MAGNETIC RESONANCE IN CHEMISTRY, VOL. 35, 477–481 (1997)

^{19}F NMR Studies of the Recombinant Human Transferrin *N*-Lobe and Three Single Point Mutants

Linda A. Luck,[1]* Anne B. Mason,[1] Kerry J. Savage,[2] Ross T. A. MacGillivray[2] and Robert C. Woodworth[1]

[1] Department of Biochemistry, University of Vermont College of Medicine, Burlington, Vermont 05405, USA

[2] Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC V6T 1Z3, Canada

^{19}F NMR was utilitized to study human serum transferrin, which is a bilobal protein with a high-affinity metal-binding site in each lobe. The *N*-terminal lobe of the recombinant protein (residues 1–337; hTF/2N) and three single point mutants, H207E, W8Y and W128Y, were expressed in baby hamster kidney cells grown in media supplemented with 5-fluorotryptophan (5-F-Trp). The three tryptophan residues gave three well resolved ^{19}F NMR resonances, which were assigned by site-directed mutagenesis of two of the three Trp residues to Tyr. It was found that conformational changes are induced by metal binding to hTF/2N and a site-directed mutant H207E which has a higher binding affinity for Fe(III). Shifts in the ^{19}F NMR spectra indicated changes when proteins bound Fe(III) or Ga(III) along with a synergistic anion, e.g. oxalate or carbonate. The resonance for 5-F-Trp 264 did not change frequency during titration with either metal in hTF/2N or the H207E mutant. Two resonances corresponding to Trp 128 and Trp 8 showed high-field shifts upon metal binding. These studies have shown that the fluorine nucleus is sensitive to local conformational changes in the binding pocket when the synergistic anion is changed from carbonate to oxalate. In addition, the fluorine nucleus can identify areas of the protein which experience secondary structural change when ligand binds. The solution NMR studies showing dynamic changes are complementary to the crystal structures of this family of Fe(III) binding proteins. © 1997 by John Wiley & Sons, Ltd.

Magn. Reson. Chem. **35**, 477–481 (1997) No. of Figures: 6 No. of Tables: 1 No. of References: 25

Keywords: ^{19}F NMR; metalloprotein; 5-fluorotryptophan; conformational change

Received 20 December 1996; revised 21 February 1997; accepted 25 February 1997

INTRODUCTION

^{19}F NMR spectroscopy is increasingly used to probe structural and conformational features of proteins too large for conventional NMR methods.[1] Because fluorine can be biosynthetically incorporated into proteins without deleterious effects on structure, ^{19}F NMR has been also shown to be a valuable tool for mechanistic studies.[2] Typically, fluorine labeled proteins are grown in media containing fluorinated amino acids in *E. coli* or yeast systems. Expression of large human proteins which require post-translational modification and that contain many disulfide bonds often result in very low yields of functional protein in bacterial systems. Thus, we have developed methods for 5-fluorotryptophan (5-F-Trp) incorporation into the *N*-terminal lobe of human transferrin and three single point mutant proteins expressed in baby hamster kidney cells in tissue culture.

Human serum transferrin is a *ca.* 80 kDa glycoprotein comprising two homologous lobes each with the ability to bind reversibly a single Fe(III) ion together with carbonate as synergistic anion. The function of the protein is to sequester and transport iron throughout the body and to prevent the occurrence of free iron in the blood.[3–5] Structural studies of three homologous proteins, human lactoferrin, rabbit serum transferrin and human serum transferrin, have shown that each of the two lobes (joined by a short bridging peptide) is made up of two domains with a single high-affinity binding site located in the inter-domain cleft.[6–8] In each binding site, the Fe(III) ion is coordinated to the side-chains of two tyrosines, one histidine, an aspartic acid and two oxygens from carbonate [see Fig. 1(A) and (B)].[9] Numerous studies have shown that the site can be occupied by many different metal ions[10,11] and that a number of substituted carboxylates can serve the role of the synergistic anion.[12,13] Biophysical studies indicate that significant conformational changes accompany metal binding.[14–16] These changes appear to be critical for receptor recognition, binding and subsequent transport of iron into cells. Crystallographic studies of lactoferrin detail the conformational changes in this protein at the molecular level.[17] Superposition of the apo- and iron-loaded *N*-lobe reveals a 54° domain rotation about a central point on the two anti-parallel inter-domain strands. Closure of the protein appears to involve two hinges which produce a see-saw motion. Low-angle x-ray scattering studies suggest that Asp 63 from the N1

* *Correspondence to*: L. A. Luck. Present address: Department of Biology, Clarkson University, Potsdam, NY 13699, USA.

