Binding of Phosphonate Chelating Agents and Pyrophosphate to Apotransferrin†

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ABSTRACT: Difference ultraviolet spectroscopy has been used to monitor the binding of a series of phosphonate ligands to human apotransferrin. The ligands consist of pyrophosphate as well as the phosphonic acids (aminomethyl)phosphonic acid (AMPA), (hydroxymethyl)phosphonic acid (HMP), (phosphonomethyl)-iminodiacetic acid (PIDA), *N*,*N*-bis(phosphonomethyl)glycine (DPG), and nitrilotris(methylenephosphonic acid) (NTP). Equilibrium constants have been measured for the sequential binding of two ligands per molecule of apotransferrin. In addition, site-specific equilibrium constants have been measured for the binding of AMPA, HMP, and PIDA to the vacant binding site of both forms of monoferric transferrin. Since titrations of diferric transferrin produce no difference UV spectrum, it is proposed that the primary binding site for phosphonic acids includes the protein groups that bind the synergistic bicarbonate anion that is required for formation of a stable ferric transferrin complex. It is further proposed that those ligands with two phosphonate groups can simultaneously bind to cationic amino acid side chains that extend into the cleft between the two domains of each lobe of transferrin. From an inspection of the ferric transferrin crystal structure, the most likely anion binding residues in the cleft are Arg-632 and Lys-534 in the C-terminal lobe and Lys-206 and Lys-296 in the N-terminal lobe.

Transferrin is the serum protein responsible for the transport of ferric ion in the blood between sites of uptake, utilization, and storage (Aisen, 1989; Chasteen, 1983; Bates, 1987; Harris & Chasteen, 1989). The protein consists of two homologous lobes, each containing a single high-affinity metal-binding site (Bailey et al., 1988). The two sites, designated C-terminal and N-terminal, are similar, but not identical. Both sites bind ferric ion through the phenolic groups of two tyrosines, the imidazole group of a histidine, and the carboxylic acid of an aspartate. In addition each ferric ion is coordinated directly to a synergistic (bi)carbonate anion that is also bound electrostatically to the protein (P. F. Lindley, unpublished results). The N- and C-terminal sites can be distinguished by ESR (Aisen et al., 1978; Chasteen et al., 1977; Zweier et al., 1978; Harris, 1975), NMR (Bertini et al., 1983; Butler & Danzitz, 1987), and fluorescence spectroscopy (O'Hara & Bersohn, 1982). In addition, they typically differ by a factor of 6–100 in their metal-binding affinities (Aisen et al., 1978; Martin et al., 1987; Harris, 1989) and release ferric ion to chelating agents at different rates (Baldwin et al., 1982; Bali & Harris, 1989; Thompson et al., 1986b; Kretchmar & Raymond, 1986).

The release of ferric ion from transferrin have been studied with a variety of chelating agents, including pyrophosphate (Williams et al., 1982; Cowart et al., 1986; Thompson et al., 1986a,b; Bertini et al., 1988; Harris et al., 1987; Bali & Harris, 1989), aminophosphonic acids (Harris, 1984; Harris et al., 1987; Harris & Bali, 1988; Bali et al., 1991), amino carboxylic acids (Baldwin, 1980; Baldwin & de Sousa, 1981; Baldwin et al., 1982; Bates et al., 1967), catecholates (Carrano & Raymond, 1979; Kretchmar & Raymond, 1986, 1988), and hydroxamates (Cowart et al., 1982; Konopka et al., 1982). Since the stability constants of ferric transferrin are around 10^{20} (Aisen et al., 1978), it is virtually certain that simple dissociation of ferric ion is too slow to account for the observed rates of iron exchange between transferrin and low molecular weight chelating agents. Thus, the exchange of ferric ion must proceed via some type of mixed-ligand intermediate.

A current enigma is that several systems show saturation kinetics with respect to free ligand without forming significant concentrations of any spectroscopically distinct intermediate (Cowart et al., 1982, 1986; Carrano & Raymond, 1979; Harris, 1984; Harris et al., 1987). One such group of ligands consists of pyrophosphate and aminophosphonic acids. We have shown that inorganic phosphate will bind reasonably well to apotransferrin (Harris, 1985; Harris et al., 1990). Thus, we are exploring the possibility that phosphonate ligands might form a mixed-ligand intermediate during iron removal reactions by binding as an anion to the protein, rather than by coordinating directly to the ferric ion. In this paper we report that pyrophosphate and di- and triphosphonates bind very strongly to apotransferrin.

Experimental Procedures

Materials. Iron-free apotransferrin (apoTf) was purchased from Calbiochem and purified of adventitious chelating agents by published procedures (Harris, 1986a). C-Terminal monoferric transferrin was prepared by adding 1 equiv of iron as a pH 4 solution of a 1/2 mixture of ferric ion and nitrilotriacetic acid to apotransferrin in 0.1 M *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (Hepes) at pH 7.4. N-Terminal monoferric transferrin was prepared by selective removal of iron from the C-terminal binding site as described by Baldwin and de Sousa (1981).

Nitrilotris(methylenephosphonic acid) (NTP) and *N*-(phosphonomethyl)iminodiacetic acid (PIDA) were prepared and purified as previously described (Harris et al., 1987). *N*,*N*-Bis(phosphonomethyl)glycine (DPG) was purchased and purified as previously described (Harris et al., 1987). Research grade tetrasodium pyrophosphate (PP_i), (aminomethyl)-phosphonic acid (AMPA), and (hydroxymethyl)phosphonic acid (HMP) were purchased and used as received.

Methods. All glassware was soaked in 1 M HNO_3, and all solutions were prepared with 18 mΩ water from a four-bowl Millipore system. All measurements were made in either 0.1 or 0.01 M Hepes, which was adjusted to pH 7.4 by the addition

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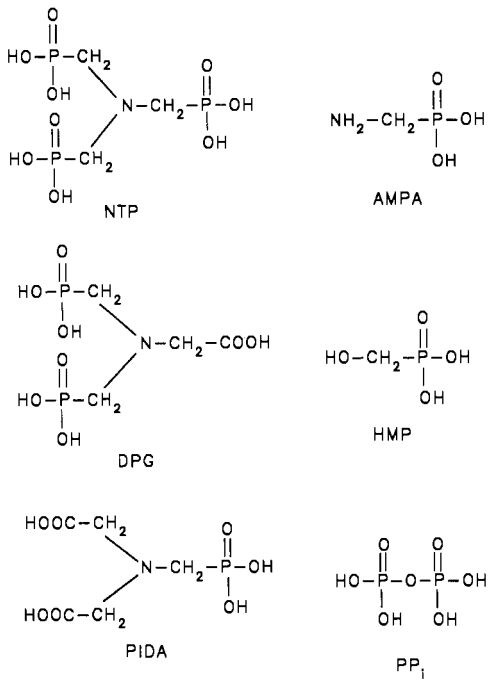
Scheme I



of concentrated NaOH. Ultraviolet spectra were recorded with the use of a Varian 2200 spectrophotometer equipped with jacketed cell holders maintained at 25 °C by an external constant temperature bath.

Stock solutions of apoTf were diluted to approximately 15 μM with Hepes buffer. The exact transferrin concentration of each sample solution was determined from the absorbance at 278 nm on the basis of a molar extinction coefficient of 93 000 M^{-1} cm^{-1} (Chasteen, 1977). Dry sample and reference cuvettes were filled with 2.0 mL of apotransferrin and a base line of protein vs protein was recorded. Sample cuvettes were titrated with 10–50-μL aliquots of the selected anion, while equal volumes of distilled, deionized water were added to the reference cuvette. Absorbance changes following the addition of anion were very rapid, and a difference spectrum from 320 to 240 nm was recorded within 5–10 min after each addition. When longer equilibration times were tested, there was no indication of slow changes in the spectra.

Equilibrium constants were calculated on the basis of the minimum in the difference UV spectrum at 245 nm. To correct for dilution effects, each absorbance value was converted to an absorptivity ($\Delta\epsilon$) by division of the absorbance by the analytical concentration of transferrin. Two macroscopic binding constants were calculated by use of the absorptivity and mass balance equations described by Harris (1985).

Crystallographic coordinates for rabbit serum transferrin were obtained from Dr. Peter Lindley and the Birkbeck College transferrin group. The coordinates reflect a slightly better refinement than that previously reported (Bailey et al., 1988), such that the atomic positions of the synergistic bicarbonate anion are now resolved. The structure was examined in detail with use of a Silicon Graphics 4D-80GT Graphics Workstation running the program Insight.

Results

The ligands used in this study are shown in Scheme I. Precise protonation constants are not available for all the ligands. The first proton from each R-PO_3H_2 moiety is invariably very acidic. Protonation constants for the R-PO_3^{2-} group are near 10^5 but vary depending on the overall net charge on the ligand. The central amino groups tend to be very basic. For example, the protonation constants for NTP are $10^{12.3}$ for the central amine, with values of $10^{6.7}$, $10^{5.5}$, and $10^{4.3}$ for the sequential protonation of the three phosphonate moieties (Martell & Smith, 1974). Thus, the ligands shown in Scheme I will be highly anionic at neutral pH, with protonated amino groups and largely deprotonated phosphonate oxygens.

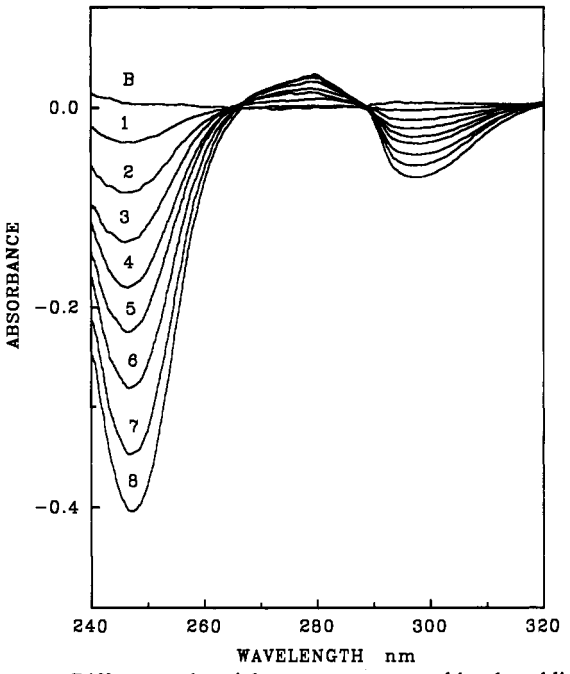


FIGURE 1: Difference ultraviolet spectra generated by the addition of aliquots of 0.5 mM NTP to 2.0 mL of 18.7 μM apoTf in 10 mM Hepes at 25 °C and pH 7.4. The spectrum labeled B is the base line of protein vs protein. The amounts of NTP added were (1) 5 μL, (2) 15 μL, (3) 25 μL, (4) 35 μL, (5) 45 μL, (6) 60 μL, (7) 80 μL, and (8) 100 μL.

All the phosphonate anions produce essentially identical difference UV spectra when they bind to apoTf. A typical family of spectra produced by the addition of aliquots of NTP to apoTf is shown in Figure 1. No detectable spectra are observed when diferric transferrin samples are titrated up to total ligand concentrations of 80 μM for PP_i or PIDA or up to 13 mM for AMPA or HMP. Thus, it is clear that ferric ion effectively blocks the specific binding of anions to the protein.

Small absorbance changes were observed when diferric transferrin was titrated with NTP and DPG. However, these spectra did not show the characteristic difference UV peaks seen in Figure 1. Instead, there was only a weak negative absorbance at 245 nm and a broad negative absorbance from 270 to 320 nm. Since these ligands have a high iron binding affinity, the spectra probably result from removal of small amounts of ferric ion from the protein.

Titration curves were prepared by plotting $-\Delta\epsilon$ at 245 nm versus the concentration of phosphonate. Titrations of apoTf and both forms of monoferric transferrin with HMP are shown in Figure 2. It is clear that phosphonate binding occurs at both the N-terminal and C-terminal binding sites. The titration data for the two forms of monoferric transferrins were used to calculate site-specific binding constants defined as

$$K_x = \frac{[A_x\text{–Tf–Fe}]}{[A][\text{Tf–Fe}]} \quad (1)$$

where [A] refers to the concentration of the free anion, [Tf–

Table I: Equilibrium Constants for the Binding of Phosphonate Anions to the Vacant Site of Monoferric Transferrins

	$Tf-Fe_N$			Fe_C-Tf		
ligand	n^a	log K_C ± 2SEM	$\Delta\epsilon_M{}^b$	n^a	log K_N ± 2SEM	$\Delta\epsilon_M{}^b$
PIDA	4	4.43 ± 0.09	5000[c]	4	5.10 ± 0.08	4900 ± 300
HMP	8	3.76 ± 0.15	5100 ± 850	8	3.85 ± 0.09	4900 ± 300
P_i[d]		3.6 ± 0.2			3.9 ± 0.2	
phosphite[d]		3.12 ± 0.05			3.50 ± 0.05	
AMPA	6	2.38 ± 0.09	5000[c]	8	2.34 ± 0.13	5000[c]

[a] Number of replicate titrations. [b] Values for molar absorptivities are expressed per anion binding site. [c] Values were estimated from data on HMP and PIDA (N-terminal). [d] Values for phosphate and phosphite are taken from Harris et al. (1990).

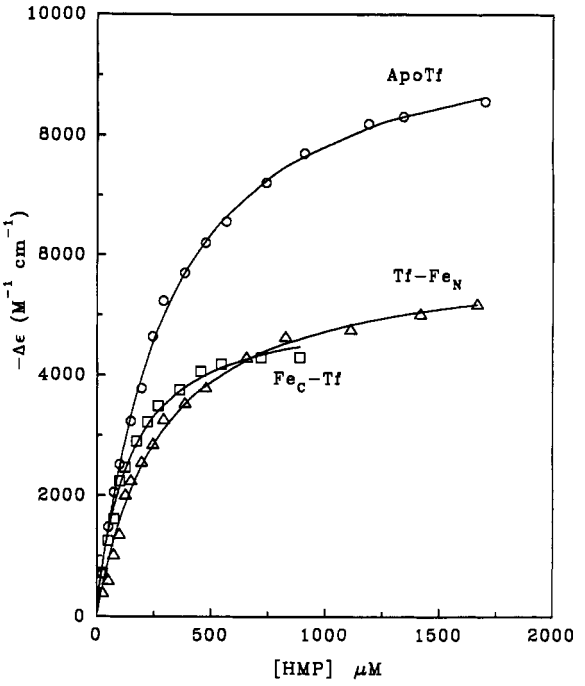


FIGURE 2: Plots of $-\Delta\epsilon$ versus the accumulated concentration of HMP for the titrations of protein in 0.1 M Hepes buffer at pH 7.4 and 25 °C with either 10 mM HMP (apoTf) or 5 mM HMP (monoferric transferrins). Values of $\Delta\epsilon$ were calculated by dividing the absorbance at 245 nm by the analytical concentration of transferrin.

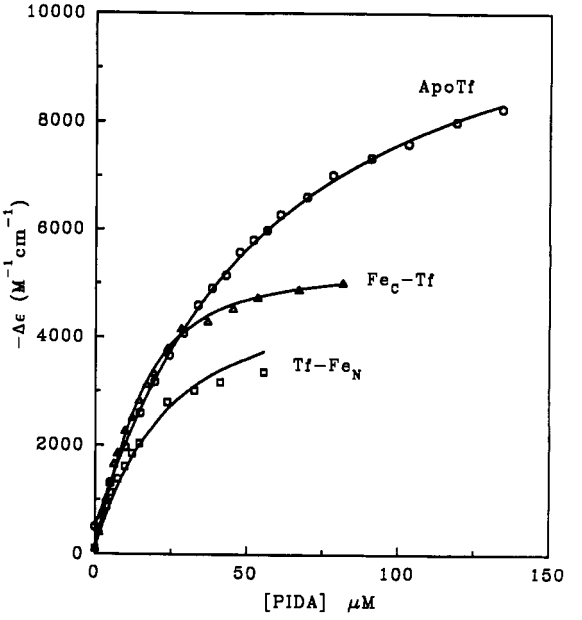


FIGURE 3: Plots of $-\Delta\epsilon$ versus the accumulated concentration of PIDA for the titration of protein in Hepes buffer at pH 7.4 and 25 °C. ApoTf in 10 mM Hepes was titrated with 10 mM PIDA. Monoferric transferrins in 0.1 M Hepes buffer were titrated with 5 mM PIDA. Values of $\Delta\epsilon$ were calculated by dividing the absorbance at 245 nm by the analytical concentration of transferrin.

Fe] refers to the molarity of the monoferric transferrin, and the x subscript denotes either the N-terminal or C-terminal binding site. There was sufficient saturation in the binding of HMP to both forms of monoferric transferrin to permit the simultaneous refinement of both K_x and the molar absorptivity ($\Delta\epsilon_M$), which are listed in Table I.

Titrations of apoTf and both forms of monoferric transferrin with PIDA are shown in Figure 3. For binding to the vacant N-terminal site of Fe_C–Tf, there was again sufficient saturation to allow the simultaneous refinement of the values of log K_N and $\Delta\epsilon_M$, which are listed in Table I.

Binding to apoTf can be described by two sequential macroscopic equilibrium constants for anion binding defined as

$$K_1 = \frac{[A\text{–}Tf]}{[A][apoTf]} \quad (2)$$

$$K_2 = \frac{[A\text{–}Tf\text{–}A]}{[A][A\text{–}Tf]} \quad (3)$$

where [A] refers to the molar concentration of the free anion and [A–Tf] and [A–Tf–A] refer to the 1:1 and 2:1 anion–transferrin complexes, respectively.

In the titrations of apoTf with PIDA and HMP, the strong negative correlation between $\Delta\epsilon_M$ and K_2 precluded the simultaneous refinement of $\Delta\epsilon_M$, K_1, and K_2. The titrations of monoferric transferrins with HMP indicated that the two binding sites have essentially identical molar absorptivities. Identical molar absorptivities for the two sites were also observed in more extensive studies of the binding of a series of inorganic anions to both forms of monoferric transferrin (Harris et al., 1990). The average molar absorptivity for the three HMP and PIDA monoferric systems described above for which $\Delta\epsilon_M$ can be calculated is 5000 ± 100 M^{-1} cm^{-1}. Successive macroscopic binding constants for the binding of

Table II: Equilibrium Constants for the Sequential Binding of Two Anions to Apotransferrin[a]

ligand	n^b	log K_1 ± 2SEM	log K_2 ± 2SEM	$\Delta\epsilon_M$ (M^{-1} cm^{-1})[c]	Δ log K
NTP	8	6.34 ± 0.28	4.78 ± 0.24	7000 ± 800	1.5
PP_i	8	6.12 ± 0.66	4.97 ± 0.19	6800 ± 700	1.2
DPG	7	6.04 ± 0.29	4.76 ± 0.14	7100 ± 770	1.3
PIDA	7	4.90 ± 0.17	4.01 ± 0.10	5500[d]	0.8
	4	5.13 ± 0.06[e]	3.34 ± 0.06[e]	5000[d]	1.8
P_i[f]		3.99 ± 0.08	3.2 ± 0.2	6100 ± 1300	0.8
HMP	8	3.94 ± 0.17[e]	2.91 ± 0.34[e]	5000[d]	1.0
phosphite[f]		3.50 ± 0.08	2.5 ± 0.2	6100 ± 800	1.0
AMPA	7	2.47 ± 0.09[e]		5000[d]	

[a] Values are for 0.01 M Hepes buffer, pH 7.4, 25 °C unless otherwise noted. [b] Number of replicate titrations. [c] Values for molar absorptivity are expressed per anion binding site. [d] Values of $\Delta\epsilon_M$ were estimated from data on corresponding monoferric transferrin as described in the text and were not allowed to vary during the calculation of K_1 and K_2. [e] Values are for 0.1 M Hepes. [f] Equilibrium constants for phosphate and phosphite are taken from Harris et al. (1990).

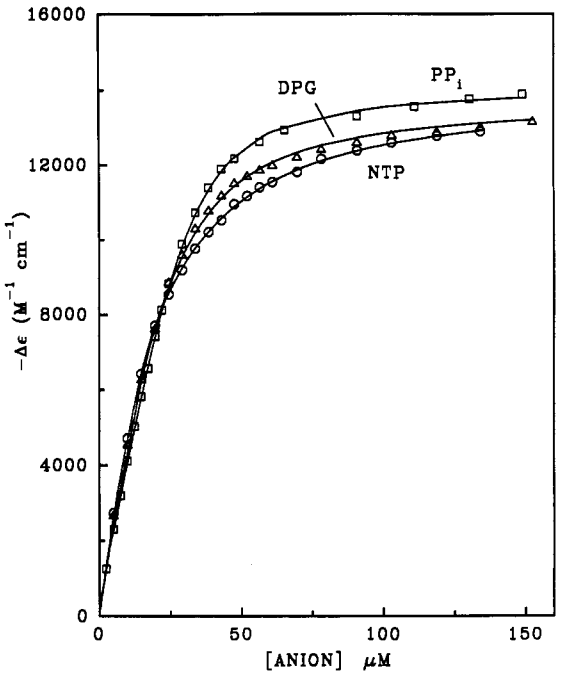


FIGURE 4: Plots of $-\Delta\epsilon$ versus the accumulated concentration of anion for the addition of 0.5 mM anion to 2.0 mL of approximately 17 μM apoTf in 10 mM Hepes buffer at 25 °C and pH 7.4. Values of $\Delta\epsilon$ are calculated by dividing the absorbance at 245 nm by the analytical concentration of transferrin.

HMP and PIDA to apoTf have been calculated with $\Delta\epsilon_M$ fixed at 5000 M^{-1} cm^{-1} and with only K_1 and K_2 as adjustable parameters. Values of log K_1 and log K_2 are listed in Table II.

It was also necessary to fix $\Delta\epsilon_M$ for the titration of N-terminal monoferric transferrin with PIDA. Removal of iron from Tf–Fe_N at higher PIDA concentrations leads to complex changes in the difference UV spectrum. There are absorbance changes associated with the loss of ferric ion from Tf, with the binding of PIDA to the vacated Tf site, and with charge transfer bands for the ferric PIDA chelate. The net result is a decrease in peak height at higher PIDA concentrations, so that one cannot directly determine the molar absorptivity of the PIDA–Tf–Fe_N complex from the plateau in the titration curve, and the binding constant must be calculated from titration data restricted to lower anion concentrations. In the case of AMPA, binding to both apotransferrin and monoferric transferrins was so weak that saturation of the binding sites was never achieved. Therefore, $\Delta\epsilon_M$ was also set to 5000 M^{-1} cm^{-1} for these systems. The complete set of binding constants for AMPA, HMP, and PIDA is shown in Tables I and II.

Titrations of apoTf with NTP, DPG, and PP_i are shown in Figure 4. The titration data were fit by nonlinear least-squares using K_1, K_2, and the molar absorptivity as adjustable parameters. The molar absorptivity was approximated by assuming that the plateau in the titration curve at high anion concentrations represents saturation of the two apoTF binding sites. The molar absorptivity was allowed to vary only within ±10% of this approximated value during the calculation of K_1 and K_2. Values of log K_1, log K_2 and $\Delta\epsilon_M$ for PP_i, DPG, and NTP are listed in Table II.

Discussion

The interaction between anions and polyprotonated macrocyclic amines has been termed anion coordination chemistry (Dietrich et al., 1981). The best-studied monophosphate is adenosine monophosphate (AMP). We have found 18 equilibrium constants in the literature for the binding of AMP to various macrocycles and to the cryptate bis(tren) (Kimura et al., 1982; Dietrich et al., 1981, 1984; Hosseini & Lehn, 1987). The binding constants for phosphate, phosphite, and HMP with apoTf are approximately 10^4. Binding constants of $\geq 10^4$ for AMP with simple macrocycles always involve at least four protonated nitrogens. In two instances macrocycle binding constants for inorganic phosphate were also measured (Kimura et al., 1982). The phosphate binding constants are smaller than the AMP binding constants by factors of 10 and 100.

Ligands with at least two phosphonate groups have transferrin binding constants of $\sim 10^6$. Equilibrium constants of 10^6 for the binding of ADP or ATP to macrocycles always involve at least five protonated nitrogens (Hosseini & Lehn, 1987; Dietrich et al., 1981, 1984). In contrast, equilibrium constants for the binding of PP_i, ADP, and ATP to simple noncyclic amines are in the range of 10^2–10^4. (Dietrich et al., 1979; Lahti et al., 1989; Nakai & Glinsmann, 1977.) With consideration of this literature on anion binding to polyamines, transferrin–anion binding constants of 10^4–10^6 can only be explained by an interaction with several positive charges or dipoles in a favorable geometric arrangement, i.e., the presence of a specific, high-affinity binding site.

There is an irregular pattern to the K_1 values for the phosphonic acids. The simplest R–PO_3^{2-} anion is phosphite, where R = –H. Since the pK_a's are 6.1 and 1.1 (Martell & Smith, 1974), phosphite is a dianion at physiological pH. Phosphite binds to apoTf with a log K_1 value of 3.5 ± 0.08 (Harris et al., 1990). Replacing the hydrogen of phosphite with an –OH group to form phosphate (primarily HPO_4^{2-} at physiological pH) increases log K_1 slightly to 3.99 ± 0.08. However, replacement of –OH with $-CH_2-NH_3^+$ to form AMPA sharply reduces the log K_1 value to only 2.47 ± 0.09. The R–PO_3^{2-} functional group of AMPA has pK_a's of 5.4 and <2 (Smith & Martell, 1976), so that the local charge around the phosphorus is still –2 at physiological pH. However, the amino group is quite basic and will be fully protonated.

To distinguish between the role of steric and electrostatic effects in reducing the binding constants of AMPA relative to phosphite and phosphate, binding constants were also measured for HMP (R = $-CH_2-OH$) and PP_i (R = $-O-PO_3^{2-}$). Because AMPA, HMP, and PP_i are approximately the same size, steric crowding would reduce the binding constant for all three molecules. However, electrostatic effects should be quite different for the three molecules on the basis of the charges of the R groups. The data in Table II show that there is a strong effect of charge on log K_1, with AMPA < HMP ≪ PP_i.

The larger chelating agents NTP, DPG, and PIDA are derivatives of AMPA. Molecular models show that when these tripodal ligands are configured so as to minimize repulsions between the anionic groups, they are essentially trigonal planar molecules. The edge distance of the triangular plane is approximately 9 Å, compared to a length of about 7 Å for pyrophosphate.

The two carboxymethyl substituents of PIDA increase the binding constant relative to AMPA from $10^{2.47}$ to $10^{4.90}$. Replacing a carboxylate group of PIDA with a second phosphonate group to form DPG produces another substantial increase in the binding constant to $10^{6.04}$. However, addition of a third phosphonate group to form NTP produces a relatively small increase of only 0.3 log units to $10^{6.34}$.

The pattern in the binding constants for NTP, DPG, PIDA, HMP, and AMPA is best explained by a two-site model: a primary binding site for the first phosphonate group, which

involves one or both of the metal binding site tyrosine residues and gives rise to the characteristic difference UV spectrum, and a secondary binding site for a second phosphonate if one is available. Simple anions like inorganic phosphate fit into the primary binding site and except for carbonate itself are competitive with metal binding. Such competition has been observed directly between phosphate and vanadate (Harris, 1985), and is strongly suggested by competition data for phosphate and sulfate with the Fe–NTA–Tf and Fe–EDTA–Tf carbonate-free ternary complexes (Rogers et al., 1977).

Whenever the ligand contains a second phosphonate functionality, as is the case for NTP, DPG, and PP_i, the binding constant increases substantially to over 10^6. We believe that this reflects a favorable interaction of the second phosphonate group with cationic amino acid residues from the interdomain cleft that constitute a secondary site. Conversely, for AMPA there is a substantial reduction in the binding constant, as would be expected due to unfavorable electrostatic interactions between the protonated amine in AMPA and the cationic protein residues of the secondary anion binding site.

Recent improvements in the crystallographic data on ferric transferrin make it possible to speculate on the nature of the proposed anion binding site. In the C-terminal lobe of ferric transferrin, the synergistic carbonate anion is hydrogen bonded to the amide N–H groups of Ala-458 (Ala-126 in the N-terminal lobe) and Gly-459 (127) and to the hydroxyl group from Thr-452 (120). It is also electrostatically bonded to Arg-456 (124). All these groups come from a continuous section of the polypeptide backbone that is at the beginning of an α helix. The residue numbers correspond to human serum transferrin as reported by Junankar et al. (1990).

Using computer graphics, we have removed the iron and carbonate anion from the transferrin structure and superimposed a P–O bond of a phosphate anion over the C–O bond of carbonate that is oriented toward the amide N–H bonding groups. One can easily rotate the phosphate around this P–O axis such that a second oxygen is oriented toward Arg-456 (124). This directs the remaining two phosphate oxygens toward Tyr-426 (95) and Tyr-517 (188), although one must remember that in the crystal structure these tyrosines are held in these positions by coordination to the ferric ion. The similar binding constants for phosphite, phosphate, and (hydroxymethyl)phosphonate suggest that the anion interacts with only one of these tyrosines when binding to the apoprotein. This hydrogen bonding to tyrosine is presumably responsible for the difference UV spectrum induced by anion binding.

We have also superimposed one of the phosphate groups of PP_i on the bicarbonate position. The resulting diagram for the C-terminal lobe is shown in Figure 5. The P1 phosphate occupies the primary binding site. The P2 phosphate can also hydrogen bond to the Arg-456 residue of the primary binding site. A second oxygen atom of P2 extends to within 3 Å of both His-585 and the Arg-632 residue in the interdomain cleft. A third oxygen atom of P2 is oriented toward Lys-534 in the interdomain cleft, although the distance to this residue appears to be about 4.5 Å.

It appears that the only adjustment in the transferrin structure required to make room for the pyrophosphate is a rotation of Tyr-517, although further study on this point is required. The cationic residues are different in the cleft of the N-terminal site. Instead of a combination of arginine and lysine, there are two lysines, Lys-296 and Lys-206, that are both about 8.2 Å away from the carbonate carbon.

Although the positions of the transferrin residues in Figure 5 are obtained from a crystal structure, one must stress that Figure 5 does not represent an experimental structure of a PP_i–Tf complex. It is a diagram generated by computer graphics, and the distances quoted should not be taken too literally. The only purpose of the figure is to give the reader a mental image of the type of binding site that is being proposed.

It must also be stressed that the crystallographic coordinates used to construct the anion binding site shown in Figure 5 are for diferric transferrin, while the equilibrium data are for apotransferrin. Viscosity measurements on apotransferrin and diferric transferrin (Rosseneu-Moutreff et al., 1971) are widely quoted as indicating a substantial conformational change in the protein accompanying iron binding. However, the crystal structure of the closely related protein apolactoferrin has recently been published and shows that there is very little change in the conformation of the C-terminal site when the metal binds (Anderson et al., 1990). There is indirect evidence that the anion binding region retains its structure in apoTf as well. As discussed above, the magnitude of the anion binding constants requires a very efficient binding site. In addition, the tetrahedral oxidants periodate and permanganate show selective oxidation of the binding site tyrosines in apoTf (Azari & Phillips, 1970; Geoghegan et al., 1980; Penner et al., 1987; Phillips & Azari, 1972), indicating that at least one of the tyrosines stays in the immediate vicinity of the bound anion.

There is no significant site selectivity for the binding of either HMP or AMPA. The values for the binding constants for the two forms of monoferric transferrin are within one standard deviation of each other, and the separation of 1.0 log units between the successive macroscopic binding constants of HMP with apoTf is only slightly greater than the statistical factor of 0.6 log units for binding to two equivalent sites. This result is consistent with the lack of a strong site selectivity in the binding of simple inorganic anions (Harris et al., 1990).

Since removal of traces of ferric ion during the titration of monoferric transferrin confounds the evaluation of anion binding, monoferric transferrins were not titrated with the stronger chelating agents DPG, NTP, and PP_i. However, the observed separations of 1.2–1.5 log units between log K_1 and log K_2 suggest that there is a significant difference between the two binding sites. This result is consistent with the observation that the cationic residues in the proposed secondary binding sites are different for the two lobes of transferrin, consisting of Arg-632 and Lys-534 in the C-terminal and Lys-206 and Lys-296 in the N-terminal lobe.

There is some inconsistency in the set of binding constants for PIDA. The most reliable indication of site selectivity for PIDA is the separation of 0.7 log units between log K_C and log K_N for the monoferric transferrins. The log K_1 values from the titration of apoTf in both 10 and 100 mM Hepes buffer are in good agreement with K_N. However, there is very poor agreement among the K_2 values and K_C. Both K_2 and K_C for PIDA were calculated by use of a fixed value of $\Delta\epsilon_M$ estimated from other titrations. We suspect that errors in the value of $\Delta\epsilon_M$ and strong negative correlations between K_2 and $\Delta\epsilon_M$ in the least-squares fits for apoTf are adversely affecting the accuracy of K_2.

The kinetics of iron removal from transferrin by the phosphonic acids have been studied (Harris, 1984; Harris et al., 1986a; Harris & Bali, 1988; Bali & Harris, 1989; Bali et al., 1991). There is strong evidence that the rate-determining step for iron removal by NTP from the N-terminal site involves the slow dissociation of an L–Fe–HCO_3–Tf intermediate (Bali et al., 1991). The primary anion binding site is blocked during iron removal by the presence of the ferric ion. Nevertheless,

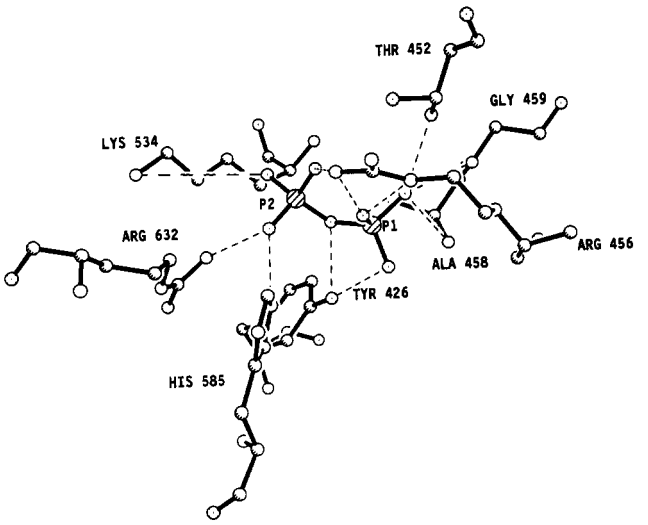


FIGURE 5: Diagram of the proposed binding site for PP_i in the C-terminal lobe of apoTf. Residue numbers correspond to human serum transferrin (Junankar et al., 1990).

with an overall binding constant for NTP in excess of 10^6 for apoTf, it is not unreasonable that binding to the secondary phosphonate binding site is strong enough to account for the observed preequilibrium between ligand and protein during iron removal.

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Registry No. AMPA, 1066-51-9; HMP, 2617-47-2; PIDA, 5994-61-6; DPG, 2439-99-8; NTP, 6419-19-8; PP_i, 14000-31-8.

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Investigation of the Solution Structure of the Human Parathyroid Hormone Fragment (1-34) by ^{1}H NMR Spectroscopy, Distance Geometry, and Molecular Dynamics Calculations†

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ABSTRACT: The structure of human parathyroid hormone fragment (1-34) in a solvent mixture of water and trifluoroethanol has been determined by ^{1}H nuclear magnetic resonance spectroscopy and a combination of distance geometry and molecular dynamic simulations. After complete assignment of the ^{1}H signals, the nuclear Overhauser enhancement data imply the existence of two α-helices, comprising residues 3-9 and 17-28, joined by a nonstructured region. The absence of any long-range NOEs and the relative magnitudes of the sequential NOEs and the $^3J(H_NH_\alpha)$ values reflect an inherent flexibