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pH Dependence of Specific Divalent Anion Binding to the N-Lobe of Recombinant Human Transferrin†

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ABSTRACT: The binding of the two synergistic anion mimics, phosphate and sulfate, and of the synergistic anions, malonate and oxalate, to the N-lobe of recombinant human serum transferrin (hTF/2N) wild-type and H207E mutant protein was assessed by difference ultraviolet (UV) spectroscopy at 246 nm as a function of pH. The absolute values of both the maximum $\Delta\epsilon_{246}$ and the K_d decreased with decreasing pH. A plot of $-\log K_d$ vs pH gave a straight line with a slope of -1.0. Furthermore, the sum of $-\log K_d$ and pH is a constant for each anion binding to each protein. We interpret these data to mean that each anion binds in divalent form along with an H^+. The binding equilibrium then appears to be $H^+ + \text{hTF/2N} + X^{2-} \overset{K'}{\rightleftharpoons} \text{H-hTF/2N(X)}$ and $\log K' = -\log K_d + \text{pH}$. A plot of $\Delta\epsilon_{246}$ vs pH was sigmoidal with a $pK_a = 7.4$ for both proteins with phosphate and sulfate. When synergistic anions were used with hTF/2N, malonate and oxalate gave pK_as of *ca.* 6.9 and 7.1 for dependence of $\Delta\epsilon_{246}$ on pH, but values of 7.3 and 7.6 for the H207E mutant protein. In an attempt to locate the anion binding site in hTF/2N, the binding of sulfate to the single point mutants of the N-lobe of human transferrin, K296E, K296Q, and K206Q, was carried out by difference UV spectroscopy at pH 7.4. In the case of K296E, sulfate binding gave $\Delta\epsilon_{246} = 0$, while for K296Q, it gave a slightly positive $\Delta\epsilon_{246}$. For K206Q, the binding gave a $\log K'$ of 10.98, which is 0.6 units less than the constant obtained from sulfate binding to hTF/2N wild-type protein. These data show that these two lysine residues have an important role in divalent anion binding.

Serum transferrin is one of a class of *ca.* 80-kDa glycoproteins, able to bind reversibly a ferric or other di- or trivalent metal ion and a synergistic anion in each of two lobes (Aisen, 1989; Harris & Aisen, 1989). The synergistic anion is required for specific metal binding. To function as a synergistic anion, a compound must have a free carboxyl group, a small size, and an electron donor group (Woodworth et al., 1975; Foley & Bates, 1988). The two lobes of transferrin are connected by a short polypeptide, and each lobe contains two domains with a high-affinity binding site in the cleft between them (Anderson et al., 1990; Sarra et al., 1990; Baker et al., 1991; Baker & Lindley, 1992). As shown by x-ray crystallographic studies of the ternary complexes, each binding site has a ferric ion octahedrally bound to the side chains of two tyrosines (Tyr 95, 188 in the hTF sequence), one histidine (His249), one aspartate (Asp63) and two oxygens from carbonate, the natural synergistic anion. In addition, in human lactoferrin and rabbit transferrin, the carbonate is H-bonded to the side chain of Arg124 and to the δ^+ end of helix 5 (Baker & Lindley, 1992). Biophysical studies have indicated that there are significant conformational changes that accompany metal-binding and release (Kilar & Simon, 1985; Baker & Lindley, 1992; Grossmann et al., 1992). Recent low-angle x-ray scattering studies appear to show that in solution, both the N- and C-lobes are open in the apo-form and closed in the diferric form for human transferrin and lactoferrin, and hen ovotransferrin (Grossmann et al., 1992).

Previous difference UV spectrophotometric studies measuring divalent anion binding to full length apo-transferrin suggested that anions bind to cationic side chains in the protein in the immediate vicinity of the metal binding site. Binding of anions gave rise to negative absorption at 246 nm and allowed estimation of binding constants that are considerably smaller than those found for the binding of metal ions (Harris, 1985, 1989). Monoferric transferrins were used to estimate the anion binding affinity for each vacant lobe (Harris, 1989; Harris et al., 1990). Phosphate and sulfate are mimics of the synergistic anions. They produce the negative ΔA_{246} effect but cannot serve as synergistic anions when metal is bound (Harris, 1985). Phosphate showed a slight preference for the N-terminal site when binding to monoferric human transferrin, while sulfate appeared to have no site selectivity. Oe et al. (1989) have determined that the two classes of anion binding sites reported by Harris for human serum transferrin appear to exist in chicken ovotransferrin and can be attributed to the individual lobes.

