Conformational stability of porcine serum transferrin

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Abstract

The conformation of porcine serum ferric transferrin (Tf) and its stability against denaturation were studied by circular dichroism. Tf was estimated to have 19–24% α -helix and 50–55% β -sheet based on the methods of Chang et al. (Chang, C.T., Wu, C.-S.C., & Yang, J.T., 1978, *Anal. Biochem.* 91, 13–31) and Provencher and Glöckner (Provencher, S.W. & Glöckner, J., 1981, *Biochemistry* 20, 33–37). Removal of the bound ferric ions (apo-Tf) did not alter the overall conformation, but there were subtle changes in local conformation based on its near-UV CD spectrum. The Tfs were stable between pH 3.5 and 11. Denaturation by guanidine hydrochloride (Gu-HCl) showed two transitions at 1.6 and 3.4 M denaturant. The process of denaturation by acid and base was reversible, whereas that by Gu-HCl was partially reversible. The irreversible thermal unfolding of Tfs began at temperatures above 60 °C and was not complete even at 80 °C. The bound irons (based on absorbance at 460 nm) were completely released at pH <4 or in Gu-HCl solution above 1.7 M, when the protein began to unfold, but they remained intact in neutral solution even at 85 °C. The NH₂- and COOH-terminal halves of the Tf molecule obtained by limited trypsin digestion had CD spectra similar to the spectrum of native Tf, and the COOH-terminal fragment had more stable secondary structure than the NH₂-terminal fragment.

Keywords: circular dichroism; conformation; denaturation; sequence-predictive method; transferrin

Mammalian Tfs can be divided into four classes according to their sources: serum Tf in blood plasma, lactoferrin in milk, ovotransferrin in egg white, and melanotransferrin in human melanoma cells (Aisen & Listowsky, 1980; Brock, 1985; Rose et al., 1986; Heubers & Finch, 1987; Bezkorovainy, 1989). Accordingly, the functions of Tfs from various sources can be quite different. For instance, serum Tf is an iron transporter in the blood, which is responsible for transporting iron from sites of absorption and heme degradation to those of storage and utilization, whereas lactoferrins are primarily bacteriostatic. CD studies for serum Tf and lactoferrin from human (Mazurier et al., 1976) and ovotransferrin (Keung et al., 1982) show that in general Tfs are α/β proteins rich in β -sheets and moderate in α -helix content. X-ray diffraction data for human lactoferrin (Anderson et al., 1987, 1989) and rabbit serum Tf (Bailey et al., 1988) further reveal that they have similar topology and are composed of two homologous lobes, each binding a single ferric ion.

In spite of numerous biochemical and biophysical studies on Tfs from various sources, the conformation of porcine serum Tf has not been studied in the past. The protein resembles human serum Tf more closely than many other mammalian Tfs based on the following facts: (1) It can bind to human serum Tf receptor with a binding capacity of about 38% of that of human serum Tf, which is higher than that of serum Tfs from other mammals (Y.-M. Feng, S. Pervais, & K. Brew, unpubl.; Zhang et al., 1991). In contrast, bovine serum Tf does not bind to the human receptor under the same conditions. (2) It can substitute for human serum Tf in the medium of serum-free cell cultures (Feng et al., 1987). Because of its possible substitution for human serum Tf in clinical use, it is important to understand the structure as well as the conformational stability of porcine serum Tf.

We report herein the conformation of porcine serum Tf in solution and its stability under various denaturation conditions. Our CD study indicates that this protein is quite stable against extreme pHs and thermal denaturation; the COOH-terminal lobe is more stable than the NH₂-terminal lobe. Furthermore, removal of the bound ferric ions virtually does not affect its overall conformation in solution.

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Abbreviations: Tf, transferrin; Fe-Tf, iron-saturated Tf; apo-Tf, iron-free Tf; NH₂-Tf and COOH-Tf, NH₂- and COOH-terminal lobes of Tf; Gu-HCl, guanidine hydrochloride; CD, circular dichroism.

