Spectrophotometric titration with cobalt(III) for the determination of accurate absorption coefficients of transferrins

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A rapid and sensitive technique, involving difference spectral titration with cobalt(III), to measure the ϵ values of chicken ovotransferrin, human serum transferrin, the N-lobe of human transferrin and several single point mutants is reported. The resulting ϵ values were compared with the values calculated from the equation proposed by Pace, Vajdos, Fee, Grimsley and Gray [(1995) *Protein Sci.* 4, 2411–2423]. The titrations with cobalt

feature sharp break-points and do not destroy the protein samples. The choice of buffer was found to be important, depending on the metal-binding avidity of the proteins. Cobalt titration should prove useful for studying the comparative metal-binding properties of transferrin and mutants of transferrin being generated by recombinant technology.

INTRODUCTION

The transferrins are a group of iron-complexation proteins that function in the transport of iron around the blood stream [1,2]. X-ray crystallographic studies have shown that the protein is bilobal, with N- and C-lobes, each of which reversibly binds a ferric ion concomitantly with a synergistic anion, carbonate [3–7]. The ferric ion is bound octahedrally to the side chains of two tyrosine residues [Tyr⁹⁵ and Tyr¹⁸⁸ in the human serum transferrin (hTF) sequence], one aspartate (Asp⁶³), one histidine (His²⁴⁹) and two oxygens from carbonate. The protein is folded such that a cleft is formed creating a favourable environment for the co-ordination of ferric ion. It has been found that this co-ordination environment is suitable not only for iron but also for other metal ions. The strength of binding seems to be dependent on the size of the metal ion, weaker when it is either smaller or larger than a ferric ion. The reversible binding of a variety of bivalent and trivalent metal ions by transferrin has been employed to help characterize the physical and chemical properties of the protein [8–16].

Recent work from a number of laboratories, including our own, has involved the expression and purification of recombinant hTF, human lactoferrin, the N-lobe of lactoferrin and the N-lobe of human serum transferrin as well as a number of single point mutants that differ in their ability to bind iron [17–24]. Accurate determination of the molar absorption coefficient, ϵ , is important for the full characterization of a protein. As reviewed recently [25], four techniques have been used to measure the ϵ values of proteins: the dry-weight method, amino acid analysis, Kjeldahl nitrogen determination, and the Edelhoch method. All of these techniques result in the destruction of the protein and do not always lead to ϵ values in good agreement. Based on a sample of 116 measured ϵ values for 80 proteins, Pace et al. [25] proposed an equation to predict the ϵ value. However, because the ϵ values for the protein chromophores depend on their environment [26,27] a specific technique is still necessary and valuable for the accurate and reliable measurement of them. We report here a rapid and sensitive approach, involving difference spectral ti-

tration with Co³⁺, to measure the ϵ values of transferrin and some of its mutants. This work is useful for study of the comparative metal binding properties of these proteins.

EXPERIMENTAL

Materials

Chemicals were of reagent grade. Stock solutions of Hepes, Tris/HCl, and other buffers were prepared by dissolving the anhydrous salts in Milli-Q purified water, and adjusting the pH to desired values (7.0–8.5) with 1 M NaOH or HCl. A standard solution of cobalt (1000 μ g/ml, AAS standard solution, Speccure) was obtained from Alfa Aesar.

Methods

Expression, isolation and purification of proteins

Full-length non-glycosylated hTF, the N-lobe of hTF and various single point mutants were expressed into the medium of BHK cells containing the relevant cDNA in the pNUT vector and were purified as described previously [17,19]. The N-lobe of human serum transferrin has recently been expressed in the methylo-trophic yeast *Pichia pastoris* (A. B. Mason, R. C. Woodworth, R. W. A. Oliver, B. N. Green, L.-N. Lin, J. F. Brandts, B. M. Tam, A. Maxwell and R. T. A. MacGillivray, unpublished work). Full-length glycosylated hTF was from Boehringer Mannheim. Ovotransferrin (oTF) was prepared from chicken egg white as described [28]. Removal of iron from the transferrin samples was accomplished by treatment of the proteins in a Centricon microconcentrator (Amicon) with 0.5 M sodium acetate, pH 4.9, containing 1 mM nitrilotriacetate and 1 mM EDTA, until no pink colour was observed. The protein was then sequentially washed with 2 ml of 0.1 M KCl, 2 ml of 0.1 M sodium perchlorate and four times with 2 ml of 0.1 M KCl. At this point the protein, still in the Centricon, was exhaustively exchanged into the buffer of choice (see below).