The recombinant N-lobe of human serum transferrin (hTF/2N)[1] and several single point mutants have been expressed in baby hamster kidney cells transfected with the plasmid pNUT containing the relevant DNA coding sequence (Woodworth et al., 1991). Each wild-type hTF/2N and mutants contain one binding site for metal ions and anions (Wood-

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[1] Abbreviations and symbols: hTF/2N, the N-lobe of recombinant human serum transferrin; hTF, human serum transferrin; hLF, human lactoferrin; rTF, rabbit serum transferrin; H207E, the 207His→Glu mutant of hTF/2N; K296E, the 296Lys→Glu mutant of hTF/2N; K206Q and K296Q, the respective Lys→Gln mutants at position of 206 and 296 of hTF/2N; $_N$hTF$_C$Fe, monoferric human transferrin with iron bound to the C-lobe; BHK, baby hamster kidney; HEPES, *N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulfonic acid]; NTA, nitrilotriacetate; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; pD, pH determined in solution of D_2O, but not corrected to H_2O; $\Delta\epsilon$, difference in molar absorptivity.

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worth et al., 1991; Wang et al., 1992 and unpublished experiments). The present study reports the binding of sulfate, phosphate, oxalate, and malonate to the wild-type hTF/2N and its H207E mutant at a series of different pH values using the technique of UV difference spectrophotometry and suggests a binding mechanism and putative binding site.

MATERIALS AND METHODS

Expression, Isolation and Purification of Recombinant Proteins. The amino-terminal half-molecule of human serum transferrin (hTF/2N) and a mutant, in which the histidine at position 207 was mutated to glutamic acid (H207E), were expressed in transformed baby hamster kidney (BHK) cells using the pNUT vector. Similar strategies were used to produce the K296E and K296Q mutants. The preparation and production of these two mutants will be described in a separate report. Expression, isolation, and purification of hTF/2N and H207E have been described in detail (Funk et al., 1990; Mason et al., 1991; Woodworth et al., 1991). Iron was removed from the iron protein by incubation in 0.5 M sodium acetate buffer, pH 4.9, containing 1 mM NTA and 1 mM EDTA. The apo-transferrin was concentrated to a minimum volume in a Centricon 10 microconcentrater (Amicon), then diluted to 2 mL and reconcentrated three times, first with 0.1 M sodium perchlorate, and then with 0.1 M potassium chloride.

Chemicals were reagent grade. Stock solutions of HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]), sulfate, phosphate, malonate, and oxalate were prepared by dissolving the anhydrous salts in Milli-Q (Millipore Corp.) purified water, and adjusting the pH to 6.5, 6.8, 7.0, 7.5, 7.8, 8.0, or 8.5 with HCl and KOH.

Anion Titrations. Solutions of approximately 8.5×10^{-6} M apo-hTF/2N or H207E, in 0.01 M HEPES at a series of pH values were placed into the sample cuvette. The reference cuvette contained 0.01 M HEPES buffer with the same volume and pH as the samples. A CARY 219 spectrophotometer under the control of the computer program Olis-219s was used to analyze the samples. A baseline of apo-protein vs. HEPES buffer was recorded from 316 to 236 nm. Anion solutions, 0.05 to 0.5 M, at the same pH as the buffer were titrated into both sample and reference cuvettes. A scan from 316 to 236 nm was taken at each step of the titration. The spectrum of the apo-protein was stored as the baseline and was subtracted from the sample spectra; the results were difference spectra. The cell holder was kept at 25 °C by an external circulating water bath. The analytical concentration of apo-transferrin in each sample solution was determined from the A_{280} and $\epsilon_{280}{}^{mM} = 38.6$ (Funk et al., 1990). $\Delta\epsilon_{246}$ was calculated after each addition of anion, as ΔA_{246}, which has been corrected for sample dilution, divided by the concentration of transferrin. Anion binding constants were calculated from direct titration data by nonlinear least-squares techniques assuming one anion binding site per transferrin half-molecule (Harris, 1985). The mass balance equations and equilibrium constants for the anion−hTF/2N system are:

$$\text{hTF/2N} + X^{2-} \overset{K}{\rightleftharpoons} \text{hTF/2N(X)} \qquad (1)$$

$$[\text{hTF/2N}]_{tot} = [\text{hTF/2N}] + [\text{hTF/2N(X)}] \qquad (2)$$

$$[\text{Anion}]_{tot} = [X] + [\text{hTF/2N(X)}] \qquad (3)$$

$$K = [\text{hTF/2N(X)}]/([X] \times [\text{hTF/2N}]) \qquad (4)$$

$$K_d = [X] \times [\text{hTF/2N}]/[\text{hTF/2N(X)}] \qquad (5)$$

$$\Delta\epsilon_{cal} = \Delta\epsilon^M \times [\text{hTF/2N(X)}]/[\text{hTF/2N}]_{tot} \qquad (6)$$

where $\Delta\epsilon^M$ is the molar absorptivity for the difference absorbance of the anion−protein complex per binding site. Then

$$\Delta\epsilon_{cal} = \Delta\epsilon^M \times [X]/(K_d + [X]) \qquad (7)$$

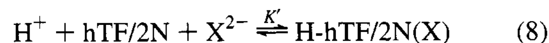
Using an iterative technique, from a set of initial guesses of $\Delta\epsilon^M$ and K_d, a nonlinear least squares fitting routine varied K_d and $\Delta\epsilon^M$ so as to minimize the sum of the squares of residuals between $\Delta\epsilon_{cal}$ and $\Delta\epsilon_{obsd}$. This procedure was carried out by the computer program AXUM 3.

RESULTS

As originally shown by Harris (1985, 1989), the binding of anions, such as sulfate, to hTF can be monitored by difference UV spectrophotometry. A typical titration of the recombinant N-lobe of human transferrin with sulfate is presented in Figure 1. The spectra are characterized by a strong negative absorption band at 246 nm and a weaker, broader minimum in the 290 to 310 nm range. There is also a shallow, broad, positive absorbance between 260 to 290 nm. As previously reported (Harris, 1989), the spectra are mirror images of those found for binding of metal ions to apo-transferrin.

As described in methods, the ΔA_{246} is converted to an apparent molar absorptivity ($\Delta\epsilon$), which takes into account the protein concentration. The accumulated anion concentration and the $\Delta\epsilon$ values were then used to estimate the binding constant K and the $\Delta\epsilon_{246}$ (the maximum of $\Delta\epsilon$ at 246 nm) for anion binding at each different pH. The results for sulfate binding to hTF/2N and the H207E mutant are given in Table 1, expressed as log*K*. The binding constants increase as pH values decrease. In addition, the sum of pH and log*K* is a constant for each anion binding to each protein.

As shown in Table 2, there are slight differences ($\alpha = 0.05$) in the sum of log*K* and pH for the different anions binding to hTF/2N and the H207E mutant. The sum was in the range of 11.1 to 12.2 for the anions binding to hTF/2N and 10.8 to 11.9 for the H207E mutant. In each case, the anions bound to hTF/2N with a slightly higher affinity than to the mutant. In all of the samples, the plots of log*K* vs. pH for the anions binding to either protein yielded straight lines with slopes of *ca.* −1.0, as shown in Table 3. These slopes indicate that one hydrogen ion participates in the binding of each divalent anion to the protein, such that at equilibrium



$$H^+ + \text{hTF/2N} + X^{2-} \overset{K'}{\rightleftharpoons} \text{H-hTF/2N(X)} \qquad (8)$$

where $\log K' = \log K + \text{pH}$. The data for phosphate and malonate fit this equation, only if adjusted for the inherent pK_as of the anions, such that the species binding is divalent. Sulfate and oxalate do not have pK_as within the pH range studied.

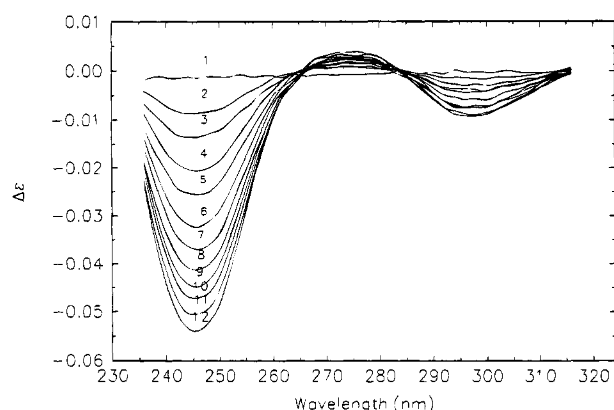


FIGURE 1: Difference UV spectra of sulfate binding to hTF/2N. A sample of apo-hTF/2N (7.7×10^{-6} M) in 0.01 N HEPES, pH 8.0, was titrated with sulfate. The spectra were generated by scanning from 316 to 236 nm for the apo sample and after each addition of sulfate. The spectrum of the apo-protein was recorded as the baseline, and further difference spectra were plotted by subtracting the baseline from each scan. The values of difference absorbance were converted into $\Delta\epsilon$. The accumulated concentrations of sulfate in the solution were (1) 0.0 μM; (2) 40 μM; (3) 80 μM; (4) 120 μM; (5) 240 μM; (6) 440 μM; (7) 635 μM; (8) 832 μM; (9) 1230 μM; (10) 1620 μM; (11) 2600 μM; (12) 7450 μM.