Results

Absorption spectra

The absorption spectrum of porcine serum Fe-Tf showed a 460-nm band associated with bound iron (data not shown), which was 10 nm lower than that reported by Pollack and Lasky (1976) for Tf from guinea pig. Apo-Tf had no band at 460 nm, but there was residual absorbance above 300 nm, probably due to light scattering. In the near-UV region there was a 280-nm band associated with aromatic groups; the band for Fe-Tf was larger (by about 24%) than that for apo-Tf, probably because of overlapping of the iron band in Fe-Tf. The ratio of absorbances, A_{280}/A_{460} , for Fe-Tf was 25.6.

CD

CD spectra of Fe-Tf and apo-Tf in the far-UV region (Fig. 1A, curves 1 and 2) were superimposable, indicating that they had a similar overall conformation. In addition, they were very close to the CD spectrum for human serum Tf (Mazurier et al., 1976) as well as that for the more distantly related ovotransferrin (Keung et al., 1982) in both shape and intensities. The estimated α -helix and β -sheet by the methods of Chang et al. (1978) (CWY) and Provencher and Glöckner (1981) (PG) in general agreed well (Table 1). The CWY method gave unusually low estimates of β -turn for both Fe-Tf and apo-Tf, which were probably not reliable given the uncertainties in β -turn estimates obtained by this method (Chang et al., 1978).

In the near-UV region, the CD spectrum of Fe-Tf showed two positive bands near 253 and 291 nm plus a broad positive band above 300 nm, and two negative bands near 277 and 285 nm (Fig. 1B, curve 1). These are largely attributable to the chromophores of aromatic side groups, although the disulfide bonds (19 in Tf) can also contribute to the CD spectrum below 300 nm. Another

Fig. 1. CD spectra of porcine serum transferrin at acid pH. **A:** Below 240 nm. Curves: 1, Fe-Tf at pH 7.5; 2, apo-Tf at pH 7.5; 3, Fe-Tf at pH 2; 4, Fe-Tf at pH 2 was adjusted to pH 7.5 through dialysis. **B:** Above 240 nm. Curves: 1, Fe-Tf; 2, apo-Tf; both at pH 7.5.

Table 1. Conformational estimates of Fe-Tf and apo-Tf by CD analysis and sequence prediction

Protein	Method	H ^a	B	T	R
Porcine serum Tf					
Fe ³⁺	CD ^b (1) PG	24	50	24	2
	(2) CWY	19	55	2	24
	SP ^c (1) GOR	39	30	21	11
	(2) CF	35	9	30	11
Apo-	CD (1) PG	23	55	22	0
	(2) CWY	18	55	1	26
Human serum Tf	CD ^d	23	62	—	—
Human lactoferrin	CD ^d	26	57	—	—
	X-ray ^e	41	24	—	35
Ovotransferrin	CD ^f	14	21	—	65

^a H, B, T, and R denote percent α -helix, β -sheet, β -turn, and unordered form, respectively.

^b CD analysis by the method of Provencher and Glöckner (1981) (PG) and Chang et al. (1978) (CWY).

^c Sequence predictive methods of Garnier et al. (1978) (GOR) and Chou and Fasman (1974) (CF).

^d Mazurier et al. (1976).

^e Anderson et al. (1989).

^f Keung et al. (1982).

broad, negative band appeared above 400 nm with a minimum around 460 nm (data not shown); this induced band corresponded to the absorption band at the same wavelength. Upon removing bound Fe³⁺ ions, the positive 290-nm band and the broad positive band above 300 nm disappeared; instead there was a distinct negative band near 295 nm (curve 2). These results may be due to changes in the local conformation around the aromatic groups, or to the overlapping iron band at 460 nm, or both. The X-ray diffraction results of rabbit serum Tf (Bailey et al., 1988) reveal that the bound Fe³⁺ ions are coordinated with the side chains of two Tyr residues and one residue each of His and Asp and can therefore affect the absorption bands of Tyr as well as the neighboring Trp residues.