Contract grant sponsor: USPHS; Contract grant number: DK21739.

CCC 0749–1581/97/070477–05 $17.50

domain (residues 1–90 and 252–320) may provide the trigger for cleft closure.[18]

The present study, using NMR and site-directed mutagenesis, was undertaken to gain further insight into the dynamic nature of the binding of metal to the *N*-terminal lobe of human transferrin and the H207E mutant (the 207His → Glu mutant of hTF/2N, where hTF/2N is the recombinant *N*-lobe of human serum transferrin). The H207E mutation in the hinge region was made to mimic the amino acid substitution found at this site in lactoferrin which binds metal ions one or two orders of magnitude more tightly than human serum transferrin.[19] Reliable probes of cleft closure in transferrin in solution have important implications for the mechanism of binding and release of iron under physiological conditions.

EXPERIMENTAL

Materials

5-Fluorotryptophan (5-F-Trp) and 3-fluorophenylalanine (3-F-Phe) were obtained from Sigma Chemical. D_2O (99.8%), purchased from Aldrich, was used as the lock solvent for the NMR studies. Oligonucleotides for DNA sequence analysis and mutagenesis were synthesized on an Applied Biosystems Model 391 DNA synthesizer. Gallium ICP/DCP standard Specpure was purchased from AESAR. The 10^4 μg ml^{-1} standard contains 143.4 mM Ga(III) in 5% HNO_3. All reagents used in the production and purification of the proteins were of analytical grade or purer. Custom-made DMEM-F12 tissue culture medium, lacking all of the aromatic amino acids and phenol red, was supplied by Gibco. The serum replacement, Ultroser G, was obtained from Gibco. More recently LPSR-1, which is the same as Ultroser G, has been purchased from Sigma.

Production of 5-Fluorotryptophan-labeled proteins

The techniques and materials used to generate the unlabeled hTf/2N and mutants have been described previously.[20,21] Production of the 5-F-Trp-labeled proteins was accomplished by providing 5-F-Trp and unsubstituted Trp at a 1 : 1 ratio in the custom-made DMEM-F12 medium at the level of 9.0 mg l^{-1}. Protein concentrations in the tissue culture media were determined by immunoassay as reported previously.[21]

Cloning and site-directed mutagenesis

To allow assignment of NMR signals to specific tryptophan residues in the amino acid sequence, mutagenesis of Trp 8 and Trp 128 to Tyr was carried out using the PCR-based method of Nelson and Long.[22] The mutagenic oligodeoxyribonucleotides 5′-AAAACTGTGAGATACTGTGCAGTGTCG-3′ (for W8Y, the Trp–Tyr mutant at position 8 of hTF/2N) and 5′-AGGTCCGCTGGGTACAACATCCCCATA-3′ (for W128Y, the Trp–Tyr mutant at position 128 of hTF/2N) were synthesized on an Applied Biosystems Model 391 DNA synthesizer and used as primers for PCR with an appropriate restriction fragment of hTF/2N cDNA cloned into pUC18 as a template. After PCR-based mutagenesis, the complete DNA sequence of the restriction fragment was determined to confirm the presence of the intended mutation and the absence of other mutations introduced by PCR. The restriction fragment containing the mutation was ligated to the remainder of the hTF/2N coding sequence in Bluescript. The mutated hTF/2N cDNA was then excised from Bluescript with *Xba*I and *Hin*dIII, made blunt-ended with the Klenow fragment of DNA polymerase I in the presence of dNTPs and ligated into the *Sma*I site of pNUT.