Abbreviations used: BHK, baby hamster kidney cells; hTF, human serum transferrin; hTF/2N, recombinant N-lobe of human transferrin comprising residues 1–337 (mutants of hTF/2N are designated by the wild-type amino acid residue, the position number and the amino acid to which the residue was mutated); oTF, chicken ovotransferrin.

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Preparation and determination of Co^{3+} solution

$\text{Na}_3[\text{Co}(\text{CO}_3)_3] \cdot 3\text{H}_2\text{O}$ was prepared as described in detail [29,30]. The dry green powder of $\text{Na}_3[\text{Co}(\text{CO}_3)_3] \cdot 3\text{H}_2\text{O}$ (0.52 g) was added to 100 ml of 1 M sodium bicarbonate. The mixture was stirred at room temperature for 2 h. After filtering through No. 1 Whatman filter paper, the filtrate was allowed to stand at room temperature for 3 days and then was filtered again to remove small amounts of precipitate. The resulting clear solution (pH 8.5) was stored at 4 °C. This stock solution was found to be stable at pH 8–10 and an ionic strength of bicarbonate of 0.1–1.0 M. It can be used for accurate spectral and kinetic measurements. The determination of Co^{3+} concentration in the stock solution (approx. 6.5 mM) was made via atomic absorbance spectra by diluting a given amount of the stock solution into an ammonium bicarbonate solution (0.1 M, pH 8.0) and comparing it with the standard solution.

Co^{3+} titration

A Cary 219 spectrophotometer under the control of the computer program OLIS-219s [31] was used to record the difference spectra. A solution of apoprotein in buffer, with an A_{280} value between 0.5 and 2.0 in a volume of 2.5 ml, was placed into the sample cuvette, which contained a small stirring bar. The reference cuvette contained the same buffer as the sample. When sodium bicarbonate was used as buffer, air/ CO_2 (19:1) was introduced into the covered cuvettes over the surface of the solution to maintain the pH. A baseline of apoprotein against buffer was recorded from 340 to 550 nm. Aliquots of Co^{3+} solutions, either 6.49 or 3.25 mM, were added to both sample and reference cuvettes. A scan from 340 to 550 nm was taken after the addition of each aliquot of Co^{3+} . An interval of 10–30 s was required for thorough mixing of samples and to reach equilibrium. The spectrum of the apoprotein, stored as the baseline, was subtracted from the sample spectra; the resulting difference spectra were analysed by plotting the absorbance maximum against the volume of Co^{3+} by using Axum, Version 3.0.

RESULTS AND DISCUSSION

At the binding site of transferrin, metal complexation to the phenolic oxygen of two tyrosine residues (Tyr^{95} and Tyr^{188}) perturbs the π to π^* transitions of the aromatic ring. The resulting absorbance changes are easily observed in the UV difference spectrum between the metalloprotein and the apoprotein. Apo-transferrin binds ferric ion stoichiometrically, which makes the measurement of the protein concentration by means of spectrophotometric titration possible. Accordingly, ferric ion should be the first choice of counterpart cation in titration. However, it takes a long time to reach equilibrium for the reaction of ferric ion and the protein, and the absorbance response is comparatively weak. The employment of other ions such as Ga^{3+} , Cu^{2+} , Zn^{2+} , Gd^{3+} , Ni^{2+} or Ca^{2+} resulted in an indefinite break-point, although they have been used for a number of studies, including determination of binding constants [8,9,11–16]. We chose Co^{3+} because it has the same tripositive charge and the same ability to co-ordinate six ligands octahedrally as Fe^{3+} . Co^{3+} also has a similar ionic radius and, like Fe^{3+} , it is a hard Lewis acid. An absorption band of medium intensity from d -electron transitions of Co^{3+} [32] can be observed clearly in the visible region (maximum at 405–415 nm) on complexation with a protein. It can be used to measure the desired difference spectra because the absorbance for all non-bonding groups in the protein is blanked out of the spectrum in this range. Meanwhile, the employment of this visible absorption band instead of the UV

