Thermodynamics of Anion Binding to Human Serum Transferrin†

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ABSTRACT: The binding of phosphate, bicarbonate, sulfate, and vanadate to human serum transferrin has been evaluated by two difference ultraviolet spectroscopic techniques. Direct titration of apotransferrin with bicarbonate, phosphate, and sulfate produces a strong negative absorbance near 245 nm, while titration with vanadate produces a positive absorbance in this region. Least-squares refinement of the absorbance data indicates that two anions of sulfate, phosphate, and vanadate bind to each transferrin molecule but that there is detectable binding of only a single bicarbonate anion. A second method used to study the thermodynamics of anion binding was competition equilibrium between anions for binding to the transferrin. The equilibrium constant for binding of the first equivalent of vanadate was determined by competition vs. phosphate and sulfate, while the equilibrium constant for binding of the second equivalent of bicarbonate was determined by competition vs. vanadate. Anion binding was described by two equilibrium constants for the successive binding of two anions per transferrin molecule: K_1 = [A–Tr]/[A][Tr] and K_2 = [A–Tr–A]/[A][A–Tr] where [A] represents the free anion concentration, [Tr] represents apotransferrin concentration, and [A–Tr] and [A–Tr–A] represent the concentrations of 1:1 and 2:1 anion–transferrin complexes, respectively. The results were the following: for phosphate, log K_1 = 4.19 ± 0.03 and log K_2 = 3.25 ± 0.21; for sulfate, log K_1 = 3.62 ± 0.07 and log K_2 = 2.79 ± 0.20; for vanadate, log K_1 = 7.45 ± 0.10 and log K_2 = 6.6 ± 0.30; for bicarbonate, log K_1 = 2.66 ± 0.07 and log K_2 = 1.8 ± 0.3. Previous studies have shown that vanadate binds to the phenolic oxygens of the metal binding sites of transferrin [Harris, W. R., & Carrano, C. J. (1984) *J. Inorg. Biochem. 22*, 201–218]. The direct competition observed between the other inorganic anions and vanadate and also the intense UV difference spectrum induced by binding of these anions to apotransferrin both indicate that the sulfate, phosphate, and bicarbonate are also interacting with these phenolic oxygens. Furthermore, binding of these anions is completely blocked in diferric transferrin. A model is proposed that involves hydrogen bonding of the anions both to essential arginine and possibly lysine residues and to the phenolic hydrogens of the tyrosine residues of the metal binding site.

It is well-known that most metal ions will not specifically bind to transferrin in the absence of a synergistic anion which is concomitantly bound and is probably directly coordinated to the metal in the resulting metal–anion–protein ternary complex. In biological systems, the anion is (bi)carbonate. The interlocking site model for this ternary complex (Bates & Schlabach, 1973; Schlabach & Bates, 1975) is now generally accepted. In this model, the anion binds to cationic protein side groups in the immediate vicinity of the metal binding site. The carbonate anion shields the metal ion from the destablizing positive charges on the protein and also provides one oxygen as a direct ligand to the metal. Arginine is frequently involved in the binding of anionic substrates to proteins (Riordan et al., 1977; Riordan, 1979), and indeed, chemical modification studies have shown that there is one essential arginine residue per metal binding site in ovotransferrin (Rogers et al., 1978). The protonated ϵ-amino group of lysine and the imidazole group of histidine have also been suggested as possible anion binding groups (Bates & Schlabach, 1973; Zweier & Aisen, 1977; Campbell & Chasteen, 1977; Zweier et al., 1981; Alsaadi et al., 1981; Chasteen, 1983).

Although metal ion, protein, and anion are all required for the formation of a stable protein complex, the mechanism of formation does not involve a true third-order reaction. Kojima & Bates (1981) have studied the effects of bicarbonate concentration on the kinetics of the binding and oxidation of ferrous ion. Saturation kinetics with respect to the bicarbonate anion were observed, and the results were interpreted in terms of the formation of a binary anion–protein intermediate species.

There have been few studies which directly address the issue of anion binding to the apoprotein in the absence of metal ion. Bicarbonate binding is rather weak, and it has been particularly difficult to obtain quantitative data on the thermodynamics of anion binding. Carbon-13 NMR spectra of apotransferrin solutions containing ^{13}C-enriched bicarbonate show a peak identified as carbonate bound to the protein (Zweier et al., 1981), but such experiments do not measure the strength of binding. From their kinetic study, Kojima & Bates (1981) reported that half-maximum velocity in the binding and oxidation of ferrous ion to transferrin was reached at a bicarbonate concentration of about 11 mM, which would correspond to a bicarbonate binding constant of about 10^2. Woodworth et al. (1975) directly measured the binding constants for carbonate with ovotransferrin by fluorescence quenching, equilibrium dialysis, and equilibrium ultracentrifugation. They reported a binding constant, expressed in terms of the carbonate dianion rather than bicarbonate, of about 10^6 at pH 9–9.5.

In this paper, the equilibrium constants for the binding of sulfate, phosphate, vanadate, and bicarbonate to human serum transferrin are reported. The binding of these anions has been measured directly by difference ultraviolet spectroscopy and indirectly by equilibrium competition of one anion vs. another. The vanadate system has recently been described in detail (Harris & Carrano, 1984), but in this previous paper, only

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the value of K_2 for vanadate binding was reported. The data in this paper do not address the issue of whether the bicarbonate binds to transferrin as bicarbonate or as the carbonate dianion. The reactions are arbitrarily expressed in terms of bicarbonate, but equivalent binding constants written in terms of carbonate can easily be derived.