NMR measurements

NMR samples contained 0.5 ml of 0.9–1.9 mM protein in 100 or 10 mM KCl and 10% D_2O, adjusted to pH* 7.0 (pH* = pH determined in solutions containing D_2O but not corrected to pure water). Samples for ^{19}F NMR spectroscopy contained 3-F-Phe as an internal standard (−38.0 ppm relative to trifluoroacetic acid at 0 ppm). Aliquots of 50 mM $Ga(NTA)_2^{3-}$ (NTA = nitrilotriacetate) were added to give the final concentrations indicted in the figure captions. To quantify the extent of fluorine incorporation, the ^{19}F NMR spectrum of a given sample was obtained with a delay of 5 times T_1. The concentration of ^{19}F nuclei was quantitated by comparing the integral of all well resolved ^{19}F protein resonances to the integral of the 3-F-Phe internal standard. Longitudinal relaxation times (T_1) of the ^{19}F NMR spectral resonances were obtained using the inversion–recovery method. ^{19}F NMR spectra were obtained at 470 mHz on a General Electric GN 500 instrument fitted with a 5 mm ^{19}F probe. Standard coupled spectral parameters were a 12 000 Hz spectral width, 16 K data points, 60° pulse width, 1.5 s relaxation delay and 25 Hz line broadening with temperature control at 25 °C. The NMR experiments averaged 2000–5000 acquisitions for each spectrum.

RESULTS

Assignment of ^{19}F resonances

The positions of the three Trp residues in hTF/2N are depicted in Fig. 1. Incorporation of 5-F-Trp into hTF/2N gives rise to three distinct ^{19}F resonances which are well resolved in the spectrum of the apoprotein (Fig. 2). Integration of each of the three resonances indicates that 5-F-Trp is incorporated equally well at each of the three positions. The amount of incorporation of the fluorine label is estimated to be 15% out of a maximum possible 50%.

Assignment of the ^{19}F NMR resonances in the spectrum to specific Trp residues in the sequence was accomplished by site-directed mutagenesis of two of the three Trp residues to Tyr. After metabolic labeling of each of the two mutants with 5-F-Trp, and isolation

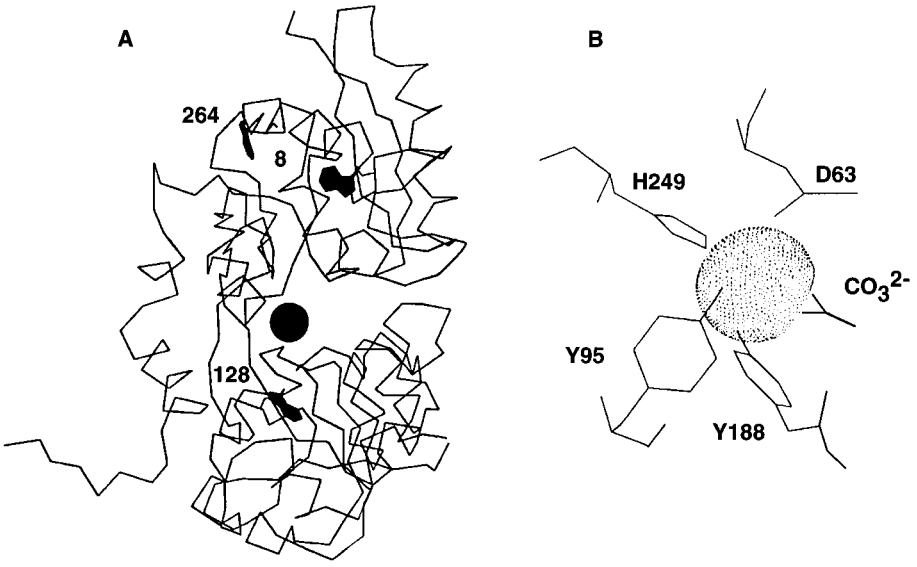

Figure 1. (A) An α-carbon backbone structure of the Fe(III) loaded *N*-lobe of human serum transferrin, showing the location of the three Trp residues in the structure.[9] (B) The ligands surrounding the Fe(III) in the binding pocket.

and purification of the protein, the spectra were recorded (Fig. 2, W8Y and W128Y). The absence of the resonance for the mutated tryptophan makes possible the unequivocal assignment of all three resonances: the low-field resonance to Trp 128, the middle one to Trp 264 and the high-field resonance to Trp 8.