Table 1: Binding Values for Sulfate–Transferrin N-Lobe Complexes

pH	$\Delta\epsilon_{246}$ (S.E.)[a] (M^{-1} cm^{-1})	Log K (S.E.) (M^{-1})	Log K + pH
		hTF/2N	
6.53	−1080 (33)	5.04 (0.10)	11.57
7.07	−2440 (27)	4.51 (0.03)	11.55
7.54	−5270 (87)	4.04 (0.03)	11.57
8.02	−7240 (110)	3.57 (0.03)	11.60
		Mean (S.E.)	11.57 (0.01)
		H207E	
6.64	−795 (29)	4.57 (0.11)	11.21
7.06	−3050 (36)	4.29 (0.03)	11.35
7.50	−4790 (28)	3.84 (0.02)	11.34
7.93	−6030 (115)	3.38 (0.04)	11.31
8.38	−7559 (87)	2.64 (0.02)	11.02
		Mean (S.E.)	11.25 (0.12)

[a] Standard errors are genrated by nonlinear least squares fitting routine.

Table 2: Sum (S.D.) of log K and pH for Anion Binding to hTF/2N and H207E

anion	N[a]	hTF/2N	N	H207E	P value (α = 0.05)
phosphate	12	12.14 (0.12)	9	11.88 (0.17)	$P = 0.001$
oxalate	8	11.82 (0.04)	7	11.26 (0.08)	$P < 0.001$
sulfate	15	11.60 (0.14)	7	11.24 (0.14)	$P < 0.001$
malonate	7	11.10 (0.10)	9	10.77 (0.13)	$P < 0.001$

[a] Number of titrations carried out at all different pH values.

Table 3: Slopes (S.E.) of log K vs pH for Anion Binding

anion	hTF/2N	H207E
phosphate	−1.02 (0.03)	−1.06 (0.07)
sulfate	−0.98 (0.02)	−0.93 (0.07)
malonate	−1.00 (0.02)	−0.76 (0.08)
oxalate	−0.98 (0.03)	−0.90 (0.08)

As shown in Figure 2, $-\Delta\epsilon_{246}$ increases with increasing pH. For binding of the various anions to both hTF/2N and H207E, Hill plots yielded the results listed in Table 4. All anions gave slopes around 1 (the range of 0.87 to 1.14 is not statistically significant). For sulfate and phosphate binding to both proteins, the pK_as were 7.4. For malonate and oxalate binding to hTF/2N, the pK_as were 6.9 and 7.1, respectively, and increased to 7.3 and 7.6 on binding to H207E.

Since the ethanesulfonic group of HEPES is negatively charged in the pH range of the experiment, it could act as a competing anion in the solution. The titration of hTF/2N and H207E by sulfate at pH 7.5 was therefore also carried out in 0.01, 0.05, and 0.1 M HEPES. The results are given in Table 5. Similar to the results reported by Harris et al. (1990) for apo- and monoferric transferrin, when the concentration of HEPES increases from 0.01 M to 0.1 M, the log$K$ for sulfate binding to hTF/2N and H207E decreases 0.1 to 0.2 units. Also a decrease in $\Delta\epsilon_{246,max}$ was observed. The plot of the K_d for sulfate vs. concentration of HEPES gave a straight line, with the slope as the inhibitory dissociation constant of HEPES (K_d^{HEPES}) and the intercept as the dissociation constant of sulfate (K_d^{sulf}) (Table 5). Evidently HEPES binds at least three orders of magnitude less well than do the divalent anions studied. Similar results were found for chloride (data not shown). No quantifiable binding of bicarbonate to hTF/2N could be detected by this methodology, consistent with similar findings in calorimetric studies (Lin et al., 1993).

In an attempt to locate the anion binding site in the N-lobe of human transferrin, some additional titrations were carried out. The K296E, K296Q, and K206Q mutants were titrated with the nonsynergistic anion sulfate at pH 7.4 in 10 mM HEPES buffer. The mutant K296E gave $\Delta\epsilon_{246} \approx 0$, whereas K296Q gave a slightly positive $\Delta\epsilon_{246}$. The mutant K206Q gave $\Delta\epsilon_{246} = -2130$ and log$K = 3.58 \pm 0.01$, i.e., log$K' = 3.58 + 7.4 = 10.98$, which is about 0.6 log units less than the constant obtained for sulfate binding to hTF/2N wild type and 0.26 log units less than binding to the H207E mutant. In addition, the K296E mutant was titrated with the synergistic anion oxalate. As with the sulfate, there was an absence of effect at $\Delta\epsilon_{246}$ implying that the lysine at position 296 is the protonation site during the binding of divalent anion, and possibly the initial binding site for divalent anions.

DISCUSSION

In the present study, the binding of divalent anions to hTF/2N and several single point mutants was measured at a number of different pHs. The use of the recombinant N-lobe of serum transferrin allows the results to be interpreted in a straightforward manner; i.e., one binding constant and one capacity factor are involved, rather than four adjustable parameters required for the full-length protein. The pH profile is important in establishing the fact that for each anion bound, an H^+ is bound. Furthermore, the use of single point mutants allows assignment of residues involved in the phenomenon.

A mutation of histidine at position 207 to glutamate was made to mimic the amino acid sequence in this region of lactoferrin (Woodworth et al., 1991). Previous work has shown that this H207E mutant binds iron approximately 20 times more tightly than does wild-type hTF/2N (Lin et al., 1993). His207 resides at the bottom of the interdomain cleft. In the pH range of our titration experiments, the side chain of this histidine residue would be uncharged or have a single

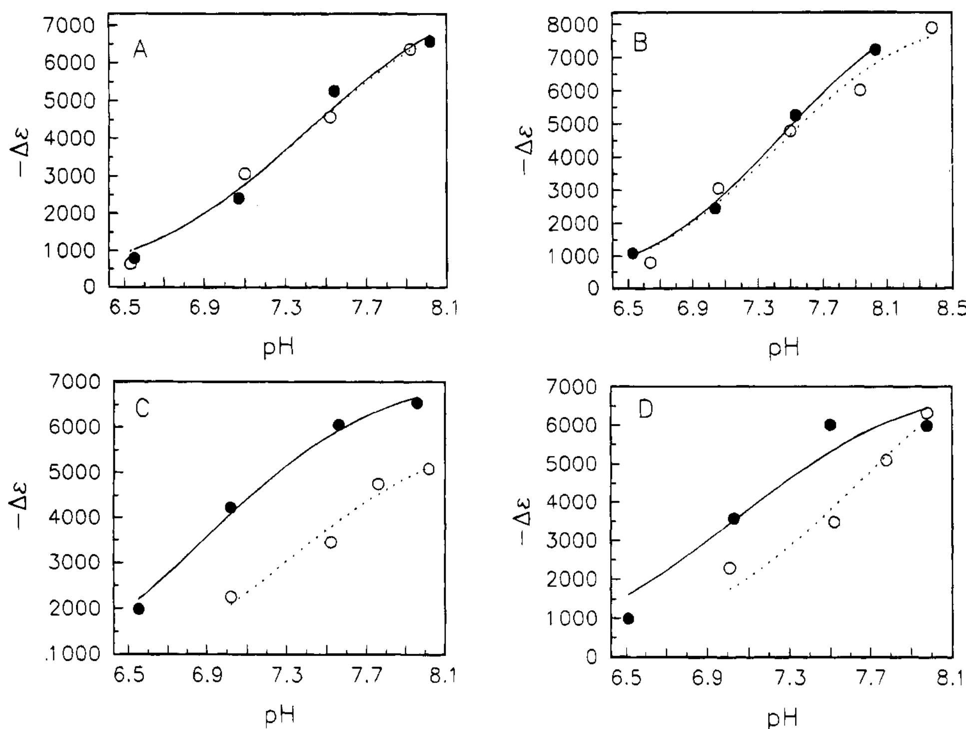


FIGURE 2: $-\Delta\epsilon_{246}$ vs pH. $-\Delta\epsilon_{246}$ was plotted as a function of pH for both hTF/2N (●) and H207E (O) when titrated with (A) phosphate; (B) sulfate; (C) malonate; (D) oxalate.

Table 4: Parameters from Hill Plots for $\log[\Delta\epsilon_{obs}/(\Delta\epsilon_{max} - \Delta\epsilon_{obs})]$ vs. pH

	hTF/2N		H207E	
anion	pK_a (S.E.)	slope (S.E.)	pK_a (S.E.)	slope (S.E.)
phosphate	7.43 (0.05)	1.07 (0.09)	7.42 (0.05)	1.14 (0.11)
sulfate	7.43 (0.02)	0.99 (0.04)	7.43 (0.09)	1.13 (0.11)
malonate	6.93 (0.05)	0.99 (0.08)	7.30 (0.06)	0.97 (0.13)
oxalate	7.12 (0.14)	1.06 (0.27)	7.60 (0.06)	0.87 (0.15)

positive charge. In the H207E mutant, a negative charge is introduced into this region of the protein. We were interested in determining the effect of this mutation on the anion binding. As shown in Table 2, the sum of log$K$ and pH = logK' decreases for the H207E mutant relative to the wild-type protein ($\alpha = 0.05$). Increased charge repulsion could play a role in this effect. The logK' decreases by 0.5 log units for oxalate, which occupies a larger volume than carbonate and can also serve as a synergistic anion for Fe^{3+} or Ga^{3+} binding to transferrin (Valcour & Woodworth, 1987; Woodworth et al., 1987; Kubal et al., 1993). For the inorganic anions, the logK' decreases by only about 0.2 log units. Also notable is the fact that when titrated with phosphate and sulfate, the pK_a of hTF/2N and H207E as derived from the Hill plots is stable at 7.4 (Table 4). In contrast, upon titration with oxalate and malonate, the pK_a is lower by *ca.* 0.5 pH unit for hTF/2N. For H207E mutant the pK_a rises by 0.2 pH units in the case of oxalate and falls 0.1 pH unit in the case of malonate. Thus, the H207E mutation had a smaller than expected effect on the binding of the synergistic anions oxalate and malonate, and almost no effect on the binding of the nonsynergistic anions phosphate and sulfate.

Clearly, as a divalent anion binds to transferrin, there is an obligate binding of an hydrogen ion (Tables 2 and 3). Thus a direct relationship exists between the binding of these two entities as expressed in eq 8. The failure to observe binding of bicarbonate (this study and Lin et al., 1993) can be rationalized on the basis that divalent carbonate is the form that binds specifically to transferrin, consistent with the results from the x-ray crystallography of transferrins (Baker & Lindley, 1992). In the pH range of our studies, the carbonate concentration will be vanishingly small as the second p$K_a \approx 10$ for carbonic acid. Even though the K_d for anion binding decreases with decreasing pH, the carbonate concentration will likewise decrease, so the two effects cancel.

When transferrin binds metal ions, $\Delta\epsilon_{246}$ is positive because the liganding tyrosyl side chains are deprotonated (Tan & Woodworth, 1969; Pecoraro et al., 1981). When divalent anions bind to transferrin, $\Delta\epsilon_{246}$ is negative. One interpretation of this phenomenon is that a critical tyrosyl hydroxyl group is H-bonded to another specific side chain, thus stretching the tyrosyl hydroxyl (OH) bond, so that it appears to be partially ionized. Upon binding the hydrogen ion associated with the binding of divalent anion, this H-bond is broken so that the OH bond relaxes to its normal, shorter state, resulting in a decrease in the $\Delta\epsilon_{246}$. If one assumes a value of $\Delta\epsilon_{246} \approx 1 \times 10^4$ for the ionization of the tyrosyl phenolic OH (Tan & Woodworth, 1969), the $\Delta\epsilon_{246}{}^{M}$ values reported in Table 1 suggest that this H-bond in apo-transferrin is quite strong. The drop in the pK_a of the proton acceptor of *ca.* 0.4 pH unit on going from the anion mimics (i.e., sulfate and phosphate) to the synergistic anions (i.e., oxalate and malonate) suggests that binding of the synergistic anions requires more steric rearrangement within the binding cleft than does binding of the mimics. Thus, breaking the H-bond in question is energetically less favored for the synergistic anions than for the mimics. It appears that this distinction is relaxed in the H207E mutant.