Sequence-predictive analysis

Conformational prediction based on the amino acid sequence of porcine serum Tf derived from cDNA (Baldwin & Weinstock, 1988) can complement the CD estimates of various conformations of porcine Tf. Table 1 lists the predicted conformations as determined by the methods of Chou and Fasman (1974) and Garnier et al. (1978). These sequence-predictive methods are also empirical, but they can provide some tentative information about the secondary structure of proteins without knowledge of their three-dimensional structures. For porcine Tf, the sequence-estimated α -helix was higher and β -sheet lower than those obtained by CD methods. The Chou-Fasman prediction led to a much lower β -sheet estimate than that from the method of Garnier et al. Previously, we found

that the Chou-Fasman method underestimated β -sheet predictions for basic fibroblast growth factor (Wu et al., 1991), epidermal growth factor, and transforming growth factor (C.-S.C. Wu, unpubl.).

The sequence prediction of porcine serum Tf by the method of Garnier et al. (1978) revealed 25 β -sheets, which roughly alternated with 19 α -helices (not shown). The X-ray diffraction of rabbit serum Tf (Bailey et al., 1988), on the other hand, identified 11 β -sheets and 10 α -helices per lobe. At this moment it is impossible to align these two results together because the sequence of rabbit protein is still unknown, but there are similarities in the predicted and the X-ray results.

Effect of pH

Far-UV CD spectrum of Fe-Tf at pH 2 (Fig. 1A, curve 3) indicated that the protein unfolded at low pHs. The magnitude of $[\theta]_{222}$ decreased and the minimum at 208 nm blue-shifted to 203 nm, approaching the negative band for unordered form. Fe-Tf lost its bound Fe^{3+} ion below pH 4 (see Fig. 3 below); thus, curve 3 in Figure 1A also represented the spectrum of apo-Tf. Acid denaturation of apo-Tf was essentially reversible merely by raising the pH of the solution to neutral through dialysis (curve 4). Addition of Fe^{3+} ions to apo-Tf solution at pH 7 did not alter the CD spectrum.

At pH 12, Fe-Tf (Fig. 2A, curve 2) showed a smaller decrease in the magnitude of CD than apo-Tf (Fig. 2B, curve 2), as compared with that at neutral pH. Alkaline denaturation of Fe-Tf was again essentially reversible upon adjusting the pH to neutral (curves 3 of Fig. 2A,B; cf. curves 1).

The absorbance at 460 nm of Fe-Tf began to drop when the pH of the solution was lowered from neutral to below 6, indicating the release of bound Fe^{3+} ions from the protein, which was complete at pH 4 (Fig. 3). This pH-absorbance curve suggested that there were two transitions

Fig. 2. CD spectra of Tf at alkaline pH below 240 nm. **A:** Fe-Tf. Curves: 1, pH 7.5; 2, pH 12; 3, sample for curve 2 adjusted to pH 7.5 through dialysis. **B:** Apo-Tf. Curves same as in A.

Fig. 3. Effect of pH on iron binding and conformation of transferrin. Bound Fe^{3+} ions as monitored by absorbance at 460 nm of 0.87 mg/mL in 1-cm cell, and conformation by CD at 222 nm. ●, Fe-Tf; ○, apo-Tf.

between pH 4 and 6, one at about pH 5 and the other at about pH 6. Previously, Lestas (1976) obtained a similar pH titration curve for human transferrin, which he attributed to two different affinities for the iron-binding sites. On the alkaline side, the bound iron remained intact even at pH 12. The reason for the small increase of absorbance between pH 9 and 12 is not known. It was not due to experimental error because repeated experiments produced the same curve.

The conformations of both Fe-Tf and apo-Tf based on $[\theta]_{222}$ was stable at pHs between 3.5 and 11 and began to lose their secondary structure at lower and higher pHs, more so for acid denaturation (Fig. 3). At pH 4 the conformation of Tf remained unchanged even though it lost the bound Fe^{3+} ; above pH 11 the bound Fe^{3+} was intact, but the conformation of Tf was slightly altered. Therefore, the presence or absence of bound iron did not affect the overall conformation of Tf. In a separate experiment the acid- or alkaline-denatured apo-Tf was adjusted to neutral pH and titrated with an Fe^{3+} ion solution. The apo-Tf bound 1.6 and 1.7 Fe^{3+} mol/mol when Tf was renatured from pH 2 and 12, respectively.