Effects of iron binding on the ^{19}F NMR spectra

^{19}F NMR spectra of iron saturated 5-F-Trp hTF/2N and the H207E mutant were recorded (Fig. 3). The synergistic anion was carbonate in both preparations. In the iron complexes, there is a complete loss of the low-field resonance associated with Trp 128 in the apo-proteins, but the resonance assigned to Trp 264 doubles in size (Fig. 3). Thus, the resonance for Trp 128 moves to the position occupied by Trp 264. Also observed in these spectra is a small shift in the resonance for Trp 8 when compared with the apo-forms of the proteins. Significantly shorter T_1 values for the Trp resonances are observed for iron-containing hTF/2N (Table 1) owing to the paramagnetic effects of the Fe(III).

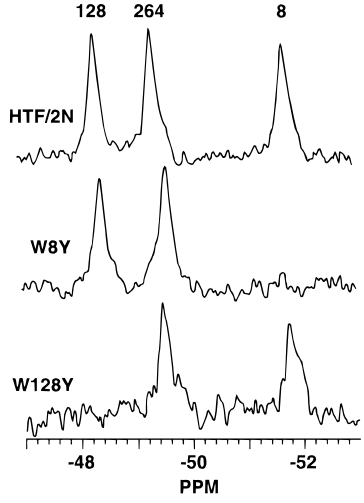

Figure 2. Assignment of the 5-F-Trp ^{19}F NMR resonances by site-directed mutagenesis for apo-hTF/2N. Shown are spectra for the hTF/2N and indicted mutant proteins, each labeled with 5-F-Trp. Resonance assignments are indicated above the top spectrum. Samples were in 100 mM KCl, 10% D_2O and 100 μM 3-F-Phe as an internal standard (not shown in spectra) at −38 ppm relative to 0.1 M trifluoroacetic acid; spectra were recorded at 470 MHz at 25 °C.

Titration of hTF/2N and H207E with Ga(III) monitored by ^{19}F NMR

Titration of 5-F-Trp-labeled hTF/2N and the H207E mutant with increasing amounts of Ga(III) was carried

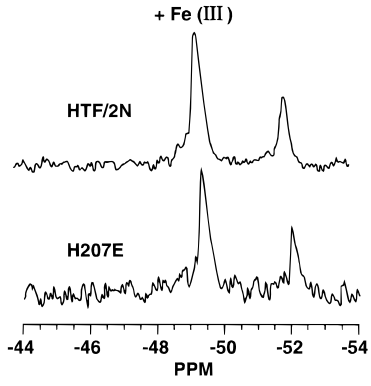

Figure 3. Effect of Fe(III) binding on the ^{19}F NMR resonances of hTF/2N and H207E. Shown are the spectra for the 5-F-Trp-labeled proteins in the presence of saturating amounts of Fe(III) and carbonate as the synergistic anion. Conditions were otherwise as in Fig. 2.

Table 1. T_1 values (±0.06 s) for 5-F-Trp residues in hTF/2N under different conditions of ligation

Metal ion	Trp 128		Trp 264	Trp 8
None	1.24		0.91	0.83
Fe(III)		0.39		0.48
Ga(III)	0.75		0.71	0.75

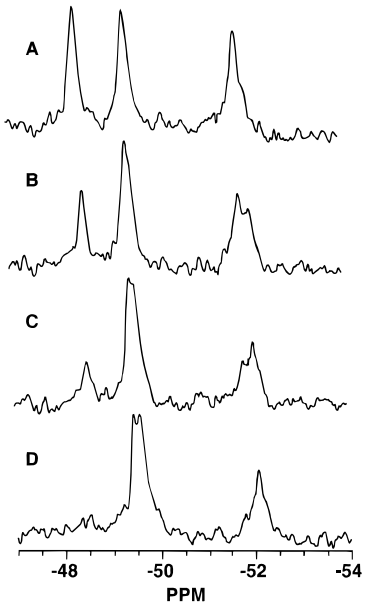

Figure 4. Titration of 5-F-Trp-labeled hTF/2N (0.9 mM) with $Ga(NTA)_2^{3-}$ in the presence of 4 mM oxalate: (A) no additions; (B) with 0.3 mM Ga(III) and 4 mM oxalate; (C) with 0.5 mM Ga(III) and (D) with 1.9 mM Ga(III).