Experimental Procedures

Materials

Apotransferrin was purchased from Sigma and purified as previously described (Harris & Pecoraro, 1983). Standard vanadate solutions were prepared by dissolution of reagent-grade ammonium vanadate. Stock solutions of the other anions were prepared from reagent-grade sodium salts. Sodium bicarbonate solutions were freshly prepared immediately before each titration.

Methods

Anion Difference Titrations. Solutions of about 1.5×10^{-5} M apotransferrin were prepared by dilution of the concentrated stock solution in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (Hepes) with additional buffer. Sample solutions were adjusted if necessary to pH 7.40 ± 0.02 immediately prior to each titration. The exact concentration of apotransferrin in each sample solution was determined from the absorbance at 278 nm by using a molar absorptivity which was measured for each batch of apotransferrin by titration with standard iron bis(nitrilotriacetic acid) [$Fe(NTA)_2$]. Molar absorptivities were typically around 100 000 M^{-1} cm^{-1}. Anion difference UV titrations were performed by recording a base line of apotransferrin vs. apotransferrin and then adding aliquots of 50–100 mM solutions of the selected anion to the sample cuvette and equal volumes of distilled water to the reference curvette. The cuvettes were maintained at 25 °C by a jacketed cell holder connected to an external constant-temperature water bath. Values of $\Delta\epsilon$ were calculated as the absorbance at λ_{max} following each addition of an aliquot of the anion divided by the analytical concentration of transferrin.

Anion binding constants were calculated from the direct titration data by nonlinear least-squares techniques. Assuming the binding of two anions per transferrin molecule, the mass balance equations for the anion–transferrin system are

$$[Tr]_{tot} = [Tr] + [A\text{–}Tr] + [A\text{–}Tr\text{–}A] \quad (1)$$

$$[A]_{tot} = [A] + [A\text{–}Tr] + 2[A\text{–}Tr\text{–}A] \quad (2)$$

where [Tr] and [A] represent concentrations of free transferrin and free anion, respectively, and [A–Tr] and [A–Tr–A] represent the concentrations of 1:1 and 2:1 anion–transferrin complexes, respectively. These equations can be restated in terms of only two components, [Tr] and [A], and two anion binding constants:

$$[Tr]_{tot} = [Tr] + K_1[Tr][A] + K_1K_2[Tr][A]^2 \quad (3)$$

$$[A]_{tot} = [A] + K_1[Tr][A] + 2K_1K_2[Tr][A]^2 \quad (4)$$

The equilibrium constants K_1 and K_2 are defined as

$$K_1 = [A\text{–}Tr]/[A][Tr] \quad (5)$$

$$K_2 = [A\text{–}Tr\text{–}A]/[A][A\text{–}Tr] \quad (6)$$

Using a set of initial guesses for K_1 and K_2, we calculated values of [A] and [Tr] for each data point by using an iterative technique which minimizes the residuals between the analytical values for the total concentrations of transferrin and anion and the values calculated from eq 3 and 4. On the basis of these values of [A] and [Tr], a value of $\Delta\epsilon$ for each data point was calculated from

$$\Delta\epsilon_{calcd} = \frac{\Delta\epsilon_M K_1[Tr][A] + 2\Delta\epsilon_M K_1K_2[Tr][A]^2}{[Tr]_{tot}} \quad (7)$$

where $\Delta\epsilon_M$ is the molar absorptivity for the difference absorption of the anion–transferrin complex per binding site. After each cycle, numerical derivatives were used to vary K_1 and K_2 so as to minimize the sum of the squares of the residuals between $\Delta\epsilon_{calcd}$ and $\Delta\epsilon_{obsd}$.

Vanadate titrations were performed as described above. However, the free vanadate ion has a UV absorbance which overlaps the difference UV peak. Therefore, vanadate titration data were converted to $\bar{v}$ values, where $\bar{v}$ is the average number of vanadium ions bound per transferrin molecule. The absorbance (A) at any point in the titration curve is given by

$$A = \epsilon_V[VO_3] + \Delta\epsilon_{VT}[VO_2\text{–}Tr] + 2\Delta\epsilon_{VT}[VO_2\text{–}Tr\text{–}VO_2] \quad (8)$$

where ϵ_V is the molar absorptivity of the free vanadate and $\Delta\epsilon_{VT}$ is the molar absorptivity of the dioxovanadium(V)–transferrin complex per binding site. Using a vanadium mass balance equation analogous to eq 2, one can substitute for the concentration of free vanadate in terms of the analytical concentration of vanadium and the concentrations of the two vanndium–transferrin species. Dividing both sides of the resulting equation by the analytical concentration of transferrin yields

$$\frac{A}{[Tr]_{tot}} = \frac{\epsilon_V([V]_{tot} - [VO_2\text{–}Tr] - 2[VO_2\text{–}Tr\text{–}VO_2])}{[Tr]_{tot}} + \frac{\Delta\epsilon_{VT}([VO_2\text{–}Tr] + 2[VO_2\text{–}Tr\text{–}VO_2])}{[Tr]_{tot}} \quad (9)$$

One can then make the following assignments:

$$\Delta\epsilon_{obsd} = A/[Tr]_{tot} \quad (10)$$

$$r = [V]_{tot}/[Tr]_{tot} \quad (11)$$

$$\bar{v} = [VO_2\text{–}Tr] + 2[VO_2\text{–}Tr\text{–}VO_2]/[Tr]_{tot} \quad (12)$$

Equation 9 can now be rewritten as

$$\bar{v} = \frac{\Delta\epsilon_{obsd} - r\epsilon_V}{\Delta\epsilon_{VT} - \epsilon_V} \quad (13)$$

which was the equation used to calculate values of $\bar{v}$. For the least-squares calculation of vanadium–transferrin binding constants, a term to account for the absorbance of the free vanadate was added to eq 7.

Vanadate Competition Titrations. The stock apotransferrin was mixed with a solution of the appropriate anion to give a final solution which contained about 1.5×10^{-5} M transferrin, 0.01 M Hepes, and concentrations of either phosphate, sulfate, or bicarbonate ranging from 1 to 150 mM. These samples were titrated with vanadate ion, and values of the vanadate binding constants were calculated by using the procedures described above. The apparent value of the vanadate binding constant varied with the anion concentration. Therefore, binding constants measured in the presence of a competing anion were designated as K_{obsd}.

Results

Main Group Anion Titrations. Apotransferrin was titrated with solutions of sulfate, phosphate, bicarbonate, perchlorate, chloride, and nitrate. The addition of sulfate, phosphate, and bicarbonate produced a novel type of difference UV spectrum,

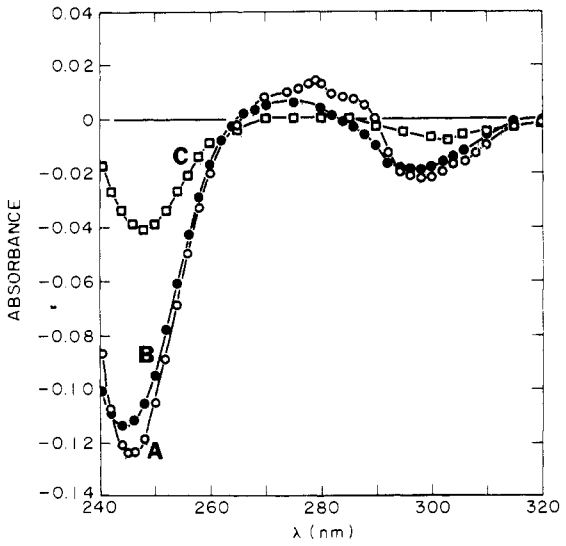

FIGURE 1: Difference UV spectra of apotransferrin–anion solutions vs. apotransferrin in 0.1 M Hepes, pH 7.4, 25 °C. [apoTr] $\simeq 1.4 \times 10^{-5}$ M. The solutions contained 0.58 mM phosphate (A), 1.2 mM sulfate (B), or 8.3 mM bicarbonate (C).

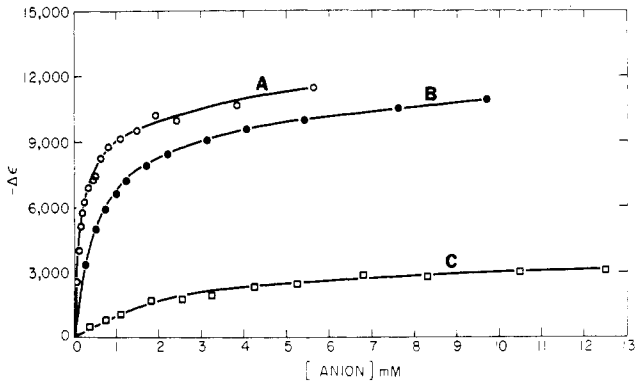

FIGURE 2: Plots of $\Delta\epsilon$ vs. [anion] for the titration of apotransferrin with phosphate (A), sulfate (B), and bicarbonate (C).

as shown in Figure 1. Phosphate and sulfate produced a strong negative absorbance around 245 nm and a weaker negative absorbance around 295 nm, as well as a very weak positive absorbance between 265 and 290 nm. The positions and relative intensities of these peaks are remarkably similar to those produced by the binding of several metal ions to apotransferrin, especially closed-shell metal ions such as gallium and zinc (Harris & Peroraro, 1983; Tan & Woodworth, 1969; Harris, 1983; Pecoraro et al., 1981). However, the anion-induced spectra are inverted relative to the metal–transferrin difference spectra, which have positive peaks at 245 and 290 nm with a broad negative absorbance around 270 nm. The spectrum produced by the bicarbonate anion is qualitatively similar to those produced by sulfate and phosphate, but less than half the intensity.

Values of $\Delta\epsilon$ were calculated as the absorbance at the minimum near 245 nm divided by the total transferrin concentration. Titration curves were prepared as plots of $-\Delta\epsilon$ vs. the accumulated anion concentration, as shown in Figure 2. Phosphate and sulfate both produce maximum $\Delta\epsilon$ values of about $-11\,000$ M^{-1} cm^{-1} at millimolar concentrations. Bicarbonate produces a different spectrum which reaches a maximum $\Delta\epsilon$ of about -3500 M^{-1} cm^{-1}. The other anions tested, perchlorate, nitrate, and chloride, all produced small negative absorbances near 245 nm, but the intensities appeared to be quite low, and no attempt was made to calculate $\Delta\epsilon$ values for these anions.

Table I: Binding Constants for Anion–Serotransferrin Complexes

anion	log K_1	method	log K_2	method	$\Delta\epsilon_M$
phosphate	4.19 ± 0.03	*a*	3.25 ± 0.21	*a*	−5500
sulfate	3.62 ± 0.07	*a*	2.79 ± 0.20	*a*	−5500
bicarbonate	2.66 ± 0.17	*a*	1.8 ± 0.30	*b*	−3500
vanadate	7.45 ± 0.17	*c*	6.6 ± 0.30	*a*, *c*, *d*	9400

[a] Direct titration. [b] Competition vs. vanadate. [c] Competition vs. phosphate. [d] Competition vs. sulfate.

The stoichiometry of anion binding was determined by using the function described by Folatjar & Chasteen (1982). The degree of saturation of the apotransferrin was defined as

$$\theta = \Delta\epsilon_i/\Delta\epsilon_{max} \quad (14)$$

An apparent association constant was defined as

$$K_a = \frac{\theta}{(1-\theta)[A]^n} \quad (15)$$

where [A] is the molar concentration of the anion at each point in the titration and n is the anion stoichiometry at each binding site. Equation 15 can be rearranged to give

$$-\log [\theta/(1-\theta)] + \log K_a = -n \log [A] \quad (16)$$

Plots of $\log [\theta/(1-\theta)]$ vs. log [A] are linear with values of n of 0.92 ± 0.04 for phosphate, 0.95 ± 0.06 for sulfate, and 1.06 ± 0.01 for bicarbonate. Thus, it appears that one anion binds per binding site. This method does not determine the number of binding sites per transferrin molecule. However, the observation that anion binding is completely blocked in diferric transferrin suggests that the anions are binding at or near the two iron binding sites of the protein.

The anion titration data were refined by nonlinear least-squares techniques as described under Experimental Procedures. The data were analyzed by using two models for anion binding. One model included a single binding site with a molar absorptivity ($\Delta\epsilon_M$) equal to the maximum $\Delta\epsilon$ value reached in Figure 2. The second model included two binding sites, each with a $\Delta\epsilon_M$ value equal to half the maximum $\Delta\epsilon$. The fits from the two methods were compared by using the R-factor ratio test as described by Hamilton (1964). The single-site model provided a satisfactory fit of the bicarbonate data with no statistically significant improvement on going to the more complex two-site model. However, a highly significant ($\alpha < 0.01$) improvement was observed by using the two-site model with both the sulfate and phosphate data. The anion binding constants K_1 and K_2 calculated from these direct titrations are listed in Table I.

Vanadate–Anion Competitions. The spectrum of the 1:1 vanadium–transferrin complex has a λ_{max} at 260 nm with a molar absorptivity of 9400 M^{-1} cm^{-1} (Harris & Carrano, 1984). This previous study concluded that the vanadium is actually bound as the dioxovanadium(V) cation to phenolic residues at the transferrin iron binding sites, which accounts for the positive difference spectrum at 245 nm compared to the negative absorbance observed at this wavelength with the other inorganic anions. When the vanadium–transferrin complex is formed in the presence of phosphate, sulfate, or bicarbonate, there is an apparent shift in both λ_{max} and $\Delta\epsilon_M$. The values are 9750 M^{-1} cm^{-1} (λ_{max} = 256 nm) in 10 mM bicarbonate, 10550 M^{-1} cm^{-1} (λ_{max} = 250 nm) in 10 mM sulfate, and 10900 M^{-1} cm^{-1} (λ_{max} = 242 nm) in 5 mM phosphate.

The titration of apotransferrin with vanadate ion was repeated in the presence of a series of increasing concentrations of phosphate, sulfate, and bicarbonate. Values of $\bar{v}$, defined in eq 12 as the average number of vanadium ions bound per transferrin molecule, are plotted in Figure 3 for a series of

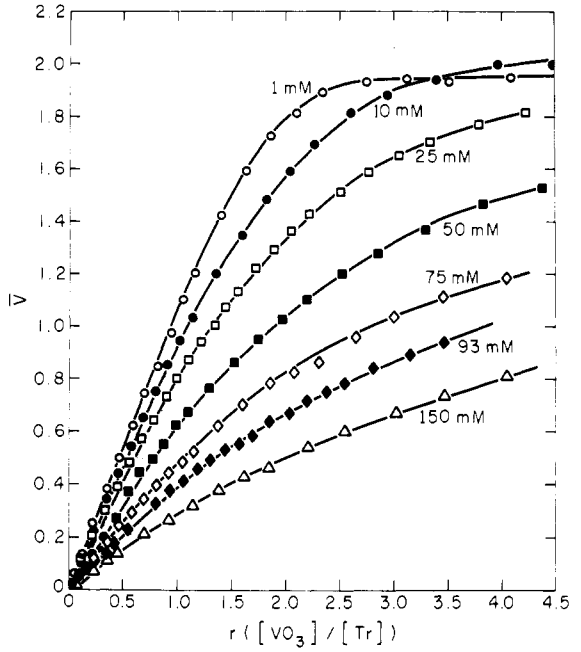

FIGURE 3: Titration of apotransferrin with vanadate in the presence of increasing concentrations of phosphate in 10 mM Hepes, pH 7.4. $\bar{v}$ is the average number of vanadium ions bound per transferrin molecule.

Table II: Apparent Vanadate–Transferrin Binding Constants at Various Phosphate Concentrations

phosphate concn (mM)	log $K_{1,obsd}$	log $K_{2,obsd}$
1		6.19 ± 0.7
2		5.91 ± 0.3
5		5.74 ± 0.09
10	6.17 ± 0.06	5.35 ± 0.05
20	5.60 ± 0.02	5.10 ± 0.01
25	5.50 ± 0.04	5.06 ± 0.02
30	5.54 ± 0.02	4.81 ± 0.01
50	5.09 ± 0.02	4.59 ± 0.01
75	4.87 ± 0.01	4.23 ± 0.01
93	4.67 ± 0.01	3.98 ± 0.01
100	4.78 ± 0.02	4.12 ± 0.01
125	4.51 ± 0.01	4.07 ± 0.01
150	4.49 ± 0.01	3.87 ± 0.02

phosphate concentrations. At low phosphate concentrations, 2 equiv of vanadium binds to apotransferrin. Starting with 1 mM phosphate, increasing phosphate concentrations result in lower values of $\bar{v}$ at a given value of r.

Apparent vanadium–protein binding constants have been calculated for a series of phosphate, sulfate, and bicarbonate concentrations. At low phosphate concentrations, only a value for the binding constant for the second equivalent of vanadium could be calculated because the first equivalent of vandium binds too tightly to the transferrin. At higher phosphate concentrations, there is measurable competition between phosphate and both equivalents of vanadate, and values for both $K_{1,obsd}$ and $K_{2,obsd}$ can be calculated. The set of binding constants calculated from phosphate competition experiments is shown in Table II. Competition from sulfate and bicarbonate is less effective than that from phosphate, so that only $K_{2,obsd}$ can be calculated even at higher anion concentrations. These $K_{2,obsd}$ values are shown in Table III.

Discussion

This is the first report that simple inorganic anions such as phosphate, sulfate, and bicarbonate induce a difference UV spectrum by binding to apotransferrin. These spectra are inverted relative to the usual metal ion induced spectra. The

Table III: Apparent Vanadate–Transferrin Binding Constants at Various Concentrations of Sulfate and Carbonate

sulfate concn (mM)	log $K_{2,obsd}$	carbonate concn (mM)	log $K_{2,obsd}$
10	6.01 ± 0.11	10	6.13 ± 0.13
20	5.99 ± 0.14	20	6.07 ± 0.07
50	5.26 ± 0.02	30	5.72 ± 0.02
100	5.00 ± 0.04	40	5.67 ± 0.05
		60	5.66 ± 0.02

anion spectra have a strong negative absorbance near 245 nm, compared to the usual positive absorbance caused by the binding of metal ions. The induction of a difference spectrum is indicative of a perturbation of aromatic groups within the protein. The changes in $\Delta\epsilon$ are quite large for certain anions and show a saturation effect with respect to anion concentration. Furthermore, this saturation is observed at relatively low anion concentrations, indicating high binding affinity, and the effect is completely blocked in diferric transferrin. These observations strongly suggest specific anion binding at or near the iron binding sites of the protein. Specific binding is also suggested by the variation in response of the transferrin to the various anions. Thus, it is proposed that the difference UV spectrum of the anion–protein complex is due to a direct interaction between the anions and the phenolic groups at the metal binding sites of the protein.

An alternative explanation could involve a general effect on protein conformation due to changes in ionic strength. Denaturation of the protein with guanidinium chloride will also cause a negative difference spectrum due to the transfer of phenolic and tryptophan residues from hydrophobic regions out into the aqueous solvent (Krysteva et al., 1976). Anions have been reported to affect the therodynamics of iron binding to transferrin (Williams et al., 1982) and the kinetics of metal ion exchange between transferrin and (EDTA) (Baldwin & DeSousa, 1981; Baldwin 1980). However, these effects have generally been observed at relatively high anion concentrations (0.1–3 M) compared to the results reported here which are observed at millimolar concentrations. Furthermore, the variation in the intensity of the difference UV absorbance for the series of anions follows the order $PO_4^{3-} > SO_4^{2-} \gg HCO_3^- > NO_3^-$, Cl^-, ClO_4^-, which is not consistent with a general conformational effect.

One needs to distinguish between vanadate and the remaining anions included in this study. Although vanadium exists in solution as the free vanadate ion, previous results have shown that it binds to transferrin as the dioxovanadium(V) cation (Harris & Carrano, 1984). Since the vanadium binds as a cation, it is not surprising that the difference UV spectrum is more similar to those due to the binding of other metal ions. The similarity is limited by the fact that there are vanadium–oxygen charge transfer bands in the same spectral region as the difference UV bands, which lead to a broader absorbance than is usually observed for metal–transferrin spectra.

Both the λ_{max} and the $\Delta\epsilon_M$ of the vanadium–transferrin complex appear to be altered in the presence of sulfate, phosphate, and bicarbonate. However, it is unlikely that these effects are due to formation of any type of ternary anion–vanadium–transferrin complex, since these anions do not normally function as synergistic anions (Schlabach & Bates, 1975), and it has been shown that the vanadium–transferrin complex forms even in the absence of a synergistic anion (Harris & Carrano, 1984). The shift in position and intensity of the vanadium–transferrin band is likely an artifact of the procedure used to measure the spectra. The apoprotein was mixed with the competing anion and then split between the

reference and sample cuvettes. The vanadium was then added to the sample cuvette, while distilled water was added to the reference. On the basis of the anion binding constants, there should be a significant concentration of the anion–transferrin complex in the cuvettes prior to the addition of any vanadate. However, the negative absorbance due to such a complex would be the same in both cuvettes and thus canceled out in the difference spectrum. The addition of vanadate would displace the competing anion, which now produces two components to the observed absorbance. Not only is there the positive absorbance due to the formation of the vanadium–transferrin complex but also there is the loss of the negative absorbance due to the destruction of the anion–transferrin complex. The observed spectrum is thus the sum of these two components. This effect is important only insofar as the correct $\Delta\epsilon_{VT}$ value must be used in the calculation of apparent vanadium binding constants.

Thermodynamic Binding Constants. Rogers et al. (1977) reported that the anions phosphate, sulfate, and bicarbonate all displace ferric–EDTA and ferric–NTA from their carbonate-free ternary ovotransferrin complexes. Conversely, chloride, nitrate, and perchlorate had essentially no effect on these transferrin complexes. It was suggested that the former anions were competing directly with the iron chelates for occupancy of the anion binding sites on the protein. Similarly, it is proposed here that even though vanadium binds as a dioxovanadium(V) cation, the anion dependence of the vanadium–transferrin binding constant is due to thermodynamic competition between vanadium and the other anions for occupation of the same binding region.

Assuming direct anion–vanadate competition, the formal equilibrium expressions for the displacement of anion by vanadate are

$$VO_3 + A\text{–}Tr\text{–}A \overset{K_{1x}}{\rightleftharpoons} VO_2\text{–}Tr\text{–}A + A + H_2O \quad (17)$$

$$VO_3 + VO_2\text{–}Tr\text{–}A \overset{K_{2x}}{\rightleftharpoons} VO_2\text{–}Tr\text{–}VO_2 + A + H_2O \quad (18)$$

The calculated values of K_{obsd} actually correspond to the values of the displacement equilibrium constant at a series of fixed [A] values. From eq 17 and 18, the anion dependence of K_{obsd} is given by

$$\log K_{obsd} = \log K_x - \log [A] \quad (19)$$

Plots of log K_{obsd} vs. log $[PO_4^{3-}]$ are shown in Figure 4. As expected, the plots are linear with slopes of 1.4 for K_1 and 1.1 for K_2 with phosphate. Similar plots have slopes of 1.1 for sulfate and 0.7 for bicarbonate. Thus, the vanadate competition reactions generally confirm the 1:1 stoichiometry of the anion–transferrin complex in agreement with the plots of eq 14.

The y intercepts of plots of eq 19 give values of log K_x, which is equal to the ratio of the transferrin binding constants for vanadate and the respective anion. From the phosphate data, one calculates a log K_{1x} value of 3.26 ± 0.12. From this and the phosphate log K_1 of 4.19, one can calculate a log K_1 for the 1:1 vanadium–transferrin complex of 7.45 ± 0.10. Both the sulfate and phosphate data were used to calculate log K_2 values for vanadate binding. The results were log K_2 = 6.31 ± 0.2 from the phosphate data and log K_2 = 6.7 ± 0.4 from the sulfate data. When combined with the value of log K_2 = 6.63 ± 0.19 from the direct titration with vanadate, the average log K_2 value for vanadium binding is 6.6 ± 0.3.

In the direct titration with bicarbonate, no appreciable binding of the second equivalent of bicarbonate was observed. However, the higher concentrations of bicarbonate used in the competition experiments were able to compete very slightly

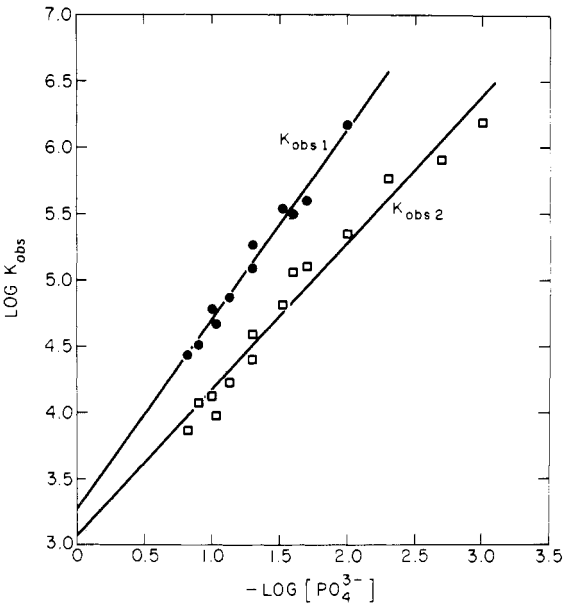

FIGURE 4: Dependence of the apparent vanadate–transferrin binding constants on the concentration of phosphate in 10 mM Hepes at pH 7.4 and 25 °C.

with vanadium binding. This competition led to a log K_{2x} value of 4.8 ± 0.3 between vanadium and bicarbonate. From this value and the average K_2 for vanadate ion, one can calculate a value of log K_2 = 1.8 ± 0.3 for bicarbonate. Woodworth et al. (1975) have suggested that the binding of the second bicarbonate anion might be cooperative with the binding of the first metal ion. Thus, one explanation for the measurable competition from bicarbonate would be that the binding of vanadium at the first site has increased the bicarbonate binding constant. However, neither the sulfate nor the phosphate data appear to support such an explanation. Instead, it is likely that the lack of binding of a second equivalent of bicarbonate in the direct titration studies is due primarily to the low bicarbonate concentrations and the rather small equilibrium constant for bicarbonate binding.

The magnitudes of the bicarbonate binding constants listed in Table I are consistent with previous reports. Kojima & Bates (1981) studied the effect of bicarbonate concentration on the kinetics of the binding and oxidation of ferrous ion. They observed saturation kinetics from which one can estimate the transferrin–bicarbonate binding constant to be about 10^2, which is intermediate between the K_1 and K_2 values listed in Table I. Woodworth et al. (1975) reported an equilibrium constant for the binding of the first equivalent of carbonate dianion to ovotransferrin at pH 9–9.5 of $10^{4.9}$. If this constant is rewritten in terms of the bicarbonate anion rather than the carbonate dianion, based on a carbonate pK_a of 10.3, the value becomes $10^{2.4}$, which is very close to the value of $10^{2.6}$ determined for transferrin in this study. There are no literature values for the specific binding of sulfate and phosphate available for comparison with the data reported here.

The data involving vanadate binding in the presence of anions have been interpreted in terms of a direct competition between vanadate and the anions. This does not mean that the diooxovanadium(V) cation and the inorganic anions bind to precisely the same set of donor groups but rather that they have certain donor groups in common and occupy essentially the same region of space so that simultaneous binding of both substrates is impossible.

Thermodynamic data cannot rule out the possibility that the effects of anions on the spectra of the vanadium–transferrin

complex are due to the binding of the anions at a different location, with a long-range effect transmitted through changes in the protein conformation. The most probable remote site for anion binding would be the "nonsynergistic" sites described by Folatjar & Chasteen (1982) for anion binding to diferric transferrin. However, there are clear indications that the reactions described here, which shall be referred to as specific binding, do not correspond to this type of nonsynergistic binding. No difference UV spectrum is observed when the anions are added to diferric transferrin. Furthermore, the stoichiometry of anion binding differs between these two processes, with 1:1 binding at the specific site and strongly cooperative 2:1 binding at the nonsynergistic site. Finally, the equilibrium constants for phosphate and sulfate described here for the specific sites are about 10^3–10^4, whereas the constants reported for these anions at the nonsynergistic site are less than $10^{0.3}$. Specific binding is strongest for phosphate and sulfate, is weaker for bicarbonate, and appears to be very weak for chloride, nitrate, and perchlorate. In contrast, the order of nonsynergistic binding constants is $ClO_4^- > Cl^- \gg PO_4^{3-}$, SO_4^{2-}, and HCO_3^-.

The proposal that the anion effects on the vanadate–transferrin spectrum are due to direct thermodynamic competition is based upon several observations. The binding of both vanadate and the anions perturbs certain tyrosine residues. This perturbation is completely blocked for diferric transferrin, indicating that vanadate and the anions bind at the iron binding sites. The spectrum of the vanadium–transferrin complex in the presence of the anions is consistent with a simple summation of the spectra produced by the binding of each substrate in the absence of the other. There is good agreement between the binding constant for the second equivalent of vanadate measured by direct titration with vanadate and by competition between vanadate and both phosphate and sulfate. Furthermore, the plots in Figure 4 show no signs of saturation of an allosteric binding site, even at anion concentrations up to 0.15 M. Finally, the kinetic studies of Rogers et al. (1977) indicate that bicarbonate and phosphate displace the weakly bound ferric–NTA and ferric–EDTA chelates from ovotransferrin to form a binary anion–ovotransferrin complex. The bicarbonate–ovotransferrin species then accepts ferric ion from the low molecular weight chelates to form the usual ternary iron–bicarbonate–ovotransferrin complex.

Nature of the Anion Binding Site. The most important anion–protein interaction is probably hydrogen bonding to the guanidinium group of an arginine residue. Arginine is essential for binding anionic substrates to a wide variety of enzymes (Riordan et al., 1977; Riordan, 1979). Freitas & Valentine (1984) have recently shown that inorganic phosphate binds to bovine copper–zinc superoxide dismutase via a single arginine at concentrations very similar to those used in this study. Finally, Rogers et al. (1978) have shown that there is an essential arginine residue associated with each metal binding site of the closely related protein ovotransferrin.