Effect of Gu-HCl

In 6 M Gu-HCl the CD spectrum of Fe-Tf (Fig. 4A, curve 2) showed a monotonic increase in the magnitude of ellipticity when the wavelength was lowered toward the 200-nm region, which is typical of the presence of an unordered form. Thus, the protein was completely denatured in this denaturant solution. Removal of Gu-HCl through dialysis restored some secondary structures (curve 3; cf. curve 1), but unlike acid or alkaline denaturation, denaturation by Gu-HCl was only partially reversible. Apo-Tf had the same CD spectrum as Fe-Tf in 6 M Gu-HCl as well as after the removal of the denaturant (data not shown).

Porcine serum Fe-Tf and apo-Tf in Gu-HCl solutions at pH 7.5 had two conformational transitions, one at 1.6 M

Fig. 4. Effect of Gu-HCl concentration on iron binding and conformation of Tf. **A:** CD spectra. Curves: 1, Fe-Tf; 2, Fe-Tf in 6 M Gu-HCl; 3, after removal of Gu-HCl through dialysis. **B:** Absorbance at 460 nm (0.75 mg/mL protein in 1-cm cell). **C:** CD at 222 nm. ●, Fe-Tf; ○, apo-Tf.

and the other at 3.4 M Gu-HCl (Fig. 4C). However, the bound Fe^{3+} ion as determined by A_{460} was completely released from the protein at about 1.7 M Gu-HCl (Fig. 4B), again indicating that bound iron had no effect on the conformation of Tf. The iron-binding curve (Fig. 4B) was not linear, suggesting that the two bound irons might be released at different Gu-HCl concentrations.

Thermal denaturation

Both Fe-Tf (Fig. 5A) and apo-Tf (data not shown) had the same CD spectrum at 86 °C (curve 2), as well as after cooling back to 25 °C (curve 3), but thermal denaturation of Tf was irreversible. Based on $[\theta]_{222}$, Fe-Tf and apo-Tf began to unfold above 60 °C and did not level off even at 86 °C (Fig. 5C). The small difference between the two curves was probably within experimental errors. The bound Fe^{3+} ions based on A_{460} were intact even at a temperature as high as 86 °C (Fig. 5B). The protein began to precipitate at elevated temperatures; the more concentrated the protein solution, the lower the temperature needed for precipitation.

Change of near-UV CD

Whereas far-UV CD monitors the overall conformation of a protein attributed to its secondary structure, near-UV CD (240–320 nm) reflects the local conformation around aromatic side groups that constitutes the tertiary structure of the protein. The unfolding of the secondary structure is often accompanied by the disruption of the tertiary structure. Thus, for acid and alkaline denaturation of Fe-Tf the CD at 277 nm (for Tyr residues) for Fe-Tf and apo-Tf began to change below pH 4 and above pH 11

Fig. 5. Thermal effect on iron binding and conformation of transferrin. **A:** CD spectra. Curves: 1, Fe-Tf at 25 °C; 2, at 86 °C; 3, sample for curve 2 cooled back to 25 °C. **B:** Absorbance at 460 nm (1.84 mg/mL protein in 1-cm cell). **C:** CD at 222 nm. ●, Fe-Tf; ○, apo-Tf.

(Fig. 6A), much like the changes observed for far-UV CD in Figure 3. The observation that Fe-Tf began to change at pH slightly above 7 was unexpected. One possible explanation, albeit speculative, is that ionization of Tyr residues might be influenced by the bound Fe^{3+} ions

Fig. 6. Effect of pH (A), Gu-HCl (B), and temperature (C) on the near-UV CD at 277 nm. ●, Fe-Tf; ○, apo-Tf.

regardless of any change in the local conformation or lack thereof. In Gu-HCl a transition occurred near 1.5 M of the denaturant (Fig. 6B), which agreed with the first transition at 1.6 M for the overall conformation (Fig. 4C). However, the second transition at 3.4 M Gu-HCl observed in the far-UV region was not seen in the near-UV spectrum. Raising the temperature of the Tf solution at pH 7.5 (without any denaturant) gradually decreased the magnitude of CD at 277 nm (Fig. 6C), which again agreed with the thermal denaturation of Tf observed in Figure 5C for the overall conformation.