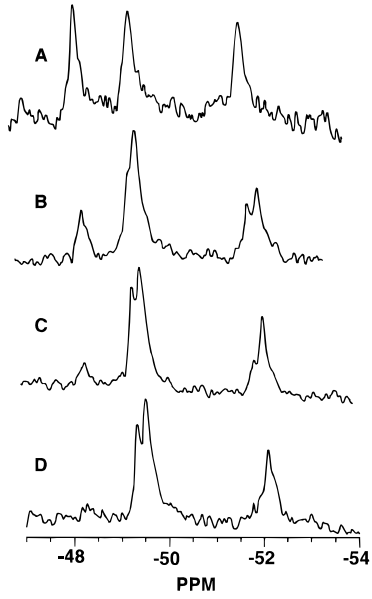

Figure 6. Titration of the 5-F-Trp-labeled H207E (1.9 mM) with $Ga(NTA)_2^{3-}$ in the presence of 4 mM oxalate: (A) no additions; (B) with 0.87 mM Ga(III) and 4 mM oxalate; (C) with 1.6 mM Ga(III) and (D) with 3.8 mM Ga(III).

out in the presence of 4 mM oxalate as the synergistic anion to monitor the effects of metal binding. The rationale for using both gallium and oxalate for NMR studies has been elaborated by Kubal *et al.*,[23] i.e. diamagnetic gallium is used in preference to paramagnetic iron, which can cause significant line broadening in the spectra. Gallium, which has a similar ionic radius to iron, has been shown to bind specifically to both lobes of human serum transferrin although with an affinity some two orders of magnitude (10^{-2}) less than that found for iron.[24] Oxalate has been used as the synergistic anion in a number of studies because it is easier to maintain at a given concentration than the physiological anion, carbonate.

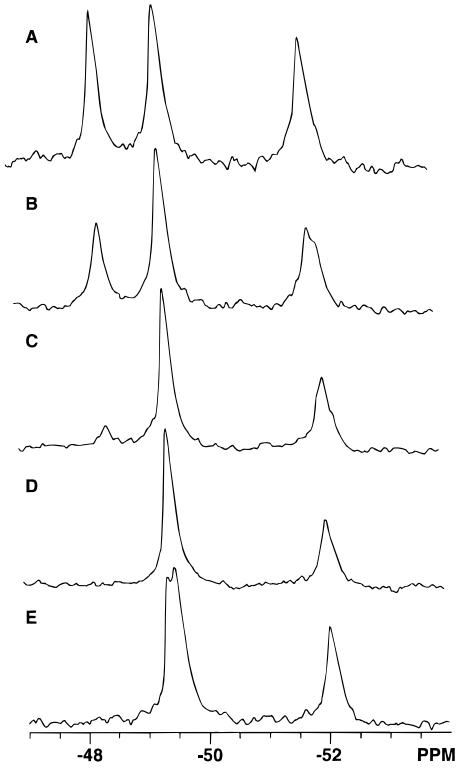

Figure 5. Titration of 5-F-Trp-labeled hTF/2N (0.9 mM) with $Ga(NTA)_2^{3-}$ in the presence of 4 mM hydrogencarbonate: (A) no additions; (B) with 0.3 mM Ga(III) and 4 mM hydrogencarbonate; (C) with 0.75 mM Ga(III); (D) with 1.9 mM Ga(III) and (E) after addition of 4 mM oxalate and 4 h of equilibration.