Recent studies indicate that there are two major structural differences between diferric human lactoferrin (hLF) and

Table 5: Sulfate Binding to hTF/2N and H207E in Different Concentrations of HEPES at pH 7.5

	HEPES	$\Delta\epsilon_{246}$ (S.E.) (M^{-1} cm^{-1})	log$K$ (S.E.) (M^{-1})	logK_d^{sul} (S.E.) (M)	logK_d^{HEPES} (S.E.) (M)
hTF/2N	10 mM	−5270 (87)	4.02 (0.03)	−4.02 (0.00)	−0.36 (0.02)
	50 mM	−4820 (82)	3.97 (0.02)		
	100 mM	−4635 (27)	3.93 (0.02)		
H207E	10 mM	−4790 (28)	3.84 (0.02)	−3.89 (0.01)	−0.85 (0.04)
	50 mM	−3750 (54)	3.76 (0.02)		
	100 mM	−2890 (58)	3.64 (0.02)		

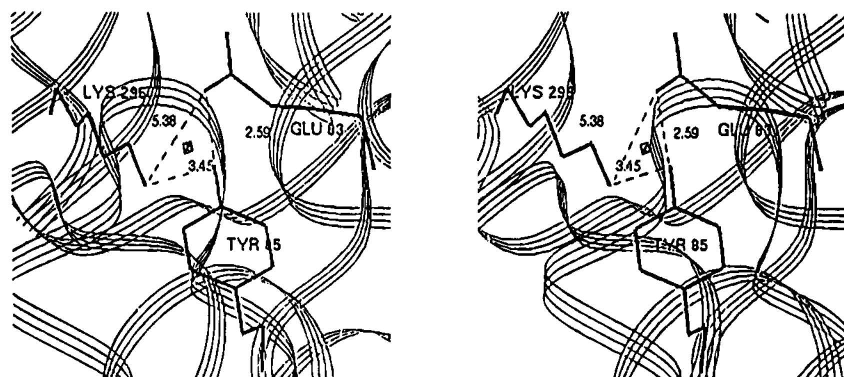


FIGURE 3: Stereo view of simulated tricenter H-bond among Lys296, Tyr85, and Glu83 in apo-hTF/2N. A pseudoproton is shown as ◊, and distances (Å) are shown in numbers. (Based on the X-ray crystallographic studies by Zuccola, 1992.)

Table 6: Distance and Angle of the Simulated Tricenter H-Bond in Apo-hTF/2N

	Angle (°)		Distance (Å)
∠NZ[a]−'H'[b]−OH[c]	118.5	NZ−'H'	2.89
∠OE1[d]−'H'−OH	80.7	OH−'H'	0.97
∠NZ−'H'−OE1	160.7	OE1−'H'	2.57

[a] NZ of Lys296. [b] The pseudoproton. [c] OH of Tyr85. [d] OE1 of Glu83.

rabbit serum transferrin (rTF), which might contribute to the different binding properties of these two proteins (Baker & Lindley, 1992). In the N-lobe of diferric rTF, Lys296 forms a close contact with Lys206 (3.2 Å), while in hLF, the corresponding Lys301 forms a salt bridge with Glu216. This salt bridge is replaced by a hydrogen bond between Ser298 and Glu212 in rTF. The Lys206−Lys296 interaction of transferrin occurs close to the iron binding site in the hinge region and has been suggested to be a possible anion binding site (Baker & Lindley, 1992). In the iron-complexed hen ovotransferrin N-lobe, the NZs of these two conserved lysines (Lys209 and Lys301) are 2.3 Å apart, forming a low-barrier hydrogen bond and possibly functioning as a pH-sensitive trigger for iron binding and release, since one or both of the lysines are thought to have an unusually low pK_a (Dewan et al., 1993). Amino-aromatic hydrogen bonds normal to the Lys−NZ, and the π-electron clouds of two tyrosyl rings also stabilize these two lysines. A similar structure exists in the iron-bound rabbit serum transferrin N-lobe (Lys206−NZ points at the ring of Tyr85, while Lys296−NZ points at the ring of Tyr95) and in iron forms of hTF/2N and H207E mutant (Sarra et al., 1990; Wang et al., 1992 and unpublished experiments). Indeed the overall structures of hTF/2N and its H207 mutant are very much alike, with minimal shifts in residue position. However, in apo-hTF/2N, the two Lys−NZ are far away from the tyrosyl rings (6−7 Å) and from each other, forming a loose pocket while the NZ of Lys296, the side chain carboxylate-OE1 of Glu83 and the hydroxyl-OH of Tyr85 form a tricenter H-bond. As shown in Figure 3, a pseudoatom is drawn according to geometric weighting of these three electronegative atoms, based on the x-ray crystallographic studies of $_N$hTF$_C$Fe (monoferric hTF with the iron in the C-site) (Zuccola, 1992). This tricenter structure is conserved in iron complexes of hTF/2N and the H207E mutant (Wang et al., 1992 and unpublished experiments). The distance is 3.45 Å between the OH of Tyr85 and the NZ of Lys296, 2.59 Å between the OH of Tyr85 and the OE1 of Glu83, and 5.38 Å between the NZ of Lys296 and OE1 of Glu83. If we assume the pseudoatom to approximate the position of the proton, 'H', the distances and angles as listed in Table 6, fit well for a tricenter H-bond in a protein or a small molecule (Jeffrey & Saenger, 1991). Also, in the side chain of Tyr85, the angle of C_4−OH−'H' is 130°. This indicates that this tricenter H-bond may not only stretch Tyr85−OH, but also bend it.

