capping interaction between His80 and Asp82, implicating it as a possible nucleation site for protein folding.

In the apoprotein, the region binding the heme (H2–H5) is partially disordered. Peptide models of the heme binding loop and its variants were examined by NMR and CD spectroscopy in the presence of TFE to determine the relative helical propensity of each region (H2–H5). CD and NMR in TFE solutions demonstrated that H2 and H3 contained small, but significant helical propensity and sequence-encoding for the intervening turn. The H4 region of the binding loop proved to have the least helical propensity of the four regions. CD experiments showed that replacement of Asp60 with an Arg residue acted to stabilize H4 through an electrostatic interaction (most likely with Glu56). Increased stability of the D60R holoprotein variant was consistent with the native-like stabilization provided by the putative side chain interaction. An Asn-to-Pro substitution at position 57 proved to have little effect on helical propensity of H4, indicating that the observed stability of the corresponding holoprotein variant was the result of complex interactions in the apo- and holoprotein states.

To model H4 and H5, covalently conjugated mesoheme-peptide compounds were synthesized. UV-visible spectroscopy revealed a strained bis-histidine coordination state in the constructs, but no appreciable helix stabilization was detected in the attached peptides. These results implied that the proximity and register of the loop termini imposed by the loop-core 2 contacts were crucial to the cofactor induced folding of core 1 in the protein. Theses studies offered insights into the relationship among local structural propensity, loop-scaffold contacts and heme binding in core 1, as well as a potential protein folding nucleation site in core 2.

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# LIST OF ABBREVIATIONS

Ala, A	alanine
Arg, R	arginine
Asn, N	asparagine
Asp, D	aspartic acid
BHC	binding loop heme conjugate
BLP	binding loop peptide
CD	circular dichroism
COSY	correlation spectroscopy
$\operatorname{cyt} b_5$	cytochrome $b_5$
DHFR	dihydrofolate reductase
DIPEA	diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethylsulfoxide
ESI	electrospray ionization
EtOH	ethanol
$f_{ m H}$	fraction helix
Fmoc	9-fluorenylmethoxycarbonyl
Gln, Q	glutamine
Glu, E	glutamic acid
Gly, G	glycine
H6P	helix 6 peptide
HATU	2-(7-aza-1Hydroxybenzotriazole-1-yl)-1,1,3,3-tetramethyluronium
	hexafluorophosphate
HBTU	2-(1Hydroxybenzotriazole-1-yl)-1,1,3,3-tetramethyluronium
IL'a II	hexafluorophosphate
HIS, H	hish norformen as liquid shremata graphy
HPLC	ingn performance inquid chromatography
lie, I	Isoleucine
Leu, L	
Lys, K	lysine
MALDI MII-	matrix-assisted laser desorption ionization
MHZ	meganertz
MKW	mean residual weighted
NHS	N-nydroxysuccinimidyl
NMK	nuclear magnetic resonance
NUE	nuclear overhauser effect
NUESY	nuclear overhauser effect spectroscopy
PAL-PEG-PS	polyamide linker - polyethylene glycol - polystyrene

PDB	protein data bank
Phe, F	phenylalanine
ppm	parts per million
Pro, P	proline
Ser, S	serine
tBu	t-butyl
TFA	trifluoroacetic acid
TFE	trifluoroethanol
Thr, T	threonine
TIPS	Triisopropylsilane
TOCSY	totally correlated spectroscopy
Trp, W	tryptophan
Tyr, Y	tyrosine
UV-vis	ultraviolet-visible
Val, V	valine
WATERGATE	water suppression by gradient-tailored excitation

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#### Chapter 1

### Introduction

### 1.1 Unfolded State Interactions in Proteins

It has been known for many decades that most proteins owe their function to a specific folded conformation. In a seminal 1973 paper, Anfinsen demonstrated that ribonuclease A reassumes a catalytically active fold after chemical denaturation.<sup>1</sup> Many water-soluble proteins have since been observed to share this ability to achieve a functional, folded conformation in vitro, demonstrating that the fold topology is encoded in the primary structure of these biopolymers. Recent years have seen a rapid increase in reporting of protein three-dimensional structures. In 2004 alone, over 5000 such structures were deposited into the protein data bank. In spite of the large number of known sequences and structures, a complete understanding of the way in which sequence governs the folding process has proven elusive. Investigators have made great strides toward correlating short amino acid sequences with specific local interactions.<sup>2,3</sup> some of which are found to be preserved in denatured proteins containing the same series of residues.<sup>4</sup> These locally coded interactions are likely to play a role early in the folding process by limiting the number of potential folding pathways, initiating formation of turns and helices, as well as positioning elements of secondary structure in proximity, thus promoting formation of tertiary interactions.

Over the last few decades, it has been recognized that the unfolded state of proteins generated via thermal or chemical means is not necessarily characterized by random backbone and side chain dihedral angles, but may harbor long-lived specific interactions. Experimental studies of residual structure in unfolded states have recently demonstrated that non-random features can influence folding and stability.<sup>4,5</sup> Furthermore, it has been shown that the disruption or enhancement of these interactions by amino acid replacements can be used to design samples with enhanced or compromised stability.<sup>6</sup>

Many proteins require association with one or more cofactors to achieve a properly folded, functional state. The heme group is one such agent. Its intrinsic hydrophobicity allows it to form strong non-specific and specific interactions with non-polar side chains, which, added to the ligation of the central iron and electrostatic interactions, can shape the binding site and have a profound influence on holoprotein structure. In some hemoproteins, heme removal results in rearrangement of the polypeptide chain ranging from increased fluctuations<sup>7</sup> or loss of tertiary structure<sup>8</sup> to near complete loss of secondary structure.<sup>9</sup> In the case of significant unfolding, close examination of the unstructured regions of the apoprotein suggests that native and non-native structural propensities may be biasing the distribution of assumed conformations. The rapid sampling of these locally folded states may play a role in adjusting the folding pathway and cofactor binding properties.

# **1.2** Structure of Cytochrome *b*<sub>5</sub>

Microsomal cytochrome  $b_5$  (cyt  $b_5$ ) is a globular, membrane-bound protein that holds a heme cofactor in a *bis*-histidine coordination state. Removal of the C-terminal membranebinding domain of cyt  $b_5$  yields a well-folded, water-soluble heme-binding domain approximately 100 residues in length.<sup>10,11</sup> The heme in the holoprotein is surrounded by a set of hydrophobic residues (core 1) supported by four short  $\alpha$ -helices (H2–H5), and  $\beta$  strands at the back of the binding site (Figure 1.1a), all of which form specific contacts with the bound cofactor. The remainder of cyt  $b_5$  consists of an irregular  $\beta$ -barrel against which two helices (H1 and H6) are packed; this region is stabilized by a second hydrophobic core (core 2). Holoprotein structures show that H6 contains a hydrogen bond between the N $\delta$  of the His80 side chain and the amide proton of Asp82, resulting in a depressed His pK<sub>a</sub> and a downfield shifted Asp82 NH resonance.<sup>12,13</sup>



**Figure 1.1**: (A) Ribbon diagram of trypsin-solubilized bovine holocytochrome  $b_5$  (Protein Data Bank entry 1cyo – crystal structure).<sup>14</sup> Helix H6 (labeled) is located farther than 20 Å from the heme iron. Hydrophobic cores 1 and 2 are indicated by dashed circles. Axial heme ligands His39 and His63 are also shown. (B) Ribbon diagram of rat microsomal apocytochrome  $b_5$  (1i8c – NMR average of 40 structures)<sup>9</sup> in an orientation similar to that of the holoprotein structure.

Heme removal from the water soluble domain of cyt  $b_5$  causes helices H3, H4, and much of H5 to become substantially disordered, effectively unfolding a large portion of core 1 (Figure 1.1b).<sup>9</sup> It is noteworthy that helices H3 and H4 are identified by IUPred,<sup>15,16</sup> a program trained to analyze sequences for their tendency to remain unstructured, as elements of the protein that are not likely to form stabilizing contacts.<sup>17</sup>

In both apo- and holoprotein solution structures, the terminal backbone atoms of the heme-binding region (Leu32 N and Thr73 C, Figure 2) are held approximately 12 Å apart by the

folded protein scaffold (Figure 2). Restraining the ends of a 42-residue disordered loop entails a large entropic contribution to the free energy of folding, estimated to be ~24 kJ/mol at room temperature using a simple Gaussian chain approximation. The influence of the heme binding loop on its supporting scaffold has been investigated by substitution for a flexible loop in photosystem I accessory protein E (PsaE).<sup>18</sup> Chemical and thermal denaturation of the chimeric protein suggests that the free energy cost of closing the H2–H5 loop in apocyt  $b_5$  is at the low end of the Gaussian chain estimate. The presence of residual structure in H2 and elsewhere may explain this lower value. An enthalpic contribution is also possible.



**Figure 1.2**: Ribbon diagrams demonstrating the proximity of residues 32 and 73 in (A) trypsinsolubilized bovine holocytochrome  $b_5$  (Protein Data Bank entry 1cyo – crystal structure)<sup>14</sup> and (B) rat microsomal apocytochrome  $b_5$  (1i8c – NMR average of 40 structures)<sup>9</sup> in an orientation similar to that of the holoprotein structure.

The effect of heme removal on core 2 is less pronounced. The irregular  $\beta$  barrel forming the hydrophobic core is not significantly altered in absence of the cofactor. In spite of the apparent lack of change in core 2, removing heme from the cyt  $b_5$  prompts H6 to interconvert slowly between at least two conformations.<sup>19</sup> The major conformer (present in ~99% abundance) has chemical shifts similar to those of the holoprotein, while the minor conformer contains NH resonances closer to random coil and a histidine residue with a  $pK_a$  expected of a freely-titrating imidazole side chain.<sup>20,21</sup>

# **1.3** Cytochrome *b*<sub>5</sub> Variants

Denaturation and heme transfer studies of both wild-type cyt  $b_5$  and variants designed to disrupt or reinforce elements of secondary structure have shown that heme affinity and apoprotein stability can be decoupled if the amino acid replacement is located in the unfolded heme binding loop. These replacements have been referred to as "Type I." (Figure 1.3). Two Type I variants with replacements in H4 were found to stabilize the holoprotein.<sup>22</sup> In one case, D60R, the effect was attributed to favorable electrostatic interactions in the folded holoprotein state. In the other case, N57P, the effect was rationalized with a reduction of entropy in the unfolded state. The disruption of H4 caused by the introduction of a proline appeared to be of little relative consequence to the native state.



**Figure 1.3**: A ribbon diagram of holocytochrome  $b_5$  (1CYO) with previously substituted residues circled. Asp60 and Asn57 are located in helix 4 within core 1. Substitution at either of these positions is termed a Type I variation. Pro81 and Ille12 are located in core2, and constitute Type II variations.

In contrast to Type I variations, core 2 alterations (referred to as "Type II" interactions) affected both apo- and holoprotein stability. An I12A variation was found to destabilize both apo- and holoproteins without perturbing fold specificity.<sup>23</sup> Replacement of helix-initiating Pro81 with alanine in H6 was shown to have a larger effect on the apoprotein than on the holoprotein.<sup>22</sup> Despite its location 20 Å from the heme iron, it appears that this particular replacement gives rise to compensating interactions when the heme is bound. This illustrates that energetic perturbations in H6 can be transmitted to the heme-binding region of the protein without significant rearrangement of core 2.

#### **1.4** Prediction of Helical Propensity Based on Sequence

Nearly one third of all residues in folded proteins adopt the conformation of an  $\alpha$ -helix or its short, tightly wound version, the 3<sub>10</sub> helix. The atomic determinants for these elements of secondary structure have been investigated since Pauling's proposal for the  $\alpha$  helix<sup>24</sup> and its experimental confirmation.<sup>25,26</sup> In 1988, a key step toward the elucidation of a stereochemical code for protein folding was made by Presta and Rose, who observed that helices are often defined by start and stop signals recognizable by examination of the primary structure.<sup>27</sup> The ends of helices in water-soluble proteins have been the subject of repeated in-depth inspection<sup>28-<sup>32</sup> since the description of these sequence signals and initial assessments of statistical preferences for certain amino acids at certain positions of helices.<sup>33,34</sup></sup>

As pointed out by Rose and coworkers, helix termini have particular compositional requirements caused in part by the interruption of regular backbone hydrogen bonding involving partners at *i* and *i*+4 ( $\alpha$  helix) or *i* and *i*+3 (3<sub>10</sub> helix). Typically, side chains within the helix provide the missing H-bond and acceptors at the N-terminus, whereas backbone amides act as donors at the C-terminus. Hydrophobic side chain–side chain interactions staple the terminal turns and provide further protection from the solvent. Extensive surveys of the detailed structure of helix termini have revealed several common N-capping motifs differing by the pattern of main chain–side chain interactions.<sup>32,35</sup>

The residues forming the start of a helix are referred to as N"-N'-(N-cap)-N1-N2-N3-.... The N-cap residue is defined as that preceding the helix proper; commonly, its backbone carbonyl is the acceptor for the backbone amide of N4 ( $\alpha$  helix) or N3 (3<sub>10</sub> helix), but the N-cap backbone dihedral angles deviate from average helical values.<sup>27</sup> Position N1 denotes the first helical residue (immediately following the N-cap) and N' designates the first extra-helical residue (immediately preceding the N-cap). The N-cap position has emerged as the most selective of all positions in both the  $\alpha$ - and 3<sub>10</sub> helices. Although N-cap motifs have been well characterized structurally, less is known about the role of the individual interactions within the N-cap motif in controlling thermodynamic properties of helices and their kinetics of folding.

# **1.5** Peptide Fragments

The subtle signatures of denatured-state interactions have prompted their investigation in peptide fragments, which can reveal local interactions, eliminate tertiary influences, and afford data sets of reduced complexity.<sup>6,36</sup> Peptide fragments can be used to inspect the behavior of loops and the tendencies they display under native conditions<sup>37</sup> For example, Padmanabhan and coworkers were able to correlate helical propensities in fragments of the phage 434 Cro protein with those observed in the protein itself.<sup>38</sup> Peptide fragments are particularly useful in the study of refolding processes<sup>39</sup> including ligand-induced refolding as it relates to the initial state of the in-vitro binding reaction.<sup>40</sup>

#### **1.6** Effect of Trifluoroethanol on Peptide Secondary Structure

Alcohol based cosolvents have found application in the study of protein folding as both denaturants and stabilizers. Perhaps the most widely used cosolvent for the study of helical propensities in peptides is TFE, which has been shown to stabilize helical structure in a sequence-dependent fashion.<sup>41</sup> The exact mechanism responsible for this stabilization is still unclear, but is generally accepted to be the result of both specific cosolvent-backbone interactions as well as alteration of bulk solvent properties. In spite of the debate over which interactions are responsible for its stabilizing effect, empirical evidence shows that TFE

preferentially promotes the formation of helix in regions of peptides predisposed to such structure.

The utility of TFE extends beyond the determination of inherent helical propensity in peptides. The nature of response to TFE addition can also yield information regarding which forces are primarily responsible for the helical tendencies observed. A concentration of 30% TFE by volume typically maximizes peptide backbone exclusion effects.<sup>42</sup> Any increase in helix content at TFE concentrations above this threshold are therefore commonly attributed to enhanced dipole or electrostatic interactions among side chains in response to the decreasing solvent dielectric constant. In this way, TFE titrations can elucidate not only helical propensity, but the nature of the stabilizing interactions in the peptide under study.

## **1.7** Peptide Fragments of Cytochrome *b*<sub>5</sub>

To understand better the role of local interactions in the fold stability of cyt  $b_5$  and its variants, peptide fragments were designed and synthesized. Analysis of the peptides using spectroscopic techniques afforded the opportunity to characterize local exchange processes and structural biases in the absence of tertiary interactions. The tendencies of each fragment were then compared to those of their representative region in cyt  $b_5$ .

The first set of samples was designed to investigate the nature of the H6 fluctuations observed in apocyt  $b_5$ . A set of three octamers comprising the N-cap motif from the protein were constructed and investigated. The goal of this study was two-fold: first, to determine whether the His80-Asp82 N-cap interaction was sampled in the absence of higher order interactions; and second, to determine the role of Pro81 isomerization (if any) in the fluctuation of this region of the apoprotein.

The second investigation probed a set of peptides representing all or part of the hemebinding loop from cyt  $b_5$  and its previously characterized variants. Analysis of the complete cyt  $b_5$  sequence with IUPred<sup>17</sup> and sequence fragments with AGADIR (this study) both suggest that the H3 and H4 regions of the binding loop are intrinsically more disordered than H2 and H5. Peptides corresponding to wild-type sequences were subjected to analysis under varying pH to determine pK<sub>a</sub> values of histidine side chains, as well as varying TFE concentrations to elucidate the helical propensities in segments of the binding loop. These results were used to draw conclusions regarding the contribution of each region to heme affinity and holoprotein stability. Similar studies were conducted on peptides corresponding to Type I variants previously studied.