Figure 1 (A) Typical difference UV spectra produced by the titration of apo-hTF/2N with Co^{3+} , and (B) titration curve for the addition of Co^{3+} to apo-hTF/2N

(A) Apo-hTF/2N (BHK) in 2.5 ml of 10 mM Hepes buffer, pH 7.5, with an A_{280} value of 1.3678 was titrated with 2 μl portions of 6.49 mM Co^{3+} . The spectra were generated by scanning from 550 to 340 nm after each addition. (B) The curve was generated by plotting the absorbance at the maximum (407 nm) against the volume of Co^{3+} added.

band for titration lessens the influence of mutating the tyrosine residues at the metal-binding site.

Under normal conditions Co^{3+} is unstable. Previous experiments [33] used H_2O_2 to oxidize Co^{2+} to Co^{3+} in the presence of the protein. This led to damage of the protein and was deemed unsuitable for the present titrations. Instead, a weak complexation form of Co^{3+} , $[\text{Co}(\text{CO}_3)_3]^{3-}$, was prepared. This form can be stabilized by dissolving its sodium salt in a NaHCO_3 solution (0.1–1.0 M). It is fortuitous that carbonate is necessary for the stoichiometric binding of Co^{3+} to transferrin and would need to be present in any case.

A typical difference UV spectrum for the recombinant N-lobe of human transferrin comprising residues 1–337 (hTF/2N) after addition of each aliquot of Co^{3+} is presented in Figure 1(A). The entire visible absorption band is shown for each titration point. A typical titration curve, the plot of the absorbance maximum in Figure 1A against the volume of Co^{3+} , is given in Figure 1(B). Both the initial and final portions of the titration curves have the desired linearity. Saturation of the transferrin (at this point, Co^{3+} has a concentration of 35.54 μM , which produces the maximum absorption) was marked by a flat line reflecting essentially no further increase in absorbance with the addition of excess Co^{3+} . Assuming that this break-point corresponds exactly to stoichiometric binding between Co^{3+} and protein (1.0 equivalent for the N- or C-lobe and 2.0 equivalents for full-length hTF or oTF), the concentrations and the ϵ values of the proteins can be calculated from the amount of Co^{3+} consumed. The ϵ values for

Table 1 Experimental and calculated millimolar absorption coefficients (ϵ) at 280 nm for different transferrins

All samples were run in 10–50 mM Hepes, pH 7.5, except as noted. Values are means $\pm$ S.D. for the number of experiments shown.

Sample	Experimental ϵ	Calculated ϵ^*	Deviation (%) †
oTF	90.83 $\pm$ 2.28 ($n = 3$)	88.17	2.93
hTF (glycosylated)	86.68 $\pm$ 0.03 ($n = 2$)	85.12*	1.80
hTF (non-glycosylated)	85.20 $\pm$ 0.25 ($n = 3$)	85.12*	0.09
hTF/2N (yeast)	38.35 $\pm$ 1.63 ($n = 2$)	38.36	–0.03
hTF/2N (BHK)	38.49 $\pm$ 0.57 ($n = 6$)	38.36	0.34
hTF/2N (M309I)	39.71 $\pm$ 0.51 ($n = 2$)	38.36	3.40
hTF/2N (H207E)	39.02 $\pm$ 0.07 ($n = 2$)	38.36	1.69
hTF/2N (D63S) ‡	40.25 $\pm$ 0.31 ($n = 2$)	38.36	4.70
hTF/2N (G65R) ‡	40.58 $\pm$ 0.09 ($n = 2$)	38.36	5.47
hTF/2N (W128Y)	36.57 $\pm$ 0.04 ($n = 2$)	34.35	6.07

* The ϵ were calculated from the equation in [25]. The value predicted in the reference for transferrin was low (83.37) because there are 19 rather than 5 Cys residues in transferrin.

† Percentage deviation is calculated as $100[\epsilon(\text{obs}) - \epsilon(\text{calc})]/\epsilon(\text{obs})$.

‡ These samples were in 10 mM NaHCO_3 buffer, pH 8.5.

Table 2 Millimolar absorption coefficients, ϵ , for recombinant wild-type hTF/2N obtained from Co^{3+} titrations in different buffers

Values are means $\pm$ S.D. for the number of experiments shown.

	Hepes	Tris/HCl	NaHCO_3
hTF/2N (BHK)	38.49 $\pm$ 0.57 ($n = 6$)	37.05 $\pm$ 0.20 ($n = 2$)	38.81 $\pm$ 1.54 ($n = 2$)
hTF/2N (yeast)	38.35 $\pm$ 1.63 ($n = 2$)	38.76 $\pm$ 1.09 ($n = 2$)	

oTF, hTF and hTF/2N, and various single point mutants of the N-lobe, are given in Table 1. For comparison, the corresponding calculated values based on the equation of Pace et al. [25] are presented. The calculation is based on the number of Trp, Tyr and Cys residues in the protein. Except for hTF/2N (yeast), small positive differences were found between the experimental and calculated ϵ values, especially for the mutants. Compared with the calculated values, the experimental values demonstrate the possible influences from the chromophores and natural environments of the different proteins.

When appropriate conditions were employed, the fast reaction between Co^{3+} and transferrin allowed most titrations to be finished within 1 h. The selection of a buffer is very important. Although the titration of hTF/2N in Tris/HCl buffer gives similar results (Table 2), reaching equilibrium takes longer, probably owing to weak reversible interaction of the buffer with Co^{3+} . Especially for the weak-binding mutants of hTF/2N, D63S and G65R [18], this kind of interaction between the buffer and Co^{3+} became dominant in solution, so that no absorption response for Co^{3+} –protein binding was observed during the Co^{3+} titrations. This same lack of reactivity occurred in Hepes buffer. In these cases, a NaHCO_3 solution (10 mM, pH 8.5) was used as buffer. The desired linearity was found for the titration curves of D63S and G65R in the NaHCO_3 solution, but the equilibration of the reactions needed 1–3 min for each point, probably owing to the competitive complexation of Co^{3+} between the protein and the large amount of bicarbonate in the solution. An analogous response delay was found in the titration of wild-type hTF/2N in NaHCO_3 although the same value ϵ was obtained (Table 2). The results for the titration of G65R are shown in Figures 2(A)

Figure 2 (A) Typical difference UV spectra produced by the titration of apo-G65R with Co^{3+} , and (B) titration curve for the addition of Co^{3+} to apo-hTF/2N

Apo-G65R (BHK) in 2.5 ml of 10 mM NaHCO_3 , pH 8.5, with an A_{280} value of 0.8619 was titrated with 2 μl portions of 6.49 mM Co^{3+} . The spectra were generated by scanning from 550 to 330 nm after each addition. (B) The curve was generated by plotting the absorbance at the maximum (410 nm) against the volume of Co^{3+} added.

and 2(B). Again the sharp end-points give confidence in the value predicted from the titration.

Numerous experiments indicate that the ideal conditions for this kind of titration are 10 mM Hepes buffer, pH 7.0–8.5, with a total A_{280} value of 0.5–2.0 in a volume of 2.5 ml, except for the weak-binding mutants, where a NaHCO_3 buffer should be employed.

Titration with Co^{3+} are suitable for kinetic and thermodynamic studies of metal binding to transferrin and offer advantages over metals previously used for such work [8,9,11,12,14–16]. Although the absorption coefficients obtained do not deviate tremendously from the calculated values, this relatively fast and non-destructive technique offers a practical experimental approach.

In summary, the Co^{3+} UV difference spectral titration for determining accurate ϵ values of transferrins described here is rapid, sensitive and non-destructive. It should prove useful for all studies characterizing isolated or recombinant transferrins and mutants thereof.

This work was supported by USPHS grant number R01 DK 21739 from the National Institute of Diabetes, and Digestive and Kidney Diseases. Q.-Y.H. was supported by the Dean's Postdoctoral Fellowship from the College of Medicine at the University of Vermont.

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