NH₂- and COOH-terminal fragments of transferrin

The combined molecular weight of NH₂-Tf (*M_r* 33,000) and COOH-Tf (*M_r* 37,000) was 10% less than that of porcine serum Tf (*M_r* 77,000), suggesting that these two fragments were partially degraded after limited trypsin digestion. Nevertheless, the CD spectra of the two fragments were close to the spectrum of native Tf (Fig. 7A), except that the positive band around 190 nm was somewhat smaller than that of native Tf (cf. Fig. 1A, curve 1). The estimated α -helix and β -sheet for NH₂-Tf were 12% and 60% based on the method of Chang et al. (1978) and 23% and 43% by the Provencher-Glückner method (1981), respectively. The corresponding α -helix and β -sheet estimates for COOH-Tf were 14% and 59% by the Chang et al. method (1978) and 18% and 57% from the Provencher-Glückner method (1981), respectively. Denaturation of the two fragments in Gu-HCl (Fig. 7B) indicated that they had different stability; NH₂-Tf and COOH-Tf had conformational transitions at about 1 M and 2 M Gu-HCl, respectively, which were slightly lower than the two transitions for the intact molecule (cf. Fig. 4C).

Discussion

Porcine serum Tf is a member of serum Tfs (Pollack & Lasky, 1976; Feng et al., 1987; Baldwin et al., 1990), which

has 70% sequence identity with human serum Tf (Baldwin & Weinstock, 1988). Among various Tfs, only rabbit serum Tf (Bailey et al., 1988) and human lactoferrin (Anderson et al., 1987, 1989) have been analyzed for their three-dimensional structures by X-ray diffraction, and both have been classified as α/β proteins. The X-ray diffraction study for human serum Tf is still incomplete (Delucas et al., 1978) and that for porcine serum Tf has not been reported. However, sequence prediction of porcine serum Tf revealed that α -helix and β -sheet segments were mixed together in the sequence (not shown). Thus, porcine serum Tf was probably also an α/β protein, like rabbit serum Tf and human lactoferrin. The estimates of α -helix and β -sheet of Tfs by CD for porcine serum Tf and human serum Tf were very close (Table 1). Although CD analysis of proteins in solution is often complemented by sequence-predictive methods, neither approach will recognize a failed analysis without X-ray diffraction results to check reliability. CD estimates of α -helix and β -sheet by the methods of Chang et al. (1978) and Provencher and Glöckner (1981) agreed well, but they differed significantly from the sequence predictions by the methods of Chou and Fasman (1974) and Garnier et al. (1978). Which values are closer to the secondary structure of Tfs in crystals must await X-ray diffraction studies. Nevertheless the CD results in Table 1 did suggest that both human and porcine serum Tf are β -rich proteins, a conclusion that supports the X-ray results of rabbit serum Tf (Bailey et al., 1988), even though the exact amounts of α -helix and β -sheet of this protein are not yet reported.

For the sake of comparison, Table 1 also includes CD estimates for various conformations of the more distantly related Tfs, human lactoferrin, and ovotransferrin. The former was also rich in β -sheet, based on its CD spectrum, but it did not agree with its X-ray results. Ovotransferrin seemed to have only moderate amounts of α -helix and β -sheet from their CD analysis. These differences among various Tfs deserve further investigation.

X-ray diffraction studies provide a direct determination of the three-dimensional structure of proteins in the crystalline state, and it is generally presumed that such structures remain intact when proteins are dissolved in aqueous solutions. However, if we can estimate or predict the folded structure of a protein, knowing only the CD spectrum and amino acid sequences of several proteins in the same class such as human and porcine serum Tf, it might help us understand protein folding, plan modifications of a protein by, say, site-directed mutagenesis, and devise means to alter the functions of a protein before having an X-ray crystalline structure of even one member of that class. Carefully interpreted, current CD analysis of proteins can provide useful information about the conformation and conformational changes of proteins in solution.

Porcine serum Tf was quite stable in acid and alkaline solution (between pH 3.5 and 11) regardless of the absence or presence of two bound Fe³⁺ ions (Fig. 3). These bound

Fig. 7. CD of NH₂- and COOH-lobe of Tf. **A:** CD spectra. Curves: 1, NH₂-Tf; 2, COOH-Tf. **B:** CD at 222 nm in Gu-HCl solutions. ●, NH₂-Tf; ○, COOH-Tf.

ferric ions were released at pH <4 (Fig. 3) where the conformation of Fe-Tf was still close to that of the native protein (Fig. 3). On the other hand, Fe-Tf retained its bound Fe^{3+} ions in alkaline solution as high as pH 12 where the protein molecule began to unfold (Fig. 3). Thus, the release of the bound Fe^{3+} does not depend on the loosening of the secondary structure of the protein molecule. This is in accord with the changes in conformation in Gu-HCl solution or at high temperatures. The protein completely lost its bound Fe^{3+} ions at 1.7 M Gu-HCl (Fig. 4B), where the protein retained much of its secondary structure (Fig. 4B). On the other hand, raising the temperature of the Fe-Tf solution to 86 °C did not release the bound Fe^{3+} ions (Fig. 5B), but the protein had already lost about 30% of its ellipticity at 220 nm (Figs. 5A, 6C).

Because Fe-Tf showed two apparent transitions in Gu-HCl (Fig. 4C), we suspect that this might be related to the stability of the two binding sites in the NH_2 - and COOH-terminal half molecules. Denaturation of NH_2 -Tf and COOH-Tf in Gu-HCl solution showed an apparent transition near 1 M and above 2 M, respectively, suggesting that they might be responsible for the two transitions in the intact molecule and that the two fragments were less stable than they were in the intact molecule against denaturation in Gu-HCl. Further, COOH-Tf was more stable than NH_2 -Tf. It is possible that the residual secondary structure of Tf at extreme pHs (Fig. 3) and at high temperature (Fig. 5C) might be due to the COOH-terminal lobe of the molecule. There are many factors that affect the stability of proteins, but the high stability of serum Tf may be due in part to its high content of disulfide bonds. In rabbit serum Tf, there are 19 disulfide bonds in the molecule; among these, 3 are the long-range bonds in the COOH-lobe that are absent in the NH_2 -lobe (Bailey et al., 1988). This type of bond may also hold the COOH-terminal lobe of porcine serum Tf together and make it more stable than the NH_2 -terminal lobe.

Aisen et al. (1978) found that the Fe^{3+} -binding site of human Tf in the COOH-terminal lobe has a higher affinity for Fe^{3+} ions than that in the NH_2 -terminal lobe. This difference may also be responsible for the pH- and Gu-HCl-dependent curves of Fe^{3+} -binding in porcine serum Tf where two transitions were observed (Figs. 3, 4B). Using pH titration, Lestas (1976) also found that the two bound iron molecules in human Tf differ in their stability in acid.

The transfer of iron from Tf to developing cells was attributed to conformational changes in the protein. Our results showed that Fe-Tf and apo-Tf had the same secondary structure and the same response to pH, Gu-HCl, and thermal denaturation. The only difference between Fe-Tf and apo-Tf was the local conformation, which was revealed by the near-UV CD spectrum (Fig. 2B). X-ray (Anderson et al., 1990) and other physical studies (Rosseneu-Motreff et al., 1971; Kilar & Simon, 1985) also show that conformational changes accompany iron bind-

ing and release. In vivo, such local conformational changes might be associated with the binding of Tf to the receptor at the cell surface and release of the bound irons.

Materials and methods

Materials

Porcine serum Tf was prepared by the method of Feng et al. (1987), and its purity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Apo-Tf was obtained by dialysis of Fe-Tf against two changes of 0.1 M citrate and 0.1 M acetate buffer, pH 4.5, and two changes of distilled water (Aisen et al., 1978). Ultrapure Gu-HCl was purchased from Schwarz/Mann Biotech. All other chemicals were of analytical grade.