The third investigation probed the effect of heme proximity on the helical propensities of H4 and H5 of cyt  $b_5$ . Covalent conjugation of ferric mesoheme propionates to peptides was performed to create samples capable of strong heme binding.<sup>43</sup> The effect of the nearby macrocycle on histidine pK<sub>a</sub> was determined, as well as peptide helical propensities when ligated to the mesoheme iron. The results were used to assess the relationship between cofactor binding and helix propensity in H4 and H5.

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#### Chapter 2

#### **Materials and Methods**

Portions of this chapter have been presented in the following publication:

Davis, RB and Lecomte, JTJ. A dynamic N-capping motif in cytochrome  $b_5$ : Evidence for a pH-controlled conformational switch. Proteins: Structure, Function and Bioinformatics. 2006; 63:336-348.

Two Dimensional Exchange Spectroscopy experiments were performed by JTJL

### 2.1 Chemicals

All chemicals were purchased from EMD Sciences, Sigma Aldrich or JT Baker unless otherwise noted. Chemicals were reagent grade, with the exception of HPLC solvents, which were HPLC grade. Fmoc-protected PAL-PEG-PS resin and HATU were purchased from Applied Biosystems. HBTU and N- $\alpha$ -Fmoc protected amino acids were purchased from NovaBiochem. Iron(III)-mesoporphyrin IX di-N-hydroxysuccinimidyl ester hydrochloride was obtained from Frontier Scientific. All reagents and solvents were used without further purification.

# 2.2 Peptide Samples

### 2.2.1 Peptide Synthesis

Each chapter of this report details the analysis of several peptide samples. The sequences of these analytes are reported in Figure 2.1. Chapter 3 details the analysis of three samples designed to probe the behavior of H6 in the apoprotein, chapter 4 concerns peptides modeling all or part of the apocyt  $b_5$  binding loop, and chapter 5 reports on

constructs designed to probe the behavior of core 1 in holocyt  $b_5$  by covalent attachment to iron (III) mesoporphyrin IX.

All peptides were synthesized manually using standard HBTU/HATU activation and Fmoc/tBu protection schemes.<sup>2</sup> PAL-PEG-PS resin was used, resulting in a C-terminally amidated samples. Fmoc removal was achieved with a solution of 20 % piperidine in dry DMF. After each deblocking step, the piperidine solution was examined by UV-visible spectroscopy to determine the success of the previous coupling. A sample deblock absorbance profile is shown in Figure 2.2.

Starting with the second amino acid coupling, unreacted amines were acylated after each coupling reaction using a freshly prepared solution of acetic anhydride (0.5 M) and 2,6-dimethylpyridine (0.5 M, Acros) in DMF. Unless otherwise noted, the peptide N-terminus was left uncapped in the completed peptide. The completed peptides were deprotected and removed from the resin by treatment with 95:2.5:2.5 TFA:TIPS:H<sub>2</sub>O (v:v:v) for 2 hours. Crude product was then precipitated in cold ether and collected by centrifugation.

H5 <sup>β2</sup> H6	70 80 90	TDARELSKTYIIGELHPDDRSKIAKPSETL	ELHPDDRS	ELHPDDRS	ELHADDRS	TDARELSKT		TDARELSKT	-DARELSKT	-DARELSKT	Q_	-DARELSET
H3 <sup>β5</sup> H4	- 90 - 20	ieevlreqaggdatenfedvghs1				I E E V L R E Q A G G D A T E N F E D V G H S I	ieevlreqagg	GDATENFEDVGHS1	GDATENFERVGHS1	GDATEPFEDVGHS1	EDVGHK1	GDATENFEDVGHK1
β4 β3 H2	20 30 40	SK STWVILHHKVYDLTKFLEEHPGGI				DDdda	P					
Structure (holo) $\frac{\beta 1}{\beta 1}$ H 1	Residue Number	Cytochrome <i>b</i> <sup>5</sup> DKDVKYYTLEEIQKHKD	Н6Р-1 <sup><i>а</i></sup>	H6P-2 <sup><i>a</i></sup>	Н6Р-З <sup>а</sup>	BLP-42 <sup>b</sup>	BLP-21N <sup>D</sup>	BLP-22C <sup>b</sup>	BLP-22C-D60R <sup>b</sup>	BLP-22C-N57P <sup>b</sup>	BLP-8 / BHC-8 <sup>c</sup>	P-22C' / BHC-22C' <sup>c</sup>

to the sequence. These samples are discussed in chapter 4. The sequences of BLP peptides are aligned below the The italicized 'H' in the H6P-3 peptide designates a D-histidine residue. (b) Binding loop peptides (BLP's) are labeled with the number of residues in the construct, the end of the binding loop sequence represented, and any alterations made protein sequence, with variations in grey. For example: BLP-22C-D60R represents the 22 C-terminal residues of the binding loop with a D60R substitution. (c) Sequences of the BLP constructs used in chapter five are shown. BHC-8 structure in the reduced holoprotein as determined by Banci et al.<sup>1</sup> Heme-ligating histidines are marked with an asterisk Figure 2.1: The sequence of the water soluble domain of rat microsomal cyt b<sub>5</sub>. Bars indicate elements of secondary (a) Samples H6P-1, H6P-2 and H6P-3 contain residues involved in initiating helix 6 and are discussed in chapter 3. was constructed using the BLP-8 peptide. BHC-22 was constructed using the BLP-22' peptide.



**Figure 2.2**: A sample histogram generated by measuring the UV-visible absorbance of deblock solutions during the synthesis of BLP-22C'. The sequence is presented in the order of the couplings from left-to-right, corresponding to the C to N direction.

# 2.2.2 Peptide Purification

Peptides were purified by gradient reverse-phase HPLC using a Waters model 600S controller and model 626 pump, a Phenomenex Luna C18 column ( $4.6 \times 250$ mm) or equivalent and a 0.1%TFA in acetonitrile/water gradient. Detection was accomplished using a Waters model 2487 dual wavelength detector operating at 214 nm and 260 nm. A small portion of the pooled eluent was re-injected to verify sample purity. The purified peptides were dried by rotary evaporation at 40 °C and re-dissolved in water with 0.1 % TFA. Samples were then desalted on a C18 column (Amersham) using water with 0.1 % TFA for loading and acetonitrile with 0.1 % TFA as an elutant. Purity of H6P samples was confirmed by 1D <sup>1</sup>H NMR spectroscopy. MALDI or ESI mass spectrometry of purified binding loop peptide (BLP) samples showed a single main peak corresponding to the expected m/z for each peptide. Expected and experimentally determined mass values are presented in Table 2.1.

Sample	Calculated Mass	Determined Mass
BLP-42	4599	4597.9
BLP-21N	2338	2297.0 <sup>a</sup>
BLP-22C	2378	2380.9
BLP-22C-D60R	2419	2419.1
BLP-22C-N57P	2363	2359.9
BHC-8	2507	2506.2
BHC-22C'	5512	5512.9

**Table 2.1**: Calculated and determined masses of BLP and BHC samples.

<sup>a</sup> Sample was analyzed by electrospray ionization mass spectrometry. The mass discrepancy is consistent with deamidation of the glutamine residue during ionization.

# 2.3 Peptide-Heme Conjugate Samples

#### 2.3.1 Peptide-Heme Conjugate Synthesis

Iron(III) mesoporphyrin IX was used in place of the natural substrate iron(III) protoporphyrin IX to eliminate potentially reactive vinyl groups from the synthetic scheme (Figure 2.3). All steps were carried out under a nitrogen atmosphere following the procedure similar to that first presented by Benson and coworkers.<sup>3</sup> DMF was dried over molecular sieves prior to use. A 25 mM solution of peptide (estimated by weight) was prepared in dry DMF (40  $\mu$ L). Diisopropylethylamine (DIPEA) (29 eq) was added to the peptide solution and mixed thoroughly to activate the lysine amino group on each peptide. Separately, an iron(III) mesoporphyrin IX chloride di-N-hydroxysuccinimidyl (NHS) ester solution of similar concentration was prepared (estimated by weight) in dry

DMF (40  $\mu$ L). The reaction was initiated by the addition of iron(III) mesoporphyrin IX di-NHS ester chloride solution (10  $\mu$ L, 0.25 eq) to the peptide/DIPEA solution, resulting in a red colored mixture. The mixture was incubated at 40 °C for 2 hrs, and then quenched by mixing with approximately 1 mL of 10 mM ammonium acetate buffer (pH 6.2).



**Figure 2.3**: Structures of (A) ferric protoporphyrin IX and (B) ferric mesoheme. The ferric proroporphyrin IX contains two vinyl groups at positions 2 and 4 of the macrocycle. Ferric mesoheme contains less reactive ethyl groups at these locations.

#### 2.3.2 Peptide-Heme Conjugate Purification

Peptide-heme conjugates were purified by injecting the quenched reaction mixture into a reverse-phase HPLC system using a Waters model 600S controller, model 626 pump and a Restek Viva C4 column ( $4.6 \times 50$  mm). A mobile phase gradient of aqueous 10 mM ammonium acetate and acetonitrile was used. Detection was accomplished using a Waters model 2487 dual wavelength detector operating at 214 nm and 380 nm. The purified samples were dried by rotary evaporation at 40 °C. Attempts to desalt the purified construct using Amersham C18 columns resulted in loss of product

because of irreversible binding to the hydrophobic stationary phase. To prevent loss of product, heme-peptide conjugates were initially desalted by HPLC using a C4 column and a water/acetonitrile gradient containing 0.1 % TFA. Subsequently, samples were desalted as needed by gel filtration (G25) prior to analysis. The identity of the purified, desalted BHC samples was confirmed by MALDI mass spectrometry (Table 2.1)

#### 2.4 NMR Experiments

#### 2.4.1 Sample Preparation

Unless otherwise noted, NMR samples were prepared at ~1-4 mM (estimated by weight) in 99.9 atom % D<sub>2</sub>O or in a mixture of H<sub>2</sub>O:D<sub>2</sub>O, 9:1 (v:v). The pH of samples was determined using a Beckman  $\phi$ -71 pH meter and glass electrode calibrated to appropriate reference buffers in H<sub>2</sub>O. pH adjustments were carried out with 0.1 M DCl or 0.1 M NaOD in D<sub>2</sub>O or H<sub>2</sub>O. TFE-containing samples were prepared by dissolution of the peptide in H<sub>2</sub>O (300 µL, pH 2.9), followed by addition of TFE(*d*<sub>3</sub>) or TFE(*d*<sub>2</sub>) (300 µL). TFE(*d*<sub>3</sub>) initially was used in sample preparation, but exchange between water and the labile deuteron of TFE(*d*<sub>3</sub>) resulted in multiple deuterium resonances. TFE(*d*<sub>2</sub>) did not suffer from this potential complication, and so was used in later experiments.

### 2.4.2 Instrumentation

Spectra were collected using a Bruker DRX-600 spectrometer operating at a proton frequency of 600.05 or 600.18 MHz. Data were processed either with XWIN-NMR (Bruker Biospin, Rheinstetten Germany) or NMRPipe.<sup>4</sup>

#### 2.4.3 **Two-Dimensional NMR Experiments**

2QF-COSY data were collected with a standard pulse sequence and presaturation of the water resonance as needed. TOCSY experiments utilized a relaxationcompensated DIPSI-2 pulse train for spin-locking.<sup>5</sup> TOCSY and NOESY data were collected with a WATERGATE sequence and 3-9-19 binomial pulse train or with presaturation of the water line.<sup>6</sup> Typical peptide acquisition and processing parameters were as follows: spectral widths were 6 kHz over 2048 complex points in the direct dimension and 6 kHz over 256 to 512 real points in the indirect dimension.

#### 2.4.4 pH Titrations

600  $\mu$ L samples of the peptides of interest were prepared at a concentration of ~2 mM in D<sub>2</sub>O. Samples were titrated from low to high pH using 1–2  $\mu$ L aliquots of 0.1 M NaOD in D<sub>2</sub>O. Small pH adjustments were also made using 1  $\mu$ L aliquots of 0.1 M DCl in D<sub>2</sub>O as needed. pH Readings were taken before and after NMR data collection using a Beckman  $\phi$ -71 pH meter and glass electrode calibrated to appropriate reference buffers in H<sub>2</sub>O. The latter pH value was used in data analysis. NFIT (Island Products, Galveston, TX) was used to fit the chemical shift data to a modified Henderson-Hasselbalch equation:

$$\delta = \delta_{\text{His}} - (\delta_{\text{His}+} - \delta_{\text{His}}) (10^{n(pKa-pH)} / [1 + 10^{n(pKa-pH)}])$$
(eq 2.1)
where  $\delta$  represents the chemical shift at any pH,  $\delta_{\text{His}}$  represents the shift of interest at the high pH limit and  $\delta_{\text{His}+}$  represents the shift of interest at the low pH limit; *n* is the Hill coefficient. The equation is valid when the neutral and protonated species are in fast exchange on the chemical shift time scale and yields an apparent pK<sub>a</sub> value corresponding to the pH at the midpoint of the transition.

All pH titrations were performed in  $D_2O$  and the pH is reported without correction for isotope effects. For the purposes of this study, it is assumed that any correction from the apparent pH in deuterated solutions (pH\*) to pD is cancelled out by decreased acidities of acids in  $D_2O$ ; this treatment is appropriate near neutrality.<sup>7</sup> Values of pH\* are therefore reported as pH in this work. The uncorrected pK<sub>a</sub> values are adequate for comparison within and across the peptides studied here.

## 2.4.5 Inversion Magnetization Transfer Experiments

Inversion transfer analysis was performed on samples in D<sub>2</sub>O with the pulse sequence  $(\pi/2)_{\pm x} - \Delta - (\pi/2)_{\pm x} - \tau - (\pi/2)_{\pm x\pm y}$  – acquisition<sup>8</sup> to determine the rate of *cistrans* proline isomerization in the peptides mimicking helix H6. These peptide contain the His-Pro sequence, and the intensity of the histidine C $\delta$ H resonance (a multiplet with small *J* values not interfering with the measurement) was followed. The carrier frequency was set on the *trans* isomer resonance; the delay  $\Delta$ , corresponding to  $(2|v_{cis} - v_{trans}|)^{-1}$  where v represents the frequency of the resonance in Hz, varied between 7 ms and 50 ms depending on the peptide and the pH;  $\Delta$  was negligible compared to T<sub>1</sub>. The transfer time  $\tau$  was set to values ranging from 20 ms to 20 s; a total number of points in excess of 30 were collected. To achieve good resolution, the spectral width was limited to 2600 Hz. Digital filtering eliminated the rest of the spectrum without generating any artifacts. Magnetization transfer is observed provided that isomer lifetimes are sufficiently short compared to the spin-lattice relaxation times. For the peptides considered here, it was necessary to perform the experiments at 313 K to bring the exchange rate into a suitable range. The measurements were performed at pH 3.3 and 8.2.

Within a two-state framework, the interconversion between *cis*- and *trans*-isomers is represented with the first order rate constants  $k_1$  and  $k_{-1}$ :

$$cis \xrightarrow{k_1} trans$$
 (eq 2.2)

The apparent equilibrium constant ( $K_{eq}$ ) was determined by simulation of the *cis* and *trans* His C $\delta$ H peaks in one-dimensional spectra. DMFit software<sup>9</sup> was used to deconvolute spectra in which resonances partially overlapped.  $K_{eq}$  for the *cis*-to-*trans* equilibrium was calculated by the equation:

$$K_{eq} = A_t/A_c = [t]/[c] = k_1/k_{-1}$$
 (eq 2.3)

where  $A_t$  and  $A_c$  are the measured areas of the *trans* and *cis* isomers, respectively, and [t] and [c] represent the sum of the concentrations of all *trans* species and all *cis* species, respectively, regardless of the state of protonation of the various functional groups.

Areas and chemical shifts for an individual resonance contain contributions from all species in fast exchange with each other.

Within the two-state framework of Equation (2), and ignoring NOE contributions, the modified Bloch equations describing the time course of the magnetization<sup>10,11</sup> have the solutions

$$\mathbf{M}_{\mathbf{Z}}^{\mathbf{c}} = \mathbf{A}\mathbf{e}^{\lambda_{+}t} + \mathbf{B}\mathbf{e}^{\lambda_{-}t} + \mathbf{M}_{\infty}^{\mathbf{c}}$$
(eq 2.4)

$$M_{z}^{t} = \frac{A(\rho_{c} + k_{1} + \lambda_{+})e^{\lambda_{+}t}}{k_{-1}} + \frac{B(\rho_{c} + k_{1} + \lambda_{-})e^{\lambda_{-}t}}{k_{-1}} + M_{\infty}^{t}$$
(eq 2.5)

$$\lambda_{\pm} = \frac{1}{2} \left( -(\rho_{c} + k_{1} + \rho_{t} + k_{-1}) \pm \left\{ \left[ (\rho_{c} + k_{1}) - (\rho_{t} + k_{-1}) \right]^{2} + 4k_{1}k_{-1} \right\}^{\frac{1}{2}} \right)$$
 (eq 2.6)

where  $M_z^i$  is the magnetization at any time t (i stands for c (*cis*) or t (*trans*));  $M_{\infty}^i$  is the magnetization at equilibrium;  $\rho_i$  is the longitudinal relaxation rate constant; and A and B are factors determined by the initial conditions. In the inversion-transfer experiments, A ~ -B.

# 2.4.6 Inversion Recovery Experiments

Apparent  $\rho$  values were determined for the C $\delta$ H proton of each isomer with a standard non-selective inversion recovery sequence. A total of 22 to 34 data points were collected to cover the relaxation profile. The repetition rate was set to values larger than

five  $T_1s$ , typically > 15 s. Histidine C $\delta$ H peak heights were fit as a function of the recovery time using the standard equation:

$$M_{z} = M_{\infty} [1 - 2 C \exp(t/T_{1})]$$
 (eq 2.7)

where C is a factor indicating the quality of the inversion (C = 1.0 for perfect inversion). The recovery curves could be fit with a single exponential recovery throughout the whole time course. The  $T_1$  value so obtained, however, contained a contribution from chemical exchange as per Equations 4–6.

Rigorous evaluation of rates from Equations 4–6 requires the determination of multiple parameters. When one of the species in equilibrium prevails, its recovery after non-selective inversion is practically unaffected by chemical exchange. The A term then dominates and an estimate of  $\lambda_+$  is obtained for use in a non-linear fit of the inversion-transfer data for the *cis* isomer (Equation 4). Rates constants are extracted using K<sub>eq</sub> and  $1/T_1$  as an estimate of  $\rho$  values. The parameters so determined are in turn used to calculate the inversion-transfer curve for the *trans* isomer and the inversion recovery curves for both isomers. Rate constants were reported when it was possible to reproduce all four experimental traces with a consistent set of parameters.

## 2.4.7 Two-dimensional exchange spectroscopy

The exchange process in apocytochrome  $b_5$  was monitored through NOESY spectra with mixing times ranging between 10 ms and 140 ms. Exchange cross-peaks were identified with TOCSY data collected under the same conditions and by comparison

with spectra collected at 500 MHz<sup>12</sup>. Intensities were obtained with NMRPipe.<sup>4</sup> Curves were constructed with equations derived by Jeener and coworkers<sup>10</sup> for the twodimensional NOE/exchange experiment. No attempt was made to fit the data to yield rate constants.

#### 2.5 **Optical Spectroscopy**

## 2.5.1 Sample Preparation

Analyte solutions of 5–15  $\mu$ M were prepared from stock solutions of known concentration. Each peptide studied in chapter 4 contained at least one aromatic residue, allowing their approximate concentrations to be determined by UV-visible spectroscopy. The concentration of each peptide stock solution was initially estimated by sample absorbance at 257 nm (pH 7,  $\epsilon = 200 \text{ M}^{-1} \text{ cm}^{-1}$  per phenylalanine). Spectra were collected at room temperature in a 1cm cell using a Beckman model DU 650 spectrophotometer.

The stock solution concentrations were then accurately determined by addition of a standardized tryptophan solution (pH 7,  $\varepsilon = 5500 \text{ M}^{-1} \text{ cm}^{-1}$  at 280 nm) to a volumetric amount of stock, followed by collection of 1D NMR spectra in H<sub>2</sub>O:D<sub>2</sub>O (9:1) with presaturation of the water line. Integration of the C $\varepsilon$ 3H resonance of the tryptophan and of well-resolved peptide methyl proton resonances was used to determine peptide concentrations reliably. The concentration error using this procedure is estimated at 10%. Concentrations of heme-conjugated samples were determined by addition of stock solution in the ferric state to 1.25 M imidazole in 1:1 EtOH:water ( $\varepsilon = 139,000 \text{ M}^{-1} \text{ cm}^{-1}$ ). TFE-containing peptide samples were prepared by mixing of unbuffered aqueous peptide solutions at pH 3.5 with 80% TFE peptide solutions of equal peptide concentration. The concentration of TFE ranged from 0 to 80% by volume. The apparent pH of TFE-containing solutions was estimated using contact pH paper. TFE-containing heme-conjugate samples were prepared as described above, with the exception that aqueous samples were buffered to pH 7.9 using 20 mM sodium phosphate. After mixing, samples were allowed to equilibrate for approximately 5 min before spectra were acquired.

## 2.5.2 Circular Dichroism Spectroscopy

Overall helical content of samples was estimated using far-UV CD spectroscopy. In order to generate data suitable for comparison to NMR results, the BLP samples described in chapter 4 were investigated at a pH of approximately 3-5. Samples investigated in chapter 5 were buffered to a pH of 7.9 to ensure that iron ligation was preserved in the BHC constructs. All CD data were collected using a JASCO model J-810 spectropolarimeter. CD spectra were acquired in triplicate with an averaging time of 1-4 s per point. Reported values are averages of the three scans. Ellipticity was measured over a range of 200 nm to 250 nm in a 1-cm cuvette, and the data were converted to mean residual ellipticity ( $[\Theta]_{MRW}$ ). Estimates of global helical content in each helix were calculated as fraction helix ( $f_{\rm H}$ ) using the equation:

$$f_{H} = \left[\Theta\right]_{MRW,222} \frac{1}{39,500 \times (1 - \frac{2.57}{n})}$$
(eq 2.8)

where  $[\Theta]_{MRW,222}$  is the molar residual ellipticity at 222 nm, in deg cm<sup>2</sup> dmol<sup>-1</sup>, and *n* is the number of residues in the peptide chain.

### 2.5.3 UV-Visible Spectroscopy

The absorbance and  $\lambda_{max}$  of the Soret,  $\alpha$ ,  $\beta$  and charge transfer bands of UVvisible spectra are frequently used to assess the ligation state of iron porphyrins.<sup>13-15</sup> In chapter 5, the heme-conjugate samples were investigated using this technique. UVvisible spectra of heme conjugate samples were collected from 260 nm to 700 nm at room temperature using an AVIV model 14-DS spectrophotometer and 1-cm quartz cuvettes. The step size was 1 nm, and the averaging time was of 0.5 or 1.0 s per point. Reported spectra are the average of three scans, with subtraction of a suitable blank spectrum.

# 2.6 Sequence Analysis

### 2.6.1 SCWRL Analysis

Side chain rotamer analysis was performed using SCWRL 3.0 software.<sup>16</sup> The backbone input was that of H4 from a solution structure of rat microsomal holo cyt  $b_5$  (pdb accession 1aqa).<sup>1</sup> Two analyses were performed on the D60R variant sequence. In the first analysis, all side chain rotamers were calculated using SCWRL. In the second calculation all residues except for D60 and E56 were locked in the state designated by the pdb file. This was done to preserve the dihedral angles induced by tertiary interactions and the nearby heme cofactor.

# 2.6.2 AGADIR Analysis

Helical propensities were calculated with AGADIR using conditions of pH and ionic strength similar to those of the CD and NMR experiments. The predicted  $f_{\rm H}$ profiles were obtained at the residue level and compared to the NOE patterns in the NMR experiments. Additionally,  $f_{\rm H}$  was predicted for peptides on a global scale for comparison to CD data.

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## Chapter 3

### A pH Controlled Conformational Switch in Helix 6 of Apocytochrome b<sub>5</sub>

Portions of this chapter have been presented in the following publication: Davis, RB and Lecomte, JTJ. A dynamic N-capping motif in cytochrome  $b_5$ : Evidence for a pHcontrolled conformational switch. Proteins: Structure, Function and Bioinformatics. 2006;

63:336-348.

Two Dimensional Exchange Spectroscopy experiments were performed by JTJL

## 3.1 Introduction

Cytochrome  $b_5$  from several sources has an H6 helix starting with the sequence LH<sup>80</sup>PD. An N-capping interaction is observed in helix H6 of holocytochrome  $b_5$ , by which the imidazole Nô of His80 (N-cap) acts as an acceptor for the NH of Asp82 (N2)<sup>1</sup> (Figure 3.1). The interaction is readily identified by <sup>1</sup>H NMR spectroscopy through a downfield shift of Asp82 NH beyond 11 ppm, a strong NOE between this NH and His80 C $\varepsilon$ H, and a His80 pK<sub>a</sub> below 5.5.<sup>1-3</sup> High-resolution X-ray structures reveal that in the crystal, the amide NH of Asp83 (N3) and the amide NH of Arg84 (N4) serve as donors to the carbonyl group of the N-cap. The NH of N4 is also within H-bonding distance of the carbonyl group of N1. This tight first turn is supported by backbone dihedral angles and interatomic distances consistent with the geometry of a "real" 3<sub>10</sub> helix, as defined by Chakrabarti and coworkers.<sup>4</sup> X-ray structures<sup>5,6</sup> further illustrate that the head group of Arg84 (N4) contacts the backbone carbonyl of Leu79 (N<sup>\*</sup>) and that the aliphatic

portion of the Arg84 side chain interacts with that of Leu79. This last feature is a staple satisfying the hydrophobic component of a helix cap, although according to multiple deposited NMR structures<sup>7</sup> these interactions may not be present in solution. In the protein, helix H6 is packed near the N-terminal  $\beta$  strand (Figure 1). The side chain of Asp83 interacts with the backbone NH of Leu9 to stabilize the fold. Contact between Pro81 and Tyr6, among others, is likely to aid in the correct placement of the helix with respect to the rest of the structure.



**Figure 3.1**: Stereoview of helix H6 and selected residues from the X-ray structure of microsomal rat cytochrome  $b_5$  (Protein Data Bank entry 1ehb<sup>6</sup>). The helix is shown in sticks from residue 79 to 84 (C-terminal residue in this particular protein sample). Also shown are Tyr6 (in contact with Pro81), Ile12, and Ile24, both participating in the folded hydrophobic core of the (apo)protein. A purple ribbon traces the backbone from residue 5 to residue 25.

When the heme is removed from the water-soluble domain of the protein (yielding apocytochrome  $b_5$ ) the heme binding site loses its well-defined structure, whereas the hydrophobic core adjacent to the heme site and helix H6 remain largely folded.<sup>8</sup> This species offers an opportunity to investigate the dynamic processes associated with the binding and release of the heme cofactor. One aspect of the considerable alteration of dynamic and energetic properties caused by heme association is manifested in the behavior of helix H6, which samples

at least two conformations in the apoprotein. The major conformation corresponds to that observed in the holoprotein and is characterized by similar chemical shift, NOEs, and His80 pK<sub>a</sub> value.<sup>9</sup> The second conformation exchanges with the major form slowly on the NMR chemical shift time scale, giving rise to two distinct resonances for each amide NH starting at Gly77 in strand  $\beta$ 2 and throughout helix H6.<sup>3</sup> In the minor form, His80 has an apparent pK<sub>a</sub> of 6.9 ± 0.3,<sup>2</sup> and the chemical shifts along the helix resemble those of the random coil. Thus, it was proposed that the His80–Asp82 N-capping interaction was broken in this species. The chemical shift separation of minor and corresponding major peaks dictates exchange rate constants slower than 120 s<sup>-1</sup>, but the appearance of exchange cross-peaks at short mixing times in NOESY experiments sets a lower limit near 1 s<sup>-1.3</sup>. The presence of a proline at position 81 (N1) suggests that *cis-trans* isomerization of the His-Pro peptide bond may be responsible for the activation energy barrier to the exchange (Figure 3.2)



**Figure 3.2**: A representation of the *cis* – *trans* interconversion of the peptidyl-prolyl bond (see eq 2.2). The N-terminal peptide chain is designated as 'Pep<sub>N</sub>" and the C-terminal chain as 'Pep<sub>C</sub>". (A) In the *cis* conformation, the N-terminal peptide chain is in a position which is sterically hindered by the C-terminal peptide chain. This effect is common to all of the 20 biologically relevant amino acids. (B) In the *trans* isomer, the C $\delta$  of the pyrrolidine ring is positioned in a sterically disfavored geometry with respect to the C-terminal peptide chain. This trans isomer destabilizing effect is unique to proline among the 20 biologically relevant amino acids and results in significant population of the *cis* isomeric state at the prolyl-peptide bond.

Kinetic analyses of Xxx-Pro bond isomerization in peptides have shown that under normal conditions of pH and temperature, the rate constant for the *cis*-to-*trans* conversion is on the order

of  $10^{-2}$ – $10^{-4}$  s<sup>-1</sup>.<sup>10</sup> For these particular bonds, steric effects reduce the preference for the *trans* isomer to about 90%, <sup>11,12</sup> although the nature of the sequence and external conditions influence the equilibrium ratio to some extent.<sup>10</sup> Compared to the rate of interconversion of the minor and major conformers in apocytochrome  $b_5$ , generic peptide rates are significantly slower. *Cis-trans* isomerization of Xxx-Pro bonds in proteins, however, does not necessarily proceed with slow kinetics. In addition to peptidyl-prolyl isomerases that can accelerate the process in an intermolecular fashion, <sup>13,14</sup> the isomerization can be mediated intramolecularly by a positive charge near the imide nitrogen of the of Xxx-Pro bonds.<sup>15,16</sup> In the case of Lys and Arg, tertiary structure is necessary for productive placement of the charged head group. In contrast, the His side chain possesses the necessary geometry to affect the conversion rates of its peptide bond to an immediately succeeding proline residue even in tetrapeptides,<sup>15</sup> highlighting that secondary or higher order structural assistance is in fact not necessary. In short peptides, protonated histidines can achieve rate enhancements of 10-fold over the same peptide containing a neutral histidine.<sup>15</sup>

The His-Pro pair in helix H6 prompted us to inspect whether a histidine-accelerated proline isomerization may be responsible for the observed exchange process in apocytochrome  $b_5$ . We reasoned that if helix H6 is decoupled from the rest of the apoprotein, it should exhibit dynamic properties similar to those of a model peptide. Alternatively, if Xxx-Pro bond isomerization is not the source of the observed process, a less trivial folding and unfolding equilibrium is at work and may report on the energetic landscape of a partially folded protein and long-range communication within the structure. At the very least the peptide should provide a tractable model system with which to investigate capping interactions and a suitable reference for thermodynamic purposes in the elucidation of the folding induced by the cofactor.

## **3.2** Peptide Design

To characterize local interactions within the N-capping region of helix H6 in cytochrome  $b_5$ , three peptides were designed. The first contained residues 78-85 of rat microsomal cytochrome  $b_5$  (ELHPDDRS-NH<sub>2</sub>, "H6P-1"). The second peptide used the same sequence with D-His instead of L-His at position 80 (ELH\*PDDRS-NH<sub>2</sub>, "H6P-2"). The last peptide was a variation of the first, with an alanine replacing the proline at position 81 (ELHADDRS-NH<sub>2</sub>, "H6P-3"). All peptides were amidated at the C terminus.

# 3.3 Results

To characterize the behavior of helix H6 in isolation, a peptide was prepared that encompassed residues 78 to 85 (H6P-1, "wild-type"). For simplicity, the protein numbering is applied to the peptides. The focus was on the N-cap residue, His80, and the next two residues. H6P-1 extended from N" over the residues found in the N-cap motif of helix H6 of cytochrome  $b_5$ . As will be described below, Peptide 1 demonstrated non-random spectral properties in the Ncapping region. H6P-2 was designed to resemble H6P-1 closely, but to perturb these interactions, which was achieved with the reversal of the stereochemistry at the  $\alpha$  carbon of His80 (H6P-2, "epimer"). Insertion of D-residues into amino acid sequences is a strategy applied in protein design<sup>17</sup> and examination of the role of residues with positive backbone dihedral  $\varphi$  angles.<sup>17,18</sup> Host-guest studies of short helical peptides indicate that D-histidine has particularly weak helix-breaking characteristics when compared to other D-amino acids.<sup>19</sup> The D-histidine substitution should present in H6P-2 inductive effects similar to those in H6P-1. A third peptide, containing an alanine instead of Pro81 (H6P-3), was also prepared for further control of ionization and conformational properties.

## 3.3.1 NMR Assignments

The imidazole ring of His80 in H6P-1 was readily recognized by its distinctive C $\delta$ H and C $\epsilon$ H signals in one-dimensional <sup>1</sup>H NMR spectra. Two sets of signals were observed, the minor set accounting for about 10% of the material. Since the peptide was chemically pure, the doubling was attributed to *cis-trans* isomerization of the His-Pro bond. Doubling was detected in H6P-2 as well, but in this case, the minor form was present at a level of about 20%. The spectrum of H6P-3 contained only one set of resonances, in agreement with the overwhelming predominance of the *trans* His-Ala peptide bond. These observations demonstrated the differential influence of the stereochemistry at the  $\alpha$  center of the Xxx residue on the equilibrium constant for Xxx-Pro bond isomerization.

<sup>1</sup>H Resonances in H6P-1 and H6P-2 were identified by residue type at pH 8.2 and pH 3.3 using TOCSY, 2QF-COSY and NOESY spectra. The two sets of signals mentioned above were best resolved for His80 and Pro81. The major isomer exhibited strong NOEs between the C $\alpha$ H of His80 and Pro81 C $\delta$ H<sub>2</sub>, confirming the *trans* identity of the His-Pro bond.<sup>20</sup> When resolved, a His80 C $\alpha$ H to Pro81 C $\alpha$ H NOE was detected in the *cis* isomer. Asp82 and Asp83 were distinguished by sequential NOEs for the pairs Pro81-Asp82 and Asp82-Asp83 in H6P-1 and H6P-2. Selected chemical shifts are reported in Table 3.1.