When hTF/2N is titrated with $Ga(NTA)_2^{3-}$, the resonance associated with Trp 128 shifts almost completely to the position of the resonance for Trp 264 (Fig. 4). Unlike the case of the Fe(III) protein observed in Fig. 3, however, the resonances do not completely overlap. Furthermore, the resonance associated with Trp 8 changes by -0.26 ppm in the fully gallium-saturated protein. Trp 8 shows a much shorter and wider resonance with distinct shoulders indicating conformational heterogeneity in that region of the protein. To investigate the temperature dependence of this conformational change in hTF/2N, we raised the temperature of the NMR experiment to 45 °C. Our results showed no change in the appearance of the spectrum (data not shown). The T_1 data for the gallium-saturated protein (Table 1) show significantly shorter relaxation times for the Trp 128 and 264 resonances and a slightly shorter T_1 for the Trp 8 resonance.

The titration of hTF/2N with gallium in the presence of hydrogencarbonate is shown in Fig. 5. These results show a similar pattern to those of the hTF/2N with oxalate except that the resonance for Trp 128 is not resolved from that for Trp 264. This result is consistent with the results found from the hTF/2N spectrum with Fe(III) and carbonate shown in Fig. 3. The replacement of carbonate by oxalate shown in Fig. 5(E) causes the resonances for Trp 128 and Trp 264 to become partially resolved as in the Ga(III) titration of hTF/2N (Fig. 4). These data suggest that Trp 128 is sensitive to the small change in structure in the binding cleft caused by the

two different anions, hydrogencarbonate and oxalate. Data from crystallographic studies on copper(II)–lactoferrin–oxalate show that, although larger than carbonate, oxalate is easily accommodated by small shifts in the position of the metal and two amino acid residues in the binding cleft with little effect on protein structure.[25] Our *solution* NMR studies have shown that the secondary elements stemming from the binding pocket are effected by this change and this may be important to the overall structural changes on the protein and recognition by the receptor.

The titration of the H207E mutant with Ga(III) is shown in Fig. 6. The results are qualitatively similar to those for hTF/2N, as we would expect since the binding of metal is greater than the wild type.

DISCUSSION

The ^{19}F NMR spectra of iron-saturated hTF/2N and H207E show that the resonance associated with Trp 128 undergoes the greatest change relative to the apo-proteins. This residue lies at the end of an α-helix (residues 129–134) in the N2 domain of the *N*-lobe of human transferrin and near the interdomain hinge. This end of the helix is associated with the synergistic anion in the binding pocket. It is not difficult to imagine that the local environment of the fluorine atom on Trp 128 would change upon closure of the interdomain cleft. The high-field shift of the Trp 128 resonance observed in the NMR spectra of hTF/2N and H207E on binding metal signifies cleft closure. These studies also show that the fluorine located on Trp 128 is sensitive to the small changes in the structure of the binding pocket when anion is changed from carbonate to oxalate. These small locals structural changes are transmitted through secondary structural elements stemming from the binding pocket.

Trp 8 is part of a β-strand in the N1 domain which comprises residues 5–11. The small shift in the position of this residue is likely due to the movement of this secondary structural element as the protein domains rotate and close upon one another. The conformational heterogeneity at Trp 8 is apparent throughout the titration of the protein with Ga(III), as indicated by the shorter wider resonance with distinct shoulders. The fluorine at this position is extremely sensitive to these changes in local environment. Thus again we observe changes in the overall structure of the protein in distal areas from the binding event. Trp 264 is in an α-helix (residues 260–273) in the N1 domain. The lack of movement of the ^{19}F resonance implies that there is no change in the local environment of this residue upon metal binding. The decrease in T_1 of all three Trp residues following metal ligation (Table 1) implies that the protein has become more rigid and that relaxation is thus more efficient than in the apo-forms, where the relative flexibilities in the regions of these residues lie in the order Trp $128 > 264 > 8$. Metal binding promotes more structural homogeneity which is consistent with recognition by the receptor.

Acknowledgements

We thank Chantal Kenney and Michael Miller for their excellent technical support. The NMR spectra were accumulated at the Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA. This investigation was supported by USPHS Grant DK21739 (to R.C.W.) and USPHS Training Grant T72 HL07594 (L.A.I. as trainee).