Lysines have also been repeatedly suggested as an anion binding residue (Bates & Schlabach, 1973; Zweier & Aisen, 1977; Campbell & Chasteen, 1977; Zweier et al., 1981). The strongest arguments in favor of lysine involve pH-dependent changes in the anion binding properties of the C-terminal site. Carbon-13 studies of anion binding indicate a release of anion from the C-terminal site at high pH (Zweier et al., 1981). Campbell & Chasteen (1977) have suggested that the pH-dependent conformational changes in the C-terminal site might be associated with deprotonation of a lysine residue. They further suggested that the C-terminal site may have two positively charged anion binding groups, while the N-terminal site appears to have only one. Thus, it is possible that lysine is involved at one site and not the other. The imidazole side group of a histidine residue has also been suggested as an anion binding group. Alsaadi et al. (1981) reported proton NMR data which indicate that the pK_a of one histidine per site is significantly raised by the addition of either oxalate or dipicolinic acid to metal-free ovotransferrin. This same set of histidine residues is also strongly affected by metal binding. Chasteen (1983) has pointed out that amino acid sequence studies indicate that three hisitdine residues are conserved in the transferrin sequence, even though only two appear to be ligands to the metal, and that structural studies place this third histidine in proximity to the arginines which are widely accepted as anion binding groups. However, the involvement, if any, of lysine or histidine in anion binding has not been firmly established.

The tendency is to categorize the binding of species to transferrin as occurring either at a metal binding site or at an anion binding site, with the inference that each site is composed of a separate and distinct set of ligands. However, the induction of a phenolic difference UV spectrum by binding of anions and their direct competition with the binding of the doxovanadium(V) cation suggest a different model. It is proposed that the ligands which collectively bind bicarbonate and ferric ion in the normal ferric–transferrin complex should be considered as a single unified binding region. In the absence of metal ion, the anions bind in this region, presumably via hydrogen bonds between their own oxo groups and arginine, the phenols that are also involved in iron binding, and possibly lysine and histidine residues. Such hydrogen bonding to the tyrosines is supported by the observation that a negative difference UV spectrum is also observed when tyrosines are exposed to a hydrogen-bonding aqueous solvent (Krysteva et al., 1976). According to this model, anion binding does not form a vacant pocket for subsequent metal binding. Instead, it forms a reaction intermediate into which the metal inserts itself, with the breaking of anion–protein bonds and the formation of stronger metal–protein bonds with some of the same protein ligating groups.

The transferrin–anion complex is much more stable than corresponding low molecular weight analogues. We observe no indication of phosphate binding to the diphenolic ligand ethylenebis[(*o*-hydroxyphenyl)glycine] (W. R. Harris, unpublished results). Cotton et al. (1977) report a binding constant of 10^5–10^6 for the bis(guanidinium)-*p*-nitrophenylphosphate complex in 95% ethanol–water, but they could detect no complex formation at all in pure aqueous solution. Clearly, one mechanism for enhancing the stability of the anion–protein complex would be formation of a hydrophobic pocket in the region around the hydrogen-bonding residues.

A second factor contributing to the stability of the anion–transferrin complex could be a favorable steric arrangement of the hydrogen-bonding groups. A similar argument has been offered to explain the enhanced rate of periodate oxidation of the binding-site phenols compared to other phenols in the protein (Geoghegan et al., 1980). Such a steric factor might also explain the variation in binding affinities between tetrahedral sulfate and phosphate and the planar bicarbonate anion.

Registry No. Phosphate, 14265-44-2; sulfate, 14808-79-8; bicarbonate, 71-52-3; vanadate, 13981-20-9; L-arginine, 74-79-3.

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Primary Structures of Ribosomal Protein YS25 from *Saccharomyces cerevisiae* and Its Counterparts from *Schizosaccharomyces pombe* and Rat Liver†

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ABSTRACT: Protein YS25 and its counterparts, SP-S28 and rat S21 [nomenclature according to Sherton, C. C., & Wool, I. G. (1972) *J. Biol. Chem. 247*, 4460–4467], from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and rat liver cytoplasmic ribosomes, respectively, were sequenced by a combination of various enzymatic digestions and/or chemical cleavage. Proteins YS25 and SP-S28 consist of 87 amino acid residues, and rat S21 consists of 83. The amino termini are all N^{α}-acetylated. The amino-terminal halves of the protein molecules are highly conserved (73–85% homologies) in contrast to the carboxy-terminal parts. Overall, rat S21 is 54% homologous to YS25 and 57% to SP-S28, despite a 76% homology between YS25 and SP-S28. Direct comparison with the available prokaryotic ribosomal protein sequences did not reveal any significant homology.

In a previous paper, we presented amino-terminal amino acid sequences of various ribosomal proteins from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and rat liver cytoplasmic ribosomes. In total, those analyses suggested that *Sc. pombe* is relatively distantly related to yeast, *Sa. cerevisiae*, and rather close to rat in phylogenetic distance, in accord with the result deduced from 5S rRNA sequences (Otaka et al., 1983). In order to examine the evolutionary relationships further, we sequenced three homologous proteins, YS25, SP-S28, and rat S21, from the same organisms. Blocked amino termini of the proteins were determined in these eukaryotic ribosomal proteins. Recently, protein YS25 was shown to react with *Escherichia coli* S6 antisera, indicating an ancestral relationship of these eukaryotes and *E. coli* (Chooi & Otaka, 1984). The sequence homologies of the those proteins will be discussed in terms of protein evolution.

Materials and Methods

Main materials used here were purchased from the sources indicated in parentheses: cellulose thin-layer plates (Macherey-Nagel, FRG), polyamide sheets (Schleicher & Schüll,

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