H6P Peptide	Residue	NH	α	β	Other
1 (trans)	L-H80	8.52	4.75	2.98, 3.06	СбН, 6.96; СєН, 7.68
	P81		4.26	2.19, 2.20	СүН, 1.86, 1.88; СбН, 3.18, 3.20
	D82	8.95	4.54	2.60, 2.68	
1 ( <i>cis</i> )	L-H80	8.43	4.89	2.93	СбН, 6.86, СєН, 7.65
	P81		4.11	1.93, 1.94	СүН, 1.72. 1.73; С8Н, 3.32, 3.42
2 (trans)	D-H80	8.56	4.91	2.91, 2.98	СбН, 6.90, СєН, 7.63
	P81		4.28	2.16, 2.17	СүН, 1.81, 1.73; СδН, 3.38, 3.67
	D82	8.21	4.56	2.55, 2.70	
2 ( <i>cis</i> )	D-H80	8.48	4.59	2.86, 2.93	СбН, 6.85, СєН, 7.66
	P81		4.92	2.08, 2.28	СүН, 1.97, 1.82; СбН, 3.44, 3.52
	D82	8.43	4.42	2.59	

**Table 3.1**: Selected <sup>1</sup>H Chemical Shifts from H6P-1 (wild-type) and H6P-2 (epimer)<sup>a</sup>

<sup>a</sup>Measured at pH 8.2, 298 K, with chemical shift referenced through the residual water line at 4.70 ppm.

In data collected at pH 3.3, amide NHs gave rise to sharp resonances, each occurring at chemical shift near random coil values. At pH 8.2, the NH signals were significantly broadened. Rapid exchange with the solvent resulted in the loss of the NH resonance from Leu79, as predicted on the basis of calculations developed by Englander and coworkers.<sup>21</sup> Random coil chemical shifts were also obtained, with one exception. In H6P-1, Asp82 NH appeared at 8.95 ppm (Figure 3.3a,b). Under the same conditions, Asp82 NH resonated closer to the normal (8.4 ppm) shift in H6P-2 (Figure 3.2c) and H6P-3 (not shown).



**Figure 3.3**: Portions of (A) TOCSY and (B) NOESY NMR data collected on H6P-1 and (C) TOCSY data collected on H6P-2 in 9:1 H<sub>2</sub>O:D<sub>2</sub>O at pH 8.2. In (A), the NH-C $\alpha$ H-C $\beta$ H<sub>2</sub> portions of His80 and Asp82 are shown at 298 K. In (B), NOEs are illustrated between the two side chains (H80 C $\epsilon$ H and Asp82 C $\beta$ H<sub>2</sub>) at 278 K. The low temperature was necessary to emphasize the effect. (C) Portion of the fingerprint region from a TOCSY spectrum of H6P-2 in 9:1 H<sub>2</sub>O:D<sub>2</sub>O at pH 8.2 and 285 K. The NH-C $\alpha$ H-C $\beta$ H<sub>2</sub> resonances of Asp82 in the major form appear approximately 0.7 ppm upfield from analogous resonances in H6P-1.

## 3.3.2 pH Titrations

A determining feature of helix H6 and its N-capping interaction in the protein resides is the ionization property of His80. pH titrations were performed on all three peptides; the curves are shown in Figures 3.3 and 3.4. The CδH and CɛH signals of His80 exhibited only a small extent

of line broadening in the middle of the transitions and the modified Henderson-Hasselbalch Equation 1 was used to obtain apparent  $pK_a$  values. A fit of C $\delta$ H and C $\epsilon$ H chemical shift as a function of pH (Figure 3.4) returned the values reported in Table 3.2. The apparent  $pK_a$  of His80 depended on the configuration about the His-Pro bond. In H6P-1, the *trans* isomer had a value higher than that of the *cis* isomer by 0.3 pH unit. The epimer (H6P-2) exhibited a  $pK_a$  difference similar in magnitude but with reverse ranking.

	Signal	pK <sub>a</sub> <sup>a</sup>	n	K <sub>eq</sub> <sup>b</sup>
H6P-1				16
trans His-Pro	СбН	$6.91\pm0.01$	$0.87\pm0.01$	
	СєН	$6.88\pm0.01$	$0.91\pm0.01$	
	Average	6.90	0.89	
cis His–Pro	СбН	$6.61\pm0.01$	$0.85\pm0.02$	
	СєН	$6.66\pm0.01$	$0.91\pm0.01$	
	Average	6.63	0.88	
H6P-2				3.2
trans His-Pro	СбН	$6.87\pm0.02$	$0.92\pm0.03$	
	СєН	$6.85\pm0.02$	$0.93\pm0.03$	
	Average	6.86	0.92	
cis His–Pro	СбН	$7.19\pm0.02$	$0.88\pm0.03$	
	СєН	$7.19\pm0.02$	$0.89\pm0.03$	
	Average	7.19	0.89	
H6P-3				_
trans His–Ala	СбН	$6.94\pm0.01$	$0.92\pm0.02$	
	СєН	$6.96\pm0.01$	$0.96\pm0.01$	
	Average	6.95	0.94	

 Table 3.2:
 pKa values for His80 in H6P peptides

<sup>a</sup>Standard deviation of the fitted parameter, which can be used for comparison of results within a peptide (Figure 3.4). Estimated accuracy of average pK<sub>a</sub> and *n* values is  $\pm 0.04$ . <sup>b</sup>Measured at pH 3.3.



**Figure 3.4**: Plots of chemical shift versus pH for C $\delta$ H and C $\epsilon$ H of histidine residues in H6P peptides. Open symbols represent the major (*trans*) isomer data. Closed symbols represent the minor (*cis*) data for H6P-1 and H6P-2. Squares and circles represent C $\delta$ H and C $\epsilon$ H data, respectively. (A) H6P-1 *cis* and *trans* isomers. (B) H6P-2 *cis* and *trans* isomers. (C) H6P-3 contains only a single isomer by virtue of the P81A substitution. Solid lines represent the least-squares fit of each data set to Equation 1 (parameters are reported in Table 3.2).

Asp82 provided further insight into the environment of His80 in each peptide. Figure 3.5 illustrates the pH response of Asp82 C $\alpha$ H in the *trans* isomers. It is noteworthy that a deviation mirroring the titration of His80 is observed only in H6P-1. In this sample, when the data were limited to pH values between 5.5 and 9.0, a pK<sub>a</sub> of ~6.7 was obtained from a fit of Asp82 C $\alpha$ H shift versus pH to Equation 1. In contrast, the same proton experienced no such effect in H6P-2 and H6P-3. A sigmoid response was obtained for Ala81 C $\alpha$ H in H6P-3, in agreement with a local sequence effect.



**Figure 3.5**: Plots of chemical shift versus pH for C $\alpha$ H of Asp82 in H6P-1 (O), H6P-2 ( $\diamond$ ), and H6P-3 ( $\Box$ ), and of C $\alpha$ H of Ala81 in H6P-3 (+).

#### **3.3.3** Peptide Conformational Properties

NOE spectra were collected on the three peptides to identify conformational preferences. Under conditions favoring formation of the His80 N $\delta$  to Asp82 NH hydrogen bond (low temperature and pH 8.2), H6P-1 exhibited NOEs not expected of a random conformation. These are shown in Figure 3.3, which illustrates the proximity of the imidazole ring of His80 to the Asp82 side chain. No such preference was detected in H6P-2. This complemented the abnormal shift of Asp82 NH in H6P-1 and the pH response of Asp82 as His80 titrates. Ring current effects from the histidine are not expected to be large even in a locked holoprotein-like conformation, and the downfield value suggests a hydrogen bonding contribution. In addition to the NOEs observed between His80 and Asp82, NOEs were observed between the C $\epsilon$ H of His80 and the C $\beta$ H/C $\gamma$ H of Pro81 in H6P-1 (data not shown). These NOEs suggested that in the major isomer of H6P-1 the histidine side chain sampled preferentially a rotameric state in which it was interacting with the pyrrolidine ring of Pro81.

## 3.3.4 Kinetics of Isomerization

An estimate of rate constants for Xxx-Pro *cis-trans* isomerization in H6P-1 and H6P-2 was obtained by the inversion-transfer method applied to the resolved CôH resonances of His80. Apparent non-selective spin-lattice relaxation rate constants were obtained under the same conditions. Values for the histidine CôH of each isomer are reported in Table 3.3. Representative inversion-transfer curves, collected at pH 8.2, are shown in Figure 3.6. Data collected at pH 3.3 were of higher quality as the exchange rate was accelerated; thus, Figure 3.6 contains a suboptimal situation to illustrate the limits of the methods. By the nature of the

experiment, the *cis* curve (resonance to which magnetization is transferred) displayed a minimum, whereas the *trans* curve followed a simple exponential recovery. Satisfactory *trans* and *cis* fits to the modified Bloch equations were obtained in all transfer and inversion recovery experiments, except those of H6P-2 at pH 8.2, even though under those conditions, retardation of the isomerization process could be noticed. The rate constants are listed in Table 3.3. In H6P-1, the rate of interconversion is accelerated when the histidine ring is positively charged; the change in  $K_{eq}$  is primarily due to an acceleration of the *cis*-to-*trans* conversion as the pH decreases. H6P-2, which favors the *cis* isomer compared to H6P-1, does so at low pH with a faster *trans*-to-*cis* rate constant.



**Figure 3.6**: Peak height (arbitrary unit) of the C $\delta$ H resonances from *cis* and *trans* isomers in H6P-1 at pH 8.2 plotted versus transfer time ( $\tau$  in s). (A) Open circles represent data for the *cis* isomer. The solid line represents the least-squares fit to Equation 5. (B) Open circles represent the data for the *trans* isomer. The solid line represents the least-squares fit to Equation 6 using  $\lambda_+$  and  $\lambda_-$  values obtained in fitting of Equation 5.

Peptide	pH*	K <sub>eq</sub>	$T_{1cis}(s)$	$T_{1trans}(s)$	$k_{-1} (s^{-1})^b$	$k_1 (s^{-1})^b$
H6P-1	3.3	13.4	3.3	3.2	0.030	0.41
	8.2	9.9	1.2	1.0	0.025	0.25
H6P-2	3.3	4.9	3.3	3.6	0.073	0.36
	8.2	5.5	1.6	2.7	_c	_

Table 3.3: His-Pro cis-trans Isomerization Parameters <sup>a</sup>

<sup>a</sup>Measured at 313 K in D<sub>2</sub>O. <sup>b</sup>As defined in Equation 2,  $k_1$  for *cis*  $\rightarrow$  *trans* and  $k_{-1}$  for *trans*  $\rightarrow$  *cis*. <sup>c</sup>Rate constants could not be determined for this sample.

## 3.3.5 Apocytochrome *b*<sub>5</sub> Exchange Rate

The exchange between major and minor isomers of helix H6 in the apoprotein was investigated for comparison with the peptide data. The protein spectrum did not lend itself to 1D magnetization transfer experiments. Instead, the time dependence of cross peak intensities for each conformer was obtained from a series of NOESY experiments. As established at 500 MHz,<sup>3</sup> exchange cross peaks rose within the first 50 ms of transfer and decreased over longer transfer times (Figure 3.7). The shared behavior of resolved cross peaks supports a concerted process linked to the protonation of His80.<sup>2</sup> Exchange manifestation was observed within H6 and for Ile76 and Trp22, which are adjacent to the C-terminal region. The data can be qualitatively interpreted with lifetimes on the order of 1 s in the major state, and milliseconds in the minor state.



**Figure 3.7**: Time course of NOE and magnetization transfer in apocytochrome  $b_5$  at pH 6.5 and 298 K. The intensity of the cross peak is plotted versus mixing time ( $\tau$  in ms):  $\blacklozenge$ , His80 C $\epsilon$ H (exchange);  $\triangle$ , NOE between Asp82NH and His80 C $\epsilon$ H;  $\blacktriangle$ , Ile76 C $\delta$ H<sub>3</sub> (exchange);  $\Box$ , Arg84 NH exchange; O, Glu78 NH (exchange);  $\blacklozenge$ , His80 NH (exchange). The four sample curves are calculated for an exchanging system with  $k_1 = 16$  and  $k_{-1} = 0.8$  and  $\rho$  of ~250 ms or NOE buildup.<sup>22,23</sup> They are drawn to guide the eye and are not the result of fits.

# 3.4 Discussion

#### 3.4.1 Histidine Ionization in Model Peptides

The capping motif adopted by helix H6 in the holoprotein is a cap'-box,<sup>4</sup> the key element of which is a hydrogen bond between the side chain of the N-cap residue and the backbone amide NH of N2. This interaction is detected in the holoprotein in solution, where it appears to be robust, and in the apoprotein as well. The  $pK_a$  of His80, when participating in the bond, could not be determined accurately.<sup>3</sup> However, the partial curves obtained in the titration of holo- and

apocytochrome were nearly superimposable, suggesting that the strength of the interaction was comparable in both protein forms. The difference in behavior between apoprotein conformers, therefore, appears to stem from tertiary interactions involving H6. The model peptides aided in elucidating this aspect of the helical structural element. The pK<sub>a</sub> value determined for His80 in the *trans* isomer of each peptide was ~6.9. The values for the *cis* isomer were different and outside of measurement error. In H6P-1, pK<sub>a</sub>(*trans*) > pK<sub>a</sub>(*cis*); the standard free energy corresponding to the difference was ~1.5 kJ/mol. A similar standard free energy difference was obtained for H6P-2, although in this case, the sign was reversed. The noticeable effect of the stereochemistry at His80 C $\alpha$  on the pK<sub>a</sub> and the histidyl-prolyl bond configuration is envisioned to be the result of distinct dipole orientations and solvation properties conferred by the covalent geometry and associated conformational preference.

Because *cis* and *trans* isomers have distinct apparent  $pK_{a}s$  within a single peptide, one expects the ratio of isomers, represented by  $K_{eq}$ , to depend on pH. The thermodynamic cycle connecting four species (abbreviated as *c*-His80-H<sup>+</sup>, *c*-His80, *t*-His80-H<sup>+</sup>, and *t*-His80) is illustrated in Figure 3.8, along with a fractional population plot using the parameters for H6P-2 (Table 3.2). At low pH, the experimental  $K_{eq}$  shifted in favor of the species with the higher  $pK_{a}$ , but the agreement was only qualitative. This was also observed in H6P-1 and in a myoglobin peptide of sequence KSHPE<sup>24</sup> (data not shown).



**Figure 3.8**: Top: A four-state model of the *cis-trans* equilibrium and histidine ionization equilibrium for a peptide containing the His-Pro dyad. The plot shows the change in fractional population of each species as a function of pH. The curves were calculated using the parameters listed in Table 3.2 for H6P-2: a, *cis* with neutral His; b, *trans* with neutral His; c, *cis* with protonated His; d, *trans* with protonated His; e, total *cis*; f, total *trans*.

In general, the measured  $pK_a$  and  $K_{eq}$  values represent aggregates of multiple equilibrium constants. For example, neutral histidines exposed to solvent exist in a mixture of two rapidly interconverting tautomeric forms that have different microscopic  $pK_a$  values.<sup>25</sup> In addition, different conformational states and charge states may be present, each experiencing distinct influences on the ionization of the ring and isomerization of the His-Pro bond. In the cytochrome and myoglobin peptides, as the pH is lowered, the carboxylic acid group of the Asp or Glu side chain following the proline undergoes protonation. It is likely that the position of the equilibrium between *cis* and *trans* isomers depends on the protonation state of the residue following Pro in addition to that of His80. A related effect was exploited by Grathwohl and Wüthrich in pD jump experiments on peptides to measure isomerization rates.<sup>10</sup> The pK<sub>a</sub> value of the histidine in H6P-1 and H6P-2 (distant by 3 pH units from that of the carboxylic acid) would not reflect this influence and therefore not be apparent in the profiles shown in Figure 3.8. Similar processes can also occur at the high pH end of the curve. In view of this, K<sub>eq</sub> was estimated as a function of pH in H6P-2. A minimum is reached near pH 5 (not shown), but as the pH was lowered further, K<sub>eq</sub> increased with a trend that mimicked the chemical shift behavior depicted in Figure 3.4. The measurement of K<sub>eq</sub> variation in H6P-1 was imprecise because of the large excess of *trans* isomer throughout.

#### **3.4.2** Local Control of Peptide Conformation

Positions N-cap through N3 of helix H6 all contain residues that are overrepresented in threelength 3<sub>10</sub> helices.<sup>4</sup> With one exception in *trans*-H6P-1, all identified NH signals resonated at chemical shifts near random-coil peptide values. The exception, Asp82, had a considerably downfield-shifted amide NH (Table 3.1), though not as perturbed as in the folded protein. It is possible that the Asp82 NH shift at basic pH is due to the peptide sampling a conformation similar to that of the major isomer of the wild-type protein. This was supported by NOEs between the ring of His80 and the side chain of Pro81 and Asp82; these effects were absent at low pH and in the other two peptides under similar conditions. Interestingly, wild-type apoprotein data collected at pH 8.2 and 25 °C revealed a chemical shift of 9.1 ppm for Asp82 in the minor conformer, and so did samples of a variant apoprotein (Ile12Ala), which is unfolded under those conditions (Mukhopadhyay and Lecomte, unpublished results). This shift was close to that of H6P-1 and suggested that the minor protein form had related conformational properties.

The rotameric state of histidine within peptides is known to depend on pH.<sup>26</sup> Formation of the 10-membered ring closed by the N-cap H-bond requires the histidine side chain to adopt a  $\chi 1$  dihedral angle of 180° (t). An analysis of the His80 C $\alpha$ H multiplet in D<sub>2</sub>O revealed a change in <sup>3</sup>*J* values with pH. In H6P-1, the variation displayed a pK<sub>a</sub> of 6.8 and an *n* value of 1. Following a Pachler three-state analysis,<sup>26,27</sup> the change in <sup>3</sup>*J* was consistent with an increase in the population of the t rotamer at high pH. In contrast, H6P-2 exhibited a smaller change in <sup>3</sup>*J*s, and the profile of both the shift and *J* value reflected an apparent pK<sub>a</sub> of 7.4 (*n* = 0.55) distinct from that obtained with the imidazole ring.

Wu and Raleigh have proposed that aromatic residues immediately preceding a proline in short peptides may participate in a weak interaction with the amide proton of the residue following the proline.<sup>28</sup> It is likely that a similar interaction occurs with histidine in *trans*-H6P-1, as suggested by the NOEs and *J* pattern. In the same study it was observed that Tyr, Phe, and Trp side chains interact favorably with a subsequent proline residue in the *cis* conformation. A possible explanation of the equilibria in helix H6 is that both H6P-1 and H6P-2 experience stabilization of the *cis* isomer via an interaction between the side chain of His80 and the pyrrolidine ring of Pro81, whereas only H6P-1 possesses the specific geometry required to favor the side chain t  $\chi$ 1 via His80-Asp82 interaction.

## 3.4.3 Rates of cis-trans Interconversion

The rates of *cis-trans* interconversion in H6P-1 and H6P-2 were measured at pH 3.3 and 8.2. The latter value was one pH unit above the highest measured His80  $pK_a$  and the former insured complete protonation of the side chain. At high pH and 298 K, isomer interconversion proved to be too slow on the NMR timescale for the collection of reliable rate data; the temperature was therefore raised to 313 K. The rate constants (Table 3.3) were similar in magnitude to those reported by Reimer and coworkers in their comprehensive study of intramolecular catalysis in short peptides.<sup>15</sup> The effect of pH was also reproduced. Specifically, in the case of H6P-1, the *cis*-to-*trans* conversion was accelerated approximately 2-fold under acidic conditions, while the *trans*-to-*cis* conversion was accelerated only marginally.

In the case of H6P-2, the inversion transfer data at pH 8.2 reflected a deceleration of the interconversion compared to low pH conditions. A suitable fit to inversion-transfer and inversion recovery data, however, was not possible. The reason for this is not clear, but it is possible that deviation from a two-state behavior interfered. This was also noted through the His80 C $\alpha$ H spectral properties. Nevertheless, a comparison of H6P-1 and H6P-2 showed that protonated D-His and L-His are both capable of accelerating the isomerization. The observed difference in K<sub>eq</sub> for these peptides is primarily attributable to a destabilization of the *trans* isomer in H6P-2 compared to H6P-1. This is to be contrasted with the effect of D-His in thyrotropin-releasing hormone, where an excess of the *cis* isomer is also observed when a D-His is present, but attributed to the formation of a H-bond stabilizing this configuration.<sup>29</sup>

### 3.4.4 Relationship to the Protein

Apocytochrome  $b_5$  is partially unfolded at 313 K.<sup>30</sup> For this reason, a direct comparison of peptide and apoprotein data was not made. At 298 K and pH 6.5, however, the major and minor conformers of apocytochrome  $b_5$  interconvert at rates well in excess of normal *cis-trans* proline

interconversion rates, and faster than H6P-1 and H6P-2 at pH 3.3 and 313 K. Matching the temperature and pH would only increase the discrepancy between the two types of samples.

The peptide data suggested that a His80-accelerated proline isomerization alone did not account for the observed protein rates. Other possible factors accelerating an isomerization include reduced solvent accessibility as water competes with the charged histidine for the imide nitrogen of proline.<sup>15</sup> This explanation is unlikely because the ordered structural properties of the major apoprotein form would result in a preferential increase of the *trans*-to-*cis* rate. Another possibility is that Arg84 contributes significantly to catalyzing the interconversion; this requires proximity of the arginine head group to the isomerizing bond, which is not borne by solution data. The combined evidence leads to the conclusion that the minor form of the apoprotein contains a *trans* His-Pro bond. The fraction of *cis* His-Pro in equilibrium with *trans* in the minor form is expected to be well below the limits of detection.

Because a *cis-trans* process can be eliminated, the simplest mechanism for the conformational exchange in the protein near neutral pH includes three states as schematized in Figure 3.9. At high pH, the predominant form is the N-capped helix packed against the hydrophobic core of the protein (*t*-His80-c). A small fraction of the population samples an uncapped helix (*t*-His80). This loss of hydrogen bonding entails a disruption along the helix that is sensed by multiple protons. As the pH is lowered, protonation of the histidine occurs in the *t*-His80 state to form *t*-His80-H<sup>+</sup>. Protonation and deprotonation rates for an exposed imidazole ring near its pK<sub>a</sub> are typically rapid on the chemical shift time scale pK<sub>a</sub> (~2000 s<sup>-1</sup>)<sup>31,32</sup> and a fast-exchange titration profile is obtained. As *t*-His80 binds protons, it is replenished from the *t*-His80-c form. This constitutes the rate limiting step and accounts for the observation of two sets of resonances, one from *t*-His80-c and one from *t*-His80 and *t*-His80-H<sup>+</sup>.



**Figure 3.9**: A three-state model of the conformational equilibrium taking place in the apoprotein. The scheme allows for a sequential model (solid arrows) and a triangle (solid and dashed arrows). The plot shows the change in fractional population of each species as a function of pH. The curves were calculated using arbitrary parameters:  $K_{eq} = 40$  and  $pK_a$  of the minor form  $(pK_{am}) = 6.9$  (dictating  $pK_a$  of the major form  $(pK_{aM}) = 5.3$  in the triangular model); a, *trans* with capping neutral His; b, *trans* with non-capping neutral His; c, *trans* with non-capping protonated His. During the titration of the uncapped form, a single resonance is obtained for the protonated and unprotonated versions.

A three-state model connecting directly *t*-His80-c and *t*-His80-H<sup>+</sup> (*i.e.*, one that assumes a single protonated form, Figure 3.9) can also be considered. The connection provides an alternative pathway for the interconversion of *t*-His80-c and *t*-His80. At sufficiently low pH, as the rate of protonation increases, this will result in fast exchange among all three species on the NMR time scale and the averaging of the resonances. In previous experiments, distinct

resonances were obtained for the major and minor forms at pH 6, possibly before the averaging threshold is reached. Below pH 6, the protein resonances broaden as acid unfolding occurs.

The 4-state and 3-state treatments predict an increase of the total minor species concentration as the pH of the solution is lowered. Direct quantification of the minor species population is difficult because of overlap with other titrating histidine resonances; on the other hand, the data did not show a measurable decrease of the *t*-His80-c population down to pH 6,<sup>3</sup> the pH at which the models predict combined populations of *t*-His80 and *t*-His80-H<sup>+</sup> near 20% (Figure 3.8). Thus, models treating the system with a small number of local processes (regional unfolding and single residue protonation) are expected to fail because of the unavoidable contribution of multiple other simultaneous events including global unfolding. The low apparent pK<sub>a</sub> of *t*-His80-c reflects coupled events that cannot be captured with simple schemes.

The origin of the barrier for the local unfolding process is likely to be found in the energy required to disrupt multiple interactions packing the helix on the rest of the structure. This explains the loss of stability of the cooperative nucleus once the cap and therefore the helix are disrupted. Amino acid replacements in the first turn of the helix (H80A, P81A) alter the stability of the cooperative core of the apoprotein,<sup>30,33</sup> supporting that the behavior of this element of secondary structure is also controlled by interactions with the rest of the protein.

The potential for local helix fluctuations to have global consequences in proteins has been demonstrated. In a recent report, Dirr and coworkers noted that disruption of helix H9 in human glutathione transferase (hGST A1-1) via a mutation of its N-cap residue caused local conformational fluctuations on the mutated helix, but with little overall destabilization or deactivation of the enzyme.<sup>34</sup> Even so, fluorescence of the lone Trp residue in the protein 18 Å away was affected by the change. Much like the fluctuations of H6 in cytochrome  $b_5$  in response

to heme removal, such an example further illustrates that local structural fluctuations can impact distant sites in the protein without significant gross structural changes.

The N-cap interaction in helix H6 illustrates the difficulty in dissecting the role of individual components in a cooperative network. When apocytochrome  $b_5$  binds the heme, extensive reorganization of the protein occurs and the binding energy is redistributed throughout. The pK<sub>a</sub> of His80, in the capping state, is low in both the holo and apo form, but a small difference in pK<sub>a</sub>, as required to explain the detection of the minor form in the apoprotein and not in the holoprotein, may not be apparent in partial titration data. A possible interpretation is that in the context of the protein the helix acts as a cooperative unit, extending from hydrophobic core 2, including Trp22 and Ile76, to the end of the polypeptide. The N-cap serves to stabilize the folded form. In contrast, the holoprotein provides a network of tertiary interactions that increase the standard free energy difference between the local folded and unfolded state. In essence, the apoprotein offers a glimpse of the break down of cooperativity of hydrophobic core 2.

The  $3_{10}$  helix has been proposed to serve as a nucleation site for  $\alpha$  structure.<sup>4,35-38</sup> The slight non-random tendency exhibited by the H6 peptide and its behavior in the context of the apoprotein support a role for elements of local structure in the folding of helices. Additional studies will be required to assess the overall conformational preference of the His-Pro motif. It is possible that this pair of residues is used only when a certain set of tertiary interactions is also present to stabilize a single conformation and avoid the pH effects observed in the apocytochrome. The converse, i.e., the use of the His-Pro dyad for pH control of conformation, is likely in other cases, and presents an attractive tool for design purposes.

#### 3.5 Conclusions

The combination of protein and peptide studies based on helix H6 of cytochrome  $b_5$  revealed the following experimental facts. (1) *Cis* and *trans* isomers of the His-Pro bond impart different ionization properties to the histidine side chain and therefore give rise to isomer populations that are dependent on pH. (2) A D-His at the position prior to the proline destabilizes the *trans* isomer and affects the imidazole pK<sub>a</sub> via subtle perturbations of dipole orientation and solvation. (3) Both D-His and L-His accelerate the *cis-trans* interconversion of the histidyl-prolyl bond when in the protonated state. (4) The L-His-Pro-Asp motif is predisposed to form an N-capping interaction when the His-Pro bond is *trans*. (5) The minor form of helix H6 in the marginally stable apocytochrome  $b_5$  behaves as the isolated peptide in the *trans* configuration. (6) The conformational equilibrium observed in the apoprotein is not due to *cis-trans* proline isomerization but rather is coupled to the folding and unfolding of helix H6 containing a *trans* His-Pro bond. (7) Removal of the heme group reveals H6 to be a possible nucleation site for folding. (8) Tertiary interactions are responsible for the barrier to the nucleation and for the differential stabilization of helix H6.

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# **Chapter 4**

# Structural Propensities in the Isolated Binding Loop of Apocytochrome b<sub>5</sub> and its Variants.

# 4.1 Introduction

In spite of substantial characterization of cyt  $b_5$ , the inherent structural propensities of the binding loop in the apoprotein state remain experimentally undefined. The observations of low energetic cost for loop closure and Type I stability alteration, combined with the encoded preference for disordered structure in H3 and H4,<sup>1</sup> warrant an inspection of conformational properties in the H2–H5 region. In this study, peptides were prepared using the primary structure of wild-type and variant rat microsomal cyt  $b_5$ 's. Each sequence was designed to mimic all or part of the H2–H5 loop with the intent of defining structural predispositions in the absence of long-range restraints imposed by the supporting scaffold.

The significant  $\alpha$ -helical content of core 1 in the holoprotein and the increased stability of the D60R and N57P holoproteins led us to use 2,2,2-trifluoroethanol (TFE) to reveal local structural propensities. TFE is known to favor helix formation in sequences with intrinsic helical tendencies,<sup>2,3</sup> particularly in the case of amphipathic helices.<sup>4</sup> Inspection of holo cyt  $b_5$  structures shows that each of the four short H2–H5 helices consists of a hydrophobic face in contact with the heme and a solvent-exposed, hydrophilic face (Figure 4.1), making them excellent candidates for studies of TFE-induced folding. Circular dichroism and NMR experiments were used to survey the global and local helical content of peptides in various mixtures of TFE and water. The data distinguished the four helical segments and provided insight into the existence of native and non-native interactions in the cytochrome heme-binding loop.



**Figure 4.1**: (A) A stereo-view of residues 32 through 73 of oxidized rat microsomal cyt  $b_5$  (pdb accession number 1AQA)<sup>5</sup> viewed along the  $\alpha$ - $\gamma$  meso axis of the heme group. The protein backbone is shown as a grey ribbon. Side chains of residues responsible for formation of the hydrophobic core 1 are also shown. (B) A helical-wheel projection of H2–H5 of holocyt  $b_5$  in the same orientation. The heme group is represented with a black bar. Helix alignments approximate their orientation in the holoprotein. Solid circles indicate hydrophobic residues contacting the heme in the holoprotein, demonstrating the amphipathic nature of each helix. Dashed circles indicate Q49 and N57, two polar residues that complete core 1 via interactions between the hydrophobic portions of their side chains and the heme group.

# 4.2 Peptide Design

Five peptides, referred to as BLP's for binding loop peptides, were prepared. Figure 2.1 (chapter 2) depicts the sequences of the BLP's with regions of secondary structure in the bovine holoprotein provided for reference;<sup>6</sup> they match closely those of the rat microsomal holoprotein in solution.<sup>5,7</sup> Three peptides were designed to comprise all or part of the heme binding loop of rat microsomal cyt  $b_5$ : BLP-42 contained residues Leu32 through Thr73; BLP-21N contained the 21 N-terminal residues (Leu32 to Gly52); and BLP-22C the 22 C-terminal residues (Gly52 to Thr73). The replacements D60R and N57P were introduced individually into the sequence of BLP-22C (BLP-22C-D60R and BLP-22C-N57P, respectively) to probe the local effects of substitutions previously demonstrated to stabilize the holoprotein. Each fragment sequence was selected to preserve start and stop signals for H3 or H4. Thus, the behavior of these helices in the peptides is expected to approximate that of similar regions in the binding loop.

The synthetic H2–H5 peptide (BLP-42) differs from the corresponding region of the apoprotein in two important ways. First, the termini of BLP-42 are not constrained to a fixed distance of 12 Å. Second, the absence of the supporting scaffold (module 2) eliminates any long range conformational influence from this region of the protein. The N- and C-terminal halves of BLP-42 each contain one of the two disordered regions of the binding loop (H3 and H4, respectively). In isolation, these fragments report on the individual tendencies of the two helices to develop holoprotein-like structure.

### 4.3 Results

#### 4.3.1 Helical Propensities of Binding Loop Peptides – CD studies

Figures 4.2A and 4.2B present the CD spectra of BLP-22C and BLP-21N in water with varying proportions of TFE. The  $[\Theta]_{MRW,222}$  data were converted into fraction of helix ( $f_H$ ) as a function of TFE using equation 8 (presented in chapter 2) to yield the curves shown in Figure 4.3A. Although a small negative  $[\Theta]_{MRW,222}$  was detected in aqueous solutions of BLP-22C and BLP-21N, the calculated helix content under these conditions proved to be small in both cases (~5%). As the TFE concentration increased, the samples showed increasing negative  $[\Theta]_{MRW,222}$  concomitant with a shift in the minimum of the spectrum from below 200 nm to a value of 206 nm. These features demonstrated helix stabilization in the peptides as TFE was added. An isodichroic point at 203 nm for BLP-22C was in agreement with a two-state coil-helix transition.<sup>8</sup> BLP-21N spectra showed a modest excursion from a typical helix-coil isodichroic under conditions of 10% TFE, likely caused by turn propensity conveyed by the Pro-Gly pair.<sup>9</sup> Cosolvent concentrations beyond 10% resulted in typical helix-coil spectra.



**Figure 4.2**: Far-UV CD spectra of (A) BLP-22C and (B) BLP-21N in various water/TFE mixtures. Spectra were collected at 5 °C with peptide concentrations of 30  $\mu$ M. (C) Fraction helicity,  $f_{\rm H}$ , as a function of TFE concentration at 5 °C.  $f_{\rm H}$  Values were calculated using eq 8 (chapter 2). BLP-22C (squares) and BLP-21N (diamonds).

Although each peptide appeared to sample a helical conformation more frequently in the presence of TFE than in its absence, the nature of the responses to solvent composition differed. For example, under all but the most helix-inducing of conditions,  $[\Theta]_{MRW,222}$  of BLP-21N was found to be more negative and  $[\Theta]_{MRW,200}$  less negative than the corresponding ellipticities in BLP-22C. Also, the CD spectrum of BLP-21N remained unchanged beyond 30% TFE, whereas

those of BLP-22C and BLP-42 continued to show increasing helical character up to 80% TFE. A TFE concentration of 30% is generally regarded to be the amount needed to maximize hydrophobic and peptide-backbone desolvation effects.<sup>4</sup> BLP-42 and BLP-22C undergo a further coil-helix equilibrium shift at higher TFE concentrations, indicating an effect due in part to electrostatic interactions among side chains. Candidates for this interaction are Glu69 and Lys72 in a i,i+3 salt bridge.<sup>10</sup>

To assess the additivity of helical content in BLP-22C, BLP-21N and BLP-42, the CD spectra were converted to  $[\Theta]$ . Figure 4.4 displays the summation of the data for BLP-21N and BLP-22C. Within error, the sums corresponded well with the spectrum for BLP-42 at lower TFE concentrations, but showed an increasing discrepancy as the TFE concentration was raised. At 50% TFE,  $[\Theta]_{222}$  of BLP-42 was greater than the sum of  $[\Theta]_{222}$  of BLP-22C and BLP-21N by a factor of 1.17. This difference was only slightly greater than expected owing to the varying lengths of the peptides (1.08) (see equation 8, chapter 2). Beyond 50% TFE, however, the discrepancy between these values increased beyond the magnitude predicted from chain length and concentration uncertainty. This difference may be attributable to the structural propensity at the union of BLP-21N and -22C (residues 50–53), which was not captured in either of the shorter peptides and the length correction.



**Figure 4.3**: Far UV  $[\Theta]$  of BLP-22C, BLP-21N and BLP-42 at 5 °C. The sum  $[\Theta]$  of samples BLP-22C and BLP-21N is also shown. (A) 0% TFE; (B) 50% TFE; (C) 80% TFE.

# 4.3.2 Helical Propensities of Variant Peptides – CD studies

AGADIR output generated at the residue level suggested that the substitution of a proline residue at position 57 would result in complete disruption of helical character in H4. A similar analysis of the BLP22C-D60R sequence predicted increased helix propensity over the wild-type

sequence in H4 with a strong increase in helix content above pH 4 as a result of glutamic and aspartic acid deprotonation.

The response of each variant peptide to TFE addition was examined and is included in Figures 4.2C and 4.2D for comparison with the wild-type peptide. It is noteworthy that the CD spectra of BLP-22C-N57P did not contain a well-defined isodichroic point. Under conditions of 50% TFE, the spectrum presented a minimum at 206 nm (compared to 203 nm for BLP-22C), the relative intensity of which was lower than anticipated for a helix-coil equilibrium. Both of these spectral features are consistent with sampling of a turn in the peptide.<sup>11</sup> Based on this observation, the  $f_{\rm H}$  results for BLP-N57P should be regarded as an upper limit. Plots of  $f_{\rm H}$  vs % TFE for BLP-22C and its variants are depicted in Figure 5B. In the absence of TFE, both variant peptides displayed a helical content similar to that of BLP-22C. The proline-containing variant BLP-22C-N57P responded to increasing TFE concentration in a fashion similar to that of BLP-22C and reached a maximum  $f_{\rm H}$  of ca. 0.35 at 80% TFE. This suggested either that H4 was not formed in BLP-22C and BLP-22C-N57P or that the boundaries of secondary structure were different in the two peptides. Increasing TFE concentrations caused a more dramatic shift toward helix formation in BLP-22C-D60R than in the wild-type peptide. At 80% TFE, a  $f_{\rm H}$  of ca. 0.55 was reached, approximately 1.5 times the helical content of its wild-type counterpart. At neutral pH, the enhanced stability of H4 in this sample is likely the result of a salt bridge between Glu56 and Arg60. Side chain rotameric state analysis in SCWRL<sup>12</sup> confirmed that the two residues could readily be positioned for favorable electrostatic interaction when supported by a helical backbone.



**Figure 4.4**: Far-UV CD spectra of (A) BLP-22C-D60R and (B) BLP-22C-N57P in various water/TFE mixtures. Spectra were collected at 5 °C with sample concentrations of 30  $\mu$ M. (C) Fraction helicity,  $f_{\rm H}$ , as a function of TFE concentration at 5 °C.  $f_{\rm H}$  Values were calculated using eq 1. BLP-22C-D60R (circles) and BLP-22C-N57P (triangles) and BLP-22C (squares) for comparison.

# 4.3.3 Conformational Properties in Aqueous Solutions – NMR studies

NMR spectroscopy was used to investigate structural propensities at the residue level in individual helices. The <sup>1</sup>H spectrum of each of the peptides was assigned with 2QF-COSY, TOCSY, and NOESY data. Of note, the signals from the lone proline residue in BLP-42 and BLP-21N (Pro40) were readily recognized; a strong NOE between Pro40 CδHs of the major Pro

isomer and the C $\alpha$ H of His39 indicated that the *trans* configuration of the Xxx-Pro bond dominated in both samples.<sup>13</sup> Integration of 1D spectra obtained at various pH demonstrated that the proportion of the *cis* form remained constant at ~10% between pH 3 and 9, values which bracketed the experimentally determined pK<sub>a</sub> of the preceding histidine residue (His39).

In the absence of TFE, the NOESY data of BLP-21N showed no evidence for the formation of regular secondary structure. A series of strong to medium NN(i,i+1) NOESY cross peaks could be traced for Asp60–Ser64 in BLP-22C and Asp60–Thr65 in BLP-42 at 5 °C. The strongest sequential effects were Val61 NH to Asp60 NH, Asp60 C $\alpha$ H, and Gly62 NH. Additionally, a series of  $\alpha$ N(i, i+2) cross peaks were observed in H4. The longer range effects commonly associated with long-lived  $\alpha$ -helices were not detected under these conditions. These observations led us to propose that, on the timescale of the experiment, residues in the aforementioned regions of each peptide occasionally sampled dihedral angles consistent with a helical conformation. The few  ${}^{3}J_{NH-\alpha H}$  values that could be determined were consistent with conformational averaging, further underscoring the transient nature of the helix content in these samples. The data were consistent with observations in the apoprotein, except for the Asp60– Gly62 region, which showed weaker NOEs in the protein than in the peptide.

### 4.3.4 Conformational Properties in TFE Solutions – NMR studies

The amide region of a BLP-21N NOESY spectrum collected in the presence of 50% TFE is shown in Figure 4.5. The spectrum contained medium strength NN(i, i+1) cross peaks for residues Lys34–His39 and residues Gly42–Gly51. A few long-range effects were also detected, indicating that both H2 and H3 were formed to some extent. To determine relative contributions of each region to the overall helicity of the sample, C $\alpha$ H chemical shifts in water were subtracted

from those in 50% TFE solution (Figure 4.6A). The shifts in H2 pointed to lower helical content than in H3. This is in contrast to the observation of a folded H2 in the apoprotein structure and is attributed to the elimination of N-capping interactions in the synthetic peptide. Low helical content was also confirmed in the Pro-Gly-Gly motif. Comparatively large upfield C $\alpha$ H shifts were detected in the H3 region in the presence of 50% TFE.



**Figure 4.5**: Region of the NOESY spectrum of BLP-21N (50:50 mixture (vol:vol) of water and trifluroethanol( $d_3$ ), 100-ms mixing time, 5 °C). Sequential NOE cross peaks for residues 34 to 39 and 42 to 51 are joined by a solid line and a dashed line, respectively.



**Figure 4.6**: C $\alpha$ H chemical shift deviation of BLP-21N, BLP-22C, and BLP-22C variants. The experimental value determined in 50% TFE was subtracted from the value determined using aqueous solutions under similar conditions of temperature and pH. BLP-21N (A); BLP-22C (B); BLP22C-D60R (C); and BLP-22C-N57P (D).

In a 50% aqueous solution of TFE(d<sub>3</sub>), the NOESY data for BLP-22C and variants contained medium to strong  $\alpha$ N(i, i+1) cross peaks for all resolved residues. The NOEs and their intensities are summarized in Figure 4.7. Degenerate amide proton chemical shifts for His63 through Asp66 in some of the BLP-22 samples was responsible for incomplete NOE mapping. NOESY data for each peptide also contained NN(i, i+1) and  $\alpha$ N(i, i+3) cross peaks among residues located in H5 (Ala67–Lys72), suggesting that this helix was partially formed through TFE addition. Medium-range NOE connectivities in H4 varied significantly among the three BLP-22C peptides. A series of  $\alpha$ N(i, i+3) cross peaks were observed between residues Thr55– Ser65 of BLP-22C-D60R and between residues Phe58–Ser65 of BLP-22C-N57P. This series was not observed in BLP-22C, lending further support to the interpretation that both variations increased the helical propensities of all or part of H4.



**Figure 4.7**: A representation of sequential and medium-range cross peaks observed in NOESY spectra collected in 50% TFE at 5 °C. The thickness of the bars indicates the intensity of the cross peak as strong, medium or weak. Bars at the top represent helical regions in the holoprotein. The ligating histidine in marked with an asterisk.

BLP-22C-D60R was observed to be considerably more helical (~25% helix) than BLP-22C in the 50% mixture of TFE and water. The NOE data confirmed that an arginine at position 60 stabilized approximately two helical turns in H4, resulting in enhanced holoprotein-like structural propensity. Examination of the NOESY data collected for BLP-22C-D60R in 50%

TFE solution did not reveal any Glu56–Arg60 side chain NOEs. This, however, is not surprising considering that the corresponding protons are distant even when a salt-bridge is formed, that there is extensive conformational sampling evidenced by  ${}^{3}J$  values, and that the pH may not be well suited for a stable interaction.

Comparison of C $\alpha$ H chemical shifts for BLP-22C and BLP-22C-D60R in 50% TFE compared to those in water (Figures 4.6B and 4.6C) showed greater differences in the region of Ala54–Ser65 of the D60R variant, with the main effect observed immediately before the site of replacement in the region encompassing H4. As expected, the proline of BLP-22C-N57P disrupted helix formation in preceding residues (Figure 4.6D), but had little effect on the TFE response of shifts of following C $\alpha$ H resonances. Such a result could be explained by an N-capping interaction influenced by the proline residue. A preceding glutamic acid residue, however, is known to be a poor N-cap side chain<sup>14</sup> and casts doubt on this interpretation. In spite of this, examination of  $\alpha$ N(i,i+3) cross peaks in the NOESY of BLP-22C-N57P confirmed that the proline substitution had little effect on the C-terminal turn of H4. Of note is the large effect experienced by Val61 in all three peptides. The shift was accompanied by a weakening of the sequential NOEs.

#### 4.3.5 Histidine pK<sub>a</sub>

In order for His39 and His63 to ligate the heme iron, their imidazole side chain must be in the neutral state. For this reason, modulating the pK<sub>a</sub> of histidines in the binding loop may play a role in the heme affinity of cyt  $b_5$ . The pK<sub>a</sub> of His39 and His63 in apocyt  $b_5$  were previously determined to be 7.7 and 7.4, respectively.<sup>15</sup> In this study, the pK<sub>a</sub> of His 63 in BLP-22C was determined to be 7.1 ± 0.1 and that of BLP-22C-D60R 6.9 ± 0.1. The depression of the His63

 $pK_a$  in BLP-22C-D60R is attributable to the substitution of a basic residue for an acidic one. This  $pK_a$  depression corresponds to a shift in equilibrium toward the neutral state at physiological pH and a free energy contribution of ~5 kJ mol<sup>-1</sup>, comparable to the small stabilization in the protein. The magnitude of the  $pK_a$  shift, however, is close to measurement error.

### 4.4 Discussion

### 4.4.1 Effects of TFE at the Residue Level – General Trends

TFE mixtures are often used to expose the helical potential of fragments from folded proteins. The analysis of the results and their extrapolation back to an aqueous medium require consideration of the differential effect that TFE has on the propensities of residues to initiate, propagate, and terminate helices. Rohl and coworkers determined that proline, glycine, and polar  $\beta$ -branched residues retain their helix-breaking characteristics in 40% TFE solutions.<sup>16</sup> All strong helix starts and stops in the BLP peptides contain these residues, leading to the conclusion that these signals are not altered by TFE. In contrast, hydrophobic residues such as valine and phenylalanine lose some of their helix-breaking power in the presence of the cosolvent. Propagation of helical character through the turn region of Val61–Ser64 of BLP-22C in TFE solution may reflect the lost efficacy of valine as a helix breaker.

The mechanism by which TFE stabilizes helical structure in peptides is not completely understood. It is generally agreed, however, that the properties of the backbone are determining.<sup>17</sup> TFE mixtures are less effective than water at solvating and as a result destabilize the coil state. Since this is a backbone effect, differences in helical content among peptides in TFE can be attributed to the ability of side chains to accommodate helical backbone dihedrals. The alteration of the unfolded state stability by TFE and the low occurrence of Phe and Val

residues in the peptides of interest make comparison of helical propensities a valid approach for qualifying the intrinsic properties of the H2–H5 loop.

### 4.4.2 Implications for the Behavior of Cytochrome *b*<sub>5</sub>

The peptides in this study were expected to reveal the structural information encoded in the center of the apoprotein binding loop in the absence of end-to-end constraints. Low but detectable helical propensity existed in each of the H2 to H5 regions. The tendency of H2 was likely underestimated owing to a missing N-cap residue in the peptide; of the remaining helices, H4 had the least helical character. The region comprising the H4–H5 turn (V61-G62-H63-S64) is of special interest. In the holoprotein, the chain exits H4 with a Schellman-like geometry, is extended over His63 and Ser64, and starts H5 with serine N-capping box. In the apoprotein, sequential NOEs connecting D60, V61, and G62 are present though weak. In the peptide, these effects were stronger and the region appeared capable of sampling helical conformation when subjected to TFE. Helix propagation through the H4–H5 peptide is consistent with the absence of strong C-cap for H4 and N-cap for H5. Collectively, the results suggest that H4 refolding in the holoprotein is influenced primarily by contacts with H5 and the heme, and that the H4-H5 chain reversal is influenced by these tertiary interactions. In the apoprotein, this turn displays the smallest <sup>1</sup>H-<sup>15</sup>N NOEs (aside from the termini of the protein), and <sup>15</sup>N transverse relaxation data are indicative of slow conformational exchange.<sup>18</sup> In contrast, H2-H3 exhibited a stronger tendency to fold and maintain the intervening turn.

Ihara and coworkers have compared the role of the axial histidines in cyt  $b_5$  by individual leucine substitution.<sup>19</sup> Their kinetic studies suggest that the C-terminal half of the binding

region, which exhibits larger conformational fluctuations than the N-terminal half, <sup>18</sup> associates with the heme group first. If this is the case, two advantages can be envisioned for the local disorder observed in H3–H5: preventing collapse of the binding site on itself and increasing the probability of efficient cofactor capture.

Introducing a D60R variation to cyt  $b_5$ 's H4 increases the T<sub>m</sub> of the holoprotein by 6 °C. This is attributed to electrostatic stabilization manifested in the presence of backbone constraints enforced by bound cofactor, i.e., in the native holoprotein state. The same interaction may not be reflected in a change in its T<sub>m</sub>, since neither Glu56 nor Arg60 belong to the cooperative core. TFE titrations of BLP-22C-D60R demonstrated that the replacement enhanced the helical propensity of H4 in the free binding loop, an effect that may contribute to the holoprotein stabilization along with a small decrease in the ligating histidine pK<sub>a</sub>. Both effects correspond to a reinforcement of native holoprotein state character in the polypeptide chain.

Analysis of N57P variants of cyt  $b_5$  and of BLP-22C suggested that the alteration had the effect of disrupting the C-terminal turn of H4. This implies an effect opposite to that of the D60R replacement, i.e., disfavoring the final bound state structure. Disruption of heme-protein contacts resulting from the substitution, however, seemed mitigated by unfolded state effects. Another interpretation is that the altered preferences led to improved interactions with the heme group. Although no evidence was found for new contacts in the spectra of this particular variant holoprotein, it is conceivable that the holoprotein structure, which is optimized for multiple properties, can be modified to enhance certain features at the expense of others. Thus, the introduction of non-native interactions can have a stabilizing influence.

### 4.4.3 Assessment of the Reductionist Approach in the Cyt *b*<sub>5</sub> Binding Loop

Transient local interactions in the denatured state of proteins are thought to influence folding by acting as nucleation sites and accessing specific pathways early in the process. The shortrange nature of these interactions has prompted investigations of peptide fragments exposed to TFE. This strategy has been used to model unfolded state interactions in proteins of high helical content with great success. Padmanabhan and coworkers, for example, demonstrated that the most helical regions of the phage 434 Cro protein fragments in TFE correlated well with those of the native protein.<sup>20</sup> Likewise, regions of greatest helical character in regions of our peptides may reflect secondary structural propensities in the apoprotein binding loop.

Investigation of regions of non-helical secondary structural propensity in peptides can prove complex. Sönnichsen and coworkers explored the validity of such a comparison by examining fragments of the amino-terminus of actin, which is known to be of  $\beta$ -strand character.<sup>21</sup> The response of actin peptides to TFE addition led to the conclusion that inherently disordered regions can remain flexible in the presence of the cosolvent. In the absence of inter-strand interactions, extended structure may be disfavored compared to helical structure because of the backbone solvation effects mentioned above. The H4–H5 interhelical region in the holoprotein has an exposed backbone and dihedrals close to those expected for an extended chain. Thus, the BLPs may assume a helix-like state rather than the holoprotein-like structure owing to a similar effect.

Transient interactions may prove to be non-native, as is the case in the  $\alpha$ -helix of the protein G B1 domain.<sup>22</sup> Addition of 30% TFE to the peptide resulted in stabilization of a native-like Schellman motif at the C-terminus accompanied by formation of a hydrophobic staple not observed in the native protein fold. This interaction is thought to be responsible for promoting

specific higher order interactions, which ultimately lead to the final fold in which the staple is broken. In the BLPs, the propagation of helix through the region separating H4 and H5 might then be explained in terms of an altered helix propagation parameter for Val61 in the cosolvent. It is possible that under the influence of a lower dielectric medium (such as a collapsed hydrophobic core) the region has helical character, which must be overcome by other interactions for the native fold to form. In this way, the region may be responsible for tuning the holoprotein stability.

# 4.5 Conclusions

The experimental data collected on peptide fragments distinguished the elements of secondary structure contacting the faces of the heme cofactor in cyt  $b_5$  and helped in the assessment of two types of sequence analyses. The first, which seeks to delineate regions of intrinsic disorder in polypeptide chains, predicted that H3 and H4 should not achieve a stable fold even in the context of the entire protein, i.e., when tethered to an ordered scaffold. This tendency was borne out by the apoprotein data. The isolated peptide studies showed that the behavior was also encoded locally in H4, which in comparison to the other helices, appeared as a weak element of structure under all conditions.

The second sequence analysis, which forecasts the location of helical structure, was also partially successful. H2 (with missing N-cap) and H3 did develop a modest degree of helix in the presence of TFE. Their titration profile was typical of a system in which hydrophobic effects dominate. H4 lacked strong start and stop signals and was not expected to be predisposed toward its holoprotein conformation. It also appeared that the H4-H5 peptide had a weak tendency for chain reversal at the level of His63. This non-native propensity may contribute to the cost of induced refolding in the presence of the heme group. In the H5 region of the H4-H5 peptide, as well as in the D60R variant, electrostatic effects appeared determinant. The consequences of the introduction of a proline were not well predicted, likely because of the complexity of native state interactions.

Examination of the isolated heme-binding loop of cytochrome  $b_5$  revealed a complex system of non-uniform fluctuations governed by structural encoding at the local level. Stabilizing alterations such as D60R appear to affect short-range interactions in the apoprotein, thus influencing the tendency of its backbone to sample holo-like dihedrals. In the case of N57P, however, holoprotein stabilization may be caused in part by a change in conformational entropy of the apoprotein, as well as a more complex system of altered backbone dihedrals, disrupted native interactions and new, non-native interactions. This study underscores the influence that this local encoding is likely to have in cofactor binding, as well as the stability of the holoprotein by modulating the energetics of the apo- and denatured states.

### 4.6 References

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### Chapter 5

#### Response of the H4-H5 Region of Cytochrome b5 to Heme Binding

#### 5.1 Introduction

Many proteins undergo conformational changes in response to heme binding. Cofactorinduced structure formation has been extensively studied using both proteins and model peptides.<sup>1-3</sup> In the case of peptides, information regarding the relationship between secondary structure formation and binding properties has been obtained from samples designed to mimic naturally occurring protein binding sites<sup>4-6</sup> as well as those designed *de novo*.<sup>7-9</sup>

Cofactor-induced structural changes in the water-soluble domain of cytochrome  $b_5$  have been well characterized by study of the complete domain using NMR techniques.<sup>10-14</sup> The presence of heme has been shown to induce the binding loop residues in H3–H5 to fold and contribute side chains to core 1. Holoprotein structures show that additional stabilizing contacts with the heme are provided by H2 and residues at the back of the binding pocket. At the center of the  $\beta$  barrel, several hydrophobic residues constitute core 2. The residues comprising the barrel, as well as H1, H2 and H6 do not experience the same degree of structural change as those located in the binding loop. The cofactor-induced folding of H3, H4 and H5 in the binding loop prompted Knappenberger and coworkers to investigate its contribution to the energetics of core 2 stability using a chimeric construct consisting of the cyt  $b_5$  binding loop grafted to an alternate support protein.<sup>15</sup> Binding loop insertion was found to have a destabilizing effect on the new scaffold than was at the low end of the predicted effect based on a self-avoiding chain, suggesting that the modest stability of core 2 is an intrinsic property of the region. Denaturation of several chimeric constructs led to the conclusion that the loop-scaffold interface is capable of influencing the stability and fold topology of distant regions in the protein, to the point of overcompensating for bearing a loop.<sup>16</sup>

The present study attempts to expand upon the investigation the scaffold contacts by removing them completely. The BLP samples offered a useful system for investigation of the role played by loop end constraints in apoprotein behavior. Their inability to bind heme in solution, however, prohibited their use for assessing changes in the helical character of the loop in the holo state. Here, samples were devised and created to determine the locally encoded helical propensities of H4 and H5 in the heme-bound state.

# 5.2 Peptide-Heme Conjugate Design

Covalent attachment of peptide ligands to heme via the macrocycle propionates has proven an effective means of inducing ligation in several different constructs at concentrations suitable for spectroscopic study.<sup>17</sup> Covalent attachment of heme to biopolymers has been accomplished using propionates as the point of connection. In the case of the cyt  $b_5$  binding loop, the side chain of residue Ser64 points toward solvent, and is positioned to form a hydrogen bond with the carboxyl group of propionate-7 from the bound heme (Figure 5.1A). Banci and coworkers confirmed the stabilizing effect of propionate-7 by reconstitution of cyt  $b_5$  with protoporphyrin IX dimethylester.<sup>18</sup> The resulting disruption of H-bonds to the Ser64 OH and NH produced a holoprotein of reduced stability in which in the orientation of His63 and Ser64 were slightly altered. The apparent ability of Ser64 and heme to interact favorably in this way led to the choice of residue 64 as the point of connection. In an attempt to preserve positioning of the peptide backbone, and thus the register of the histidine with respect to the heme iron, lysine was substituted at this position. Covalent conjugation of the amine side chain via amide bond formation adds only a single bond unit to the motif, providing a modest increase in the flexibility of the linkage, but preserving the approximate distance constraints between the heme and the backbone of the modified peptide (Figure 5.1 B).



**Figure 5.1**: (A) A stereo representation of the H4–H5 region from a crystal structure of bovine cyt  $b_5$  (1cyo) in the ferric state showing the proximity of the Ser64 side chain to the heme propionate. The peptide backbone from residue Gly52 through Thr73 is represented by ribbons. The His63 and Ser64 side chains as well as the heme cofactor are shown as licorice. (B) A schematic representation of the putative hydrogen bond from bovine holocytochrome  $b_5$  (left) and of the amide linkage in the BHC-8 and BHC-22C' constructs. Heme is represented by a black bar and grey sphere. The attached peptide is represented by a grey bar.

To investigate the relationship between helix formation and heme binding in the C-terminal half of the cyt  $b_5$  binding loop, two peptides were designed using the sequence of rat microsomal cyt  $b_5$  as a template. The strategy of covalent heme conjugation was that of Benson and coworkers.<sup>8</sup> The attachment via an amide bond required that only one primary amine be present in the unconjugated peptides. Peptide sequences were therefore altered to contain a single lysine residue at a site suitable for subsequent attachment of a mesoheme cofactor. The N-termini of both peptide samples were acetylated to render them unreactive in the heme conjugation

reaction. Mesoheme was selected for this synthesis because it lacks the potentially reactive vinyl groups of protoporphyrin IX.

The first peptide synthesized (BLP-8) contained Glu59–Asp66 of the cyt  $b_5$  sequence with an S64K variation and an acetylated N-terminus. This sample contained the residues forming the turn between H4 and H5 of the holoprotein structure. A second peptide (BLP-22C') was created using the 22 C-terminal residues of the cyt  $b_5$  binding loop (Gly52–Thr73) with two alterations: S64K and K72E. Replacement of the lysine at position 72 was required to render this residue unreactive during the peptide-heme conjugation. The peptide sequences used are shown in Figure 5.2.

Structure (holo)	<u>H2</u>	_H3	_H4_	H5
Cyt b5 Binding Loop	LTKFLE	EHPGGEEVLRE	QAGGDATENFE	OVGHSTDARELSKT
BLP-8 / BHC-8			E	DVGHKTD
BLP-22C' / BHC-22C'			GDATENFE	DVGHKTDARELSET

**Figure 5.2**: Peptide sequences used in the BLP and BHC constructs discussed in this chapter. The sequences of sample peptides are aligned below the binding loop sequence, with variations in grey. The positions of H2–H5 in the holoprotein are illustrated by black bars.

Investigation holocyt  $b_5$  structures shows that the side chain of lysine at position 72 is poised to form a favorable electrostatic interaction with Glu69. This interaction is also supported by increased helix stabilization of BLP-22C in TFE concentrations greater than 30%. The apparent helix-stabilizing interaction, along with the known helical propensity of this region (H5), required careful consideration when designing the peptide sample. A Pfam alignment of 1058 cyt  $b_5$  sequences (FP00173) shows that the most common pair of residues at these positions is Glu69 and Lys72 (Figure 5.3 A). Analysis of the sequence alignments showed weaker, but significant preferences for alternative pairs at these positions, including Glu69 and Glu72. This pair contains no amine functional groups and suggests that K72E is an acceptable replacement for this construct. Substitution of the acidic Glu for the basic Lys at position 72 would be expected to eliminate any favorable local electrostatic interaction between these side chains, resulting in lower helix content at high TFE concentrations.



**Figure 5.3**: Occupancy analysis of positions (A) 69 and 72 and (B) 68 and 72 from a Pfam alignment of 1058 cyt  $b_5$  sequences. The number of occurrences for each pair is indicated by the number of concentric lines (5 counts per line). Pairs corresponding to the cyt  $b_5$  sequence are highlighted in red. Pairs from the BLP-22C' peptide are highlighted in green.

Further analysis of the Pfam alignment reveals that Glu occurs with the second highest frequency in sequences containing an Arg at position 68 (as in cyt  $b_5$ ) (Figure 5.3 B). The potential interaction between basic and acid residues at these positions is, therefore, expected to compensate partially for the lost Glu69–Lys72 interaction. Analysis of wild-type and K72E

sequences in AGADIR predicted that the impact of this substitution on helix propensity in the region of H5 is marginal, with the variant retaining ~75% of its helical character in this region.

#### 5.3 Results

#### 5.3.1 pH Titrations

BHC-8 and BHC-22C' each consist of two identical peptides covalently linked to a mesoheme, placing the histidine residue of each peptide in close proximity to the heme iron. Under such high effective concentrations, neutral histidine is likely to ligate the heme iron intramolecularly. At low pH, however, the imidazolium side chain of histidine lacks the lone pair of electrons necessary to function as a ligand. For this reason, heme iron ligation by histidine is favored only when the pH of the solution is greater than the  $pK_a$  of the ligating side chain.

Additionally, heme affinity of the histidine side chain may be affected by pH changes resulting in protonation or deprotonation of other titratable side chains within the peptides. Changes in protonation states of other side chains may alter heme-binding by inducing holoprotein-like helix formation, or by changing the overall charge of the attached peptide. Solutions of BLP-8 and BLP-22C' were monitored by CD spectroscopy as a function of pH (Figure 5.4) and the peptides were found to be predominantly random coil across a wide range of pH values.  $[\Theta]_{MRW,222}$  for both samples decreased slightly as pH was raised. BLP-8 showed a slight decrease in negative ellipticity between pH 3 and 5, and a second decrease between pH 8 and 11. These values bracket the ordinary pK<sub>a</sub> of acidic (Glu, Asp) and basic (Lys, Arg) residues, respectively. Lack of a minimum at 206 nm in all spectra also suggests that both peptides are predominantly random-coil under all conditions of pH studied.



**Figure 5.4**: Circular dichroism spectra of (A) BLP-8 and (B) BLP-22C' with varying pH. Sample concentrations were estimated to be 10  $\mu$ M. Spectra were collected at 25 °C.

BHC-8 and BHC-22C' were titrated over a similar range of pH and monitored by UV-visible spectroscopy. The resulting spectra are shown in Figure 5.5. Both samples exhibited a red-shifted  $\lambda_{max}$  with increasing pH; a characteristic of low-spin species in ferric heme complexes. The spectra, however, did not form well-defined isosbestic points, ruling out a two-state model for heme binding as a function of pH.



**Figure 5.5**: UV-visible spectra of BHC-8 (A, B) and BHC-22C' (C, D) at 25 °C under varying conditions of pH.

At pH values between 3 and 5, BHC-22C' exhibited a large shoulder at 360 nm and a slightly red-shifted charge transfer band. A similar, but less pronounced shoulder was present at 360 nm in the spectra of BHC-8, and the charge transfer band of this conjugate did not appear to be shifted. Between pH 5 and 7, the spectra contained a sharp Soret band at ca. 395 nm and the charge transfer band at the wavelength expected of a high-spin heme. Further increase in pH (pH 7–9) resulted in a red-shifted Soret band and a decreased absorbance at 620 nm in both

samples. A Q-band absorbance was detected at 521 nm with a shoulder at 556 nm (BHC-8) and at 523 nm with a shoulder at 554 nm (BHC-22C'), indicating that bis-histidine coordination had been achieved. UV-visible spectra showed no dependence over a range of concentrations (3–50  $\mu$ M) at pH 7.9, suggesting that both axial positions were occupied by intramolecular iron lgation at these concentrations. Finally, under the most basic conditions studied (pH 9–11) both samples exhibited growth of a small shoulder in the charge transfer region of the spectrum concomitant with a slight decrease in the intensity of the Soret band.

The proposed scheme of heme binding with changing pH is summarized in Figure 5.6. At low pH the UV-visible spectra of BHC-22C' exhibited both a red-shifted charge transfer band and a much larger shoulder than BHC-8 at 360 nm. ProtParam was used to estimate the pI of the BHC-22C' peptide, which is 3.95, leading to the conclusion that the spectral properties of BHC-22C' at low pH are the result of aggregation among attached peptides. Lack of aggregation in the BHC-8 sample near its pI (4.29) was attributed to the smaller size of the attached peptides. As the pH increased beyond the pI of the attached 22-mers (pH 5–7), the spectrum of BHC-22C' became more similar to that of BHC-8. The visible spectra of each sample under this pH were characteristic of high spin species, suggesting that the mixture consisted primarily of complexes with only one axial histidine. Lack of a shoulder at 360 nm combined with loss of the charge transfer band and red-shifting of the Soret band at pH 7-9 led to the conclusion that bis-histidine coordination was achieved. Increasing the pH beyond 9 in both samples resulted in a slight decrease in the Soret band intensity and recovery of a small absorption in the charge-transfer These spectral features suggested that competing hydroxide ligands were band region. displacing the histidine ligands to an appreciable extent at this pH.



**Figure 5.6:** A schematic representation of the dominant ligation state in BHC-8 and BHC-22C' samples under various conditions of pH. The heme plane is represented by a black bar, the heme iron by a sphere and the attached peptide by grey bars.

The absorbance of each sample at 402 reported in Figures 5.5A and 5.5C was used to construct a pH-response plot for BHC-8 and BHC-22C'. The resulting curves were fit to a modified Henderson-Hasselbalch equation similar to that shown in eq (1) of chapter two, with absorbances substituted for chemical shift. The results are shown in Figure 5.7. The fits returned pK<sub>a</sub> values of  $5.75 \pm 0.04$  for BHC-8 and  $5.59 \pm 0.08$  for BHC-22. This difference in pK<sub>a</sub> values is close to the estimated error associated with the experiment. It is interesting to note, however, that the slope of the transition (Hill coefficient) in each plot varies. Fitting for BHC-8 returned an n-value of  $1.2 \pm 0.1$ , and that of BHC-22C' was determined to be  $0.8 \pm 0.1$ . The quoted uncertainties represent the standard deviation of the fitted parameters and underestimate measurement errors.



**Figure 5.7:** Plots of absorbance at 402 nm vs pH for BHC-8 (circles) and BHC-22C' (squares) from spectra shown in Figure 5.4 A and 5.4 C. Lines represent fits to a modified Henderson-Hasselbalch equation.

#### 5.3.2 Helical Content of Peptides and Peptide-Heme Conjugates

It was previously determined (chapter 4) that addition of TFE to model peptides from the cyt  $b_5$  binding loop enhanced helical content in the regions of H4 and H5. It was also discovered that weak helix stop and start signals between H4 and H5 contributed to helix propagation through the intervening residues, which form a turn in the holoprotein. To explore the effects of heme binding on the helical propensity of attached peptides, samples were titrated with TFE at pH 7.9. Throughout the titration, helix content was monitored by CD spectroscopy and ligation of mesoheme iron by histidines was assessed using UV-visible spectroscopy.

To describe the effect of the attached heme on the secondary structure of the peptide, it was first necessary to account for the two residue substitutions introduced in BLP-22C to create BLP-22C'. The unconjugated peptide was titrated with TFE and monitored by CD for this purpose.

Additionally, any effect of TFE addition on the heme-binding of the sample caused directly by the solvent composition (rather than helix induction in the peptide) was accounted for by titration of BHC-8 monitored by UV-visible spectroscopy.

Figure 5.8 shows the CD response of BLP-22C' and BHC-22C' as a function of TFE concentration. Each spectrum in aqueous solution had little helical character. For both samples, increased TFE concentration resulted in a more negative ellipticity at 206 nm and 222 nm, as well as an isodichroic point at 203 nm. Under low TFE concentrations (< 50% by volume) BLP-22C' and BHC-22C' spectra are similar in shape and intensity. At 50% TFE and beyond, however,  $[\Theta]_{MRW,222}$  of BLP-22C' becomes more negative than that of BHC-22C', suggesting greater helix content in the free peptide. Samples BLP-8 and BHC-8 yielded CD spectra consistent with random-coil dihedrals across the range of TFE studied (data not shown).



**Figure 5.8**: CD spectra of (A) BLP-22C' and (B) BHC-22C' at 25 °C in 20 mM phosphate buffer (pH 7.9) with varying proportions of TFE. Sample concentrations were 8  $\mu$ M.

To compare the effect of TFE on helix content,  $f_{\rm H}$  was calculated for BLP-22C' and BHC-22C' samples under all concentrations of TFE used. Figure 5.9 shows the calculated fraction helix of both samples as a function of volume % TFE in phosphate buffer (20 mM, pH 7.9). Similar data for the wild-type sequence peptide (BLP-22C, unbuffered, pH 3.5) are included for reference. Protonation of acidic side chains in this sample is likely to affect the helix-coil equilibrium and should be considered when making a direct comparison.



**Figure 5.9**: Calculated  $f_{\rm H}$  of BLP-22C' (circles) and BHC-22C' (diamonds) vs TFE concentration. Aqueous components contained 20 mM phosphate buffer at pH 7.9.

#### 5.3.3 Heme Ligation in TFE Solutions

The effect of TFE on heme ligation was examined by UV-visible spectroscopy (Figure 5.10). BHC-8 and BHC-22C' both produced similar spectra under low TFE conditions (0-33%). Spectra contained a sharp Soret peak at 402-404 nm. The visible region also contained a  $\beta$  band at 525 nm. Both of these features are commonly seen in low-spin, hexacoordinate heme species.
TFE concentrations of 50–75% resulted in decreased intensity of both the Soret and  $\beta$  bands. The intensity of the charge transfer band, however, did not decrease. A shift in the complex BHC-22C' toward a high-spin state at these TFE concentrations is the most likely cause of these features.



**Figure 5.10**: UV-visible spectra of (A) BHC-8 and (B) BHC-22C' at 25 °C with varying proportions of TFE. The aqueous component was prepared in 20 mM phosphate buffer (pH 7.9). Sample concentrations were  $7-8 \mu$ M.

### 5.3.4 Optical Spectrum of Reduced BHC-22C'

The ligation state of BHC-22C' at pH 7.9 was probed further by collection of a visible spectrum in the presence of dithionite as an agent capable of reducing the heme iron (Figure 5.11). The Soret band in the reduced complex was red-shifted relative to the oxidized complex and exhibited a large shoulder at approximately 420 nm;  $\alpha$  and  $\beta$  bands at 550 and 520 nm, respectively, were present but poorly resolved.



**Figure 5.11:** Visible spectrum of BHC-22C' in the reduced state (20 mM phosphate, pH 7.9, 25 °C). (A) The Soret region contains a broad peak with a maximum at 410 nm. A significant shoulder at 420 nm is also present. (B) The  $\alpha$  and  $\beta$  peaks at 550 nm and 520 nm, respectively are broadened relative to those expected of a low-spin species.

### 5.4 Discussion

#### 5.4.1 Effect of Side Chain Ionization

The UV-visible spectra of BHC-8 and BHC-22C' with varying pH indicated that neither system underwent a simple two-state transition with varying pH. The pK<sub>a</sub> values obtained from pH titration curves are therefore only apparent and are considered for comparative purposes only. The lower apparent pK<sub>a</sub> of BHC-22C' hints that the presence of the H4 and H5 peptides promotes heme binding. Lack of increased helix content in BHC-22C' rules out native-like interactions between the peptide and heme as the source of this pK<sub>a</sub> depression, leading to the interpretation that non-specific interaction may occur between the attached peptide and the heme. The decreased *n* value may also be explained by this phenomenon, as additional interactions between peptide and heme may further distort the heme plane, decreasing its affinity for the second histidine.

#### 5.4.2 Effect of Iron Ligation on the Population of Helical Structure

To best approximate the heme ligation state observed in holocyt  $b_5$ , CD and UV-visible spectroscopy experiments were conducted on BHC-8 and BHC-22C' in solutions buffered to a pH of 7.9. Aqueous samples at this pH produced UV-visible spectra similar to one another, each containing a maximum absorbance at 404 nm and lacking a charge-transfer band at 630 nm. These features suggest that the complexes primarily adopt a strained, hexacoordinate ligation state at this pH. A likely source of this strain is a sub-optimal ligand geometry imposed by the short covalent linker joining the peptides to the heme propionates.<sup>19</sup> Benson and coworkers observed a similar effect in peptide-sandwiched mesoheme (PSM) constructs containing covalently attached peptides of high helical propensity.<sup>7</sup> In the case of certain PSM's, the source of the strained ligation was the inability of the constructs to accommodate both helix formation and heme ligation,<sup>7</sup> whereas in the case of BHC-8 and BHC-22C' the most likely cause is a combination of two factors, both of which stem from geometric restraints imposed by the linker. First, lack of sufficient length and flexibility in the covalent linker may result in a sub-optimal orientation between the His80 imidazole and the porphyrin. Second, the additional bond unit in the linker may reduce or eliminate the favorable interaction between the linker carbonyl and the Ser64 NH.

Hexacoordinate iron(II) porphyrin complexes bearing N-alkylimidazole ligands are known to exhibit low-spin optical characteristics including a sharp Soret peak at 410 nm and well-resolved  $\alpha$  and  $\beta$  bands at approximately 547 and 518 nm, respectively. Similar mono-histidine complexes contain a Soret band that is red-shifted by approximately 10 nm and a single, broad absorption in the  $\alpha$ - $\beta$  region of the spectrum. The reduced BHC-22C' optical spectrum appears to contain an amalgamation of these spectral features (the broad shoulder on the Soret peak and lack of well-defined minima between  $\alpha$  and  $\beta$  bands), further substantiating the interpretation that the construct exists as a mixture of bis-histidine and mono-histidine species.

It has been demonstrated previously that helix formation and heme ligation can be coupled to one another in samples designed to adopt favorable binding geometries when folded.<sup>9</sup> Altered helical propensities of the attached peptides in BHC-22C' can therefore be attributed to the conformational restraints imposed by ligation. CD data did not indicate any increase in helical content of the peptides in BHC-22C' when ligated to mesoheme in aqueous solution. The apparent lack of helix enhancement demonstrates that macrocycle proximity and conformational restraints imposed by ligation do not sufficiently promote the contacts needed to stabilize H4 and H5. It is likely that additional restraints imposed by the presence of core 2 are required to induce helix formation under these conditions.

To assess changes in the helical propensity of the attached peptide in BHC-22C', TFE was added to the sample. UV-visible data confirm that heme ligation is preserved in BHC-8 at cosolvent concentrations up to 75% and in BHC-22C' at concentrations of up to 33%. The CD spectra of BLP-22C' and BHC-22C' indicate that the helical content of the free peptide exceeds or matches that of the attached peptide under all TFE conditions studied. Reduced helical content of the attached peptide is attributable in part to the constrained dihedrals within the linker imposed by iron ligation. This motif, however, is not expected to contribute significantly to  $[\Theta]_{MRW,222}$ , leading to the conclusion that helix content in H4 and H5 remains unchanged within the error of the measurements.

Holoprotein-like contacts between H4, H5 and the attached mesoheme are only possible if the region Val61–Thr65 assumes a turn. It has been determined that this region of the BLP peptides has a tendency to sample helical dihedrals in the presence of TFE, indicating that high concentrations of the cosolvent may interfere with native-like structure formation. Reduced helical content in BHC-22C' at 50–75% TFE can be envisioned to be the result of competition between these two states. Such a situation, however, would be expected to generate CD spectra containing a third component assignable to the turn. The well-defined isodichroic casts doubt on this explanation.

Reduction of hexacoordinate character in BHC-22C' as helix content continued to increase at 50–75% TFE can be explained by helix propagation into the region containing the ligating histidine. The helical dihedrals imposed on the backbone in this region are likely to be inconsistent with those needed to maintain iron ligation, increasing the strain of the imidazoleiron bond and therefore disfavoring the hexacoordinate state. Lower helical propensity in the attached peptides under these conditions is consistent with iron ligation in competition with helix formation in the Val61–Thr65 region.

#### **5.4.3** Comparisons to Protein Behavior

Ihara and coworkers investigated H39L and H63L variants of cyt  $b_5$ .<sup>20</sup> Their conclusion was that the H4–H5 region of the binding loop is more effective at heme capture than the H2–H3 region. The authors postulated that the greater heme affinity is an enthalpic effect attributable to a larger contact area with the heme, and that the greater degree of fluctuation in H4–H5 of the apoprotein may exist to facilitate formation of these contacts. If this were the case, a nearby, properly oriented heme would be expected to promote holoprotein-like interactions in a model compound. Lack of increased helix content in ligated BHC-22C' suggests that helix formation is coupled to additional conformational restraints that exist in the protein. These restraints may be provided by core 2 in the form of loop closure, which would lower the entropic penalty of

induced refolding. Alternately, the distance and orientation of the loop termini imposed by core 2 may promote specific interactions in the binding loop, thus modulating the enthalpy of refolding. In light of the effects of loop–scaffold alterations to an SH3-like domain and of H6 disruption on apo- and holoprotein stability, it is likely that both of these factors are at work in holocyt  $b_5$ .

In addition to the absence of core 2, a second noteworthy difference between the BHC-22C' construct and the heme binding loop is the presence of two H4–H5 peptides per heme group. In the protein, each half of the binding loop forms a distinct network of protein-heme interactions. In contrast, BHC-22C' contains two C-terminal halves, each of which is expected to adopt a similar conformation upon binding. It is possible that the H2–H3 region of core 1 is optimized to create stabilizing contacts with the heme in the presence of distortions and conformational restraints imposed by the H4–H5 region. Sandwiching the heme between two H4–H5 regions could, therefore create a situation in which binding of the second peptide is less favored owing to the presence of the first ligand on the opposing face of the heme. Such a situation would be expected to result in a lowered Hill coefficient, which is seen in the BHC-22C' sample.

#### 5.5 Conclusions

Evidence of strained ligation in the constructs imposed by the covalent linker motif reveals that the BHC constructs may not ideally model the heme binding seen in holocyt  $b_5$ . Similar optical characteristics in BHC-8 and BHC-22C' suggest that the covalent linkage may be the cause of this strain. Careful design of a more appropriate covalent attachment strategy may produce a new set of BHC samples with optimized heme binding characteristics. In chapter 4, H4 of the isolated cytochrome  $b_5$  binding loop was shown to possess the greatest intrinsic disorder of the four helix-forming segments in the region. In this chapter, covalent heme conjugation to peptides was shown to promote bis-histidine heme ligation. The lack of increased helix content in the heme-ligated form of BHC-22C' underscores the importance of the loop-scaffold contacts and tertiary interactions to achieving the proper, folded holoprotein conformation.

## 5.6 References

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#### **Chapter 6**

### **Future Directions**

#### 6.1 BHC Sample Design Considerations

The lack of helix stabilization in H4 and H5 of the BHC-22C' construct may be attributable in part to several design elements of the constructs, including strained heme ligation, lack of loop end restraint, absence of contacts with the H2–H3 residues or the presence of two H4-H5 peptides. Each of these design elements can be assessed to ensure that the effect of cofactor capture on helix formation in the binding loop of cyt  $b_5$  is properly modeled.

## 6.2 Optimization of Heme-Peptide Linkage Motif

Strained ligation in BHC-8 and BHC-22C' may be the consequence of the geometry or length of the covalent linker. Investigation would require creation of a BHC-8 using ornithine in place of lysine, resulting in an equal number of bonds between the heme propionate and the ligating histidine compared to the holoprotein (Figure 6.1). An unstrained ligation in the new construct would support the former hypothesis, whereas strained ligation would imply that the latter is correct. If the new construct were to prove sub-optimal, longer linkers could be considered in their place, at the risk of introducing unintended entropic effects.



**Figure 6.1**: A proposed change to the covalent peptide linker motif. (A) The putative Ser64 OH-propionate hydrogen bond from the crystal structure of bovine holocyt  $b_5$ . (B) The lysine-propionate amide linkage used in this study. (C) The ornithine-propionate amide linkage proposed for future studies.

### 6.3 Inclusion of the H2-H3 region in BHC samples

The BHC samples used in this study were intended to model only the H4–H5 region of holocyt  $b_5$ , resulting in a C2 symmetric construct with respect to the ligand peptides. The H2–H3 and H4–H5 regions of cyt  $b_5$ , however, have distinct sequences and have been shown to interact differently with the cofactor. In light of this, investigation of the heme-bound H2–H3 region may provide additional information regarding factors stabilizing core 1 in the holoprotein.

A BHC sample containing the H2–H3 peptide could be created by substitution of a lysine or ornithine of appropriate stereochemistry for Gly41 (Figure 6.2). Ultimately, a sample consisting of one BLP-21N' and one BLP-22C' peptide attached to the same

mesoheme would provide the most holoprotein-like sample. This mixed peptide construct could be synthesized by reaction of mesoheme active ester with an equimolar mixture of BLP-21N' and BLP-22C' peptides followed by chromatographic isolation of the hetero-substituted construct. With appropriate covalent attachment, analysis of such a compound should lead to a meaningful approximation of the role played by loop residues in holoprotein fold stability.



Figure 6.2: A stereoview of the H2–H3 region of core 1 in rat microsomal cyt  $b_5$  (PDB ID 1AQA).<sup>1</sup> The peptide backbone is shown as ribbons. His63 and the heme cofactor are represented as licorice. Gly41 protons are shown to demonstrate their proximity to the heme propionates.

## 6.4 Mono-Substituted BHC Samples

The heme-binding behavior of BHC-8 and BHC-22C' proved to be non-two-state as a function of pH. It is possible that the ligation of one histidine causes distortions of the heme group or generates steric hindrances that lower the affinity for the second histidine. In order to characterize heme affinity in the absence of these factors, a construct may be devised in which only one BLP is able to bond to the heme. Attachment of a single peptide to mesoheme has been accomplished previously using stoichiometric control of active-ester coupling.<sup>2</sup> Application of this method to the synthesis of a 1:1 complex of BHC-22C' and mesoheme is expected to result in a compound that would form a single

histidine-iron bond, allowing more accurate characterization of the first binding event. Comparison to data presented in this work may provide insight into the consequence of attaching a second, identical peptide to the mesoheme.

## 6.5 Implications for Future Research

Study of peptide fragments derived from natural proteins has been applied successfully to elucidate sequence-structure relationship in proteins. Historically, the reductionist approach has been used to search for residual native structure in a search for folding nucleation sites<sup>3-5</sup> and determinants.<sup>6</sup> Only recently have researchers started to understand the contribution of intrinsic disorder and non-native interactions to unfolded state behavior.<sup>7-10</sup>

This work highlights the role of disorder and non-native (or holoprotein-like) structural propensities in the structural response of a hemoprotein to cofactor insertion. The use of peptide fragments and covalent cofactor attachment, however, need not be confined to this class of proteins. For example, the cobalamin transporter BtuB from *E. coli* was recently shown to undergo significant conformational change when bound to substrate.<sup>11</sup> Local structural propensities of small regions from the BtuB protein in absence and presence of cofactor may be elucidated by construction of model compounds analogous to those presented here. In this way, many proteins that undergo a conformational change in response to cofactor or substrate binding can be analyzed, providing insight into the thermodynamics of cofactor capture.

# 6.6 References

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## MEMBERSHIPS AND AWARDS

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