REFERENCES

1. M. A. Danielson and J. J. Falke, *Annu. Rev. Biophys. Biomol. Struct.* **25**, 163 (1996).
2. J. T. Gerig, *Prog. Nucl. Magn. Reson. Spectrosc.* **26**, 293 (1994).
3. J. J. Bullen, C. G. Ward and H. J. Rogers, *FEMS Microbiol. Lett.* **71**, 27 (1990).
4. D. C. Harris and P. Aisen, in *Iron Carriers and Iron Proteins*, edited by T. M. Loehr, pp. 239–351. VCH, New York (1989).
5. N. D. Chasteen and R. C. Woodworth, in *Iron Transport and Storage*, edited by P. Ponka, H. M. Schulman and R. C. Woodworth, pp. 68–79. CRC Press, Boca Raton, FL (1990).
6. B. F. Anderson, H. M. Baker, G. E. Norris, D. W. Rice and E. N. Baker, *J. Mol. Biol.* **209**, 711 (1989).
7. B. F. Anderson, H. M. Baker, G. E. Norris, S. V. Rumball and E. N. Baker, *Nature (London)* **344**, 784 (1990).
8. E. N. Baker, B. F. Anderson, H. M. Baker, M. Haridas, G. B. Jameson, G. E. Norris, S. V. Rumball and C. A. Smith, *Int. J. Biol. Macromol.* **13**, 123 (1991).
9. Y. Wang, J. Chen, Y. Luo, W. D. Funk, A. B. Mason, R. C. Woodworth, R. T. A. MacGillivray and G. D. Brayer, *J. Mol. Biol.* **227**, 575 (1992).
10. A. T. Tan and R. C. Woodworth, *Biochemistry* **8**, 3711 (1969).
11. V. L. Pecoraro, W. Harris, C. J. Carrano and K. N. Raymond, *Biochemistry* **20**, 7033 (1981).
12. M. R. Schlabach and G. W. Bates, *J. Biol. Chem.* **250**, 2182 (1975).
13. R. C. Woodworth, L. M. Virkaitis, R. G. Woodbury and R. A. Fava, *Proteins of Iron Storage and Transport in Biochemistry and Medicine*, edited by R. R. Crichton, pp. 39–50. North-Holland, Amsterdam (1975).
14. F. Kilar and I. Simon, *Biophys. J.* **48**, 799 (1985).
15. M. Y. Rosseneu-Motreff, F. Soetewey, R. Lamote and H. Peeters, *Biopolymers* **10**, 1039 (1971).
16. J. G. Grossmann, M. Neu, R. W. Evans, P. F. Lindley, H. Appel and S. S. Hasnain, *J. Mol. Biol.* **229**, 585 (1993).
17. M. Gerstein, B. F. Anderson, G. E. Norris, E. N. Baker, A. M. Lesk and C. Chothia, *J. Mol. Biol.* **234**, 357 (1993).
18. J. C. Grossmann, A. B. Mason, R. C. Woodworth, M. Neu, P. F. Lindley and S. S. Hasnain, *J. Mol. Biol.* **231**, 554 (1993).
19. W. R. Harris, *Biochemistry* **25**, 803 (1986).
20. R. C. Woodworth, A. B. Mason, W. D. Funk and R. T. A. MacGillivray, *Biochemistry* **30**, 10824 (1991).
21. A. B. Mason, W. D. Funk, R. T. A. MacGillivray and R. C. Woodworth, *Protein Exp. Purif.* **2**, 214 (1991).
22. R. M. Nelson and G. L. Long, *Anal. Biochem.* **180**, 147 (1989).
23. G. Kubal, A. B. Mason, S. U. Patel, P. J. Sadler and R. C. Woodworth, *Biochemistry* **32**, 3387 (1993).
24. W. R. Harris and V. L. Pecoraro, *Biochemistry* **22**, 293 (1983).
25. C. A. Smith, H. M. Baker and E. N. Baker, *J. Mol. Biol.* **219**, 155 (1991).