NH_2 - and COOH-terminal half molecules of Tf were prepared by limited trypsin digestion of Tf and separated on a Sephadex G-75 column, which showed three peaks (Y.-M. Feng, X.-T. Zhang, Y.-S. Zhang, & K. Brew, unpubl.). Judging from their molecular weights, peak I was the unhydrolyzed Tf, whereas peaks II and III each were about half the size of the intact molecule. Both fragments contained ferric ions as evidenced from their absorption bands at 465 nm. The NH_2 -terminal amino acid sequences of peak II and peak III were homologous to the COOH- and NH_2 -terminal lobes of human serum Tf, respectively. Peak II, but not peak III, bound with concanavalin A-Sepharose on a column. Because the carbohydrate chains on serum Tfs are known to be located at the COOH-terminal lobe (Bailey et al., 1988) that binds concanavalin A, peaks III and II were therefore identified as the NH_2 - and COOH-lobe, respectively. The molecular weights of NH_2 -Tf and COOH-Tf, based on SDS-PAGE, were 33,000 and 37,000, respectively, as compared with 77,000 for the native protein.

Determination of protein concentration

Tf concentrations were determined by nitrogen content using the Jaenicke (1974) method, in which the samples were ashed with perchloric acid at its boiling temperature, followed by direct photometric analysis of the ammonia ions with indophenol blue. This method required only 0.2–10 μg protein and was more sensitive than the micro-Kjeldahl method. The results agreed well with those determined by the Lowry method using bovine serum albumin as a standard (Lowry et al., 1951).

Absorption spectra

Absorption spectra were measured on a Zeiss PMQII spectrophotometer at room temperature, using a cuvette with a 1-cm pathlength at a concentration of 5.8 mg/mL for both native and Fe-free proteins.

CD

CD spectra were recorded on a JASCO J-500A spectropolarimeter equipped with a DP-500 data processor for data acquisition and an IBM PC for data analysis. The cell containing the protein solution was placed in a water-jacketed cell holder, through which water was circulated at a constant temperature. The temperature of the solution was kept at 25 °C, except for thermal denaturation experiments. Both the instrument and optical cells had been calibrated with recrystallized *d*-10-camphorsulfonic acid (Chen & Yang, 1977). The data were expressed as mean residue ellipticity, $[\theta]$, in deg cm²/dmol, which was calculated from $[\theta] = (d \times s \times M)/(c \times l)$, where *d* denotes observed ellipticity (the displacement in cm from the baseline), *s* sensitivity in mdeg/cm, *M* the mean residue weight (115 for Tf), *c* protein concentration in mg/mL, and *l* cell pathlength in mm. The Tf concentrations were kept at about 0.1 mg/mL. The CD data were analyzed by the method of Chang et al. (1978), using the reference spectra obtained from 15 proteins of known three-dimensional structure (Yang et al., 1986), as well as the method of Provencher and Glöckner (1981).

Sequence prediction

The conformation of native porcine Tf based on its sequence derived from cDNA (Baldwin & Weinstock, 1988) was predicted by the method of Chou and Fasman (1974) and that of Garnier et al. (1978). The choice of decision constants for the latter method was based on the results of CD analysis.

Denaturation

pH study

The range of pH between 2 and 10 was maintained by using constant ionic strength buffers ($\mu = 0.1$), which contained glycine (pH 2–3.5 and 9.5–12), acetate (pH 4–5.5), and phosphate (pH 6–7.5). Appropriate amounts of the pH 7.5 and pH 10 buffers were mixed to cover the range of pH 8–10 (Wu et al., 1987).

Denaturant study

Eight molar Gu-HCl was added to a protein solution to the desired concentration of protein and Gu-HCl concentration from 0 to 6 M. For renaturation study, the protein in 6 M Gu-HCl was dialyzed against a Gu-HCl solution of lower molarity (toward zero), and the procedure was repeated until Gu-HCl was completely removed.

Temperature study

The sample temperature was raised at 5 °C intervals from 6 to 86 °C, and held for 15 min at each temperature before measurements. Renaturation of the protein was attempted by slowly air cooling the solution to 25 °C in the cell holder.

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