Titration of the hTF/2N mutants K296E, K296Q, and K206Q with sulfate and oxalate appears to indicate an important role for Lys296 and Lys206 in anion binding. Since anion binding is accompanied by proton binding, we

Table 7: Comparison of Binding Constants of Divalent Anions to Various N-Lobe of Transferrin

transferrin	method	buffer	logK' = log$K$ + pH: phosphate	sulfate	oxalate
Recombinant human serum transferrin N-lobe	difference UV spec.[a]	0.01 M HEPES, series pH values	12.14	11.60	11.82
	proton NMR[b]	0.01 M KCl in D_2O, pD 7.25	N/A	N/A	11.29
$_N$TF$_C$Fe[c]	difference UV spec.	0.1 M HEPES, pH 7.4	11.39	10.83	N/A
Proteolytically derived ovotransferrin N-lobe[d]	difference UV spec.	0.1 M Tris-HCl, pH 8.0	11.1	N/A	11.0

[a] This study. [b] Kubal et al. (1993). [c] Harris, (1989). [d] Oe et al. (1989).

speculate that the NZ of Lys296 is the group protonated as the initial target, thus breaking the hydrogen-bonding network. This would allow the tyrosyl OH bond to relax to its normal length or position, resulting in the observed decrease in $\Delta\epsilon_{246}$. In the K206 and K296 mutants, the binding site has been modified, and we see decreased or no change in the $\Delta\epsilon_{246}$ and a decrease in the binding affinity. It was also found that there is no difference UV spectrum observed when titrating diferric transferrin with anions (Harris, 1985). When forming the metal–transferrin complex, the protein goes through a structural change, which also modifies the divalent anion binding site.

In the present studies of divalent anion binding to recombinant N-lobe transferrin in 0.01 M HEPES at a series of pH values, as listed in Table 7, a similar binding constant has been reported for the binding of oxalate to hTF/2N as measured by proton NMR, where $\log K + \mathrm{pD} = 11.29$ (Kubal et al., 1993) vs. 11.81 (present study). In the studies of anion binding to monoferric transferrin N-lobe ($_{N}$hTF$_{C}$Fe) shown in Table 7, the values of $\log K + \mathrm{pH}$ for dibasic phosphate and sulfate were 11.39 ($\log K = 3.99 \pm 0.13$) and 10.83 ($\log K = 3.43 \pm 0.15$) respectively (Harris, 1989), about 0.8 units lower than the results from hTF/2N (or 0.6 units lower in 0.1 M HEPES; see Table 5). In studies of anion binding to the proteolytically derived ovotransferrin N-lobe, the K_d of phosphate and oxalate binding to protein was 8.6×10^{-4} M and 1.1×10^{-3} M in 0.1 M Tris-HCl at pH 8.0; then $\log K + \mathrm{pH} = 11.1$ and 11.0, as shown in Table 7, respectively (Oe et al., 1989). The differences among the binding constants of each anion species to various proteins may be caused by the differences in the environment of anion binding sites and different buffers that were used. However, the slopes of Hill plots ($\log[\Delta\epsilon_{obs}/(\Delta\epsilon_{max} - \Delta\epsilon_{obs})]$ vs. log[Anion]) are about 1 for all the anions binding to transferrins (data not shown). This indicates that one anion binds per binding site (Harris, 1989).

CONCLUSIONS

The present study of divalent anion binding to recombinant human serum transferrin N-lobe and a number of single point mutants shows that in the mechanism of divalent anion binding to hTF/2N, a proton takes part in the process. The single point mutant H207E has a less than expected effect on the binding of the synergistic anion (oxalate and malonate), with little or no effect on the binding of the nonsynergistic anion (phosphate and sulfate). The residues Lys296 and Lys206 appear to play an important role in binding of both synergistic and nonsynergistic anions to apotransferrin, and Lys296 may be the initial site for divalent anion binding in hTF/2N prior to binding of metal ion. Concomitant with ligation of metal ion, the planar synergistic anion moves to its final position to coordinate the metal ion. Evidently the tetrahedral mimics are sterically incapable of serving this latter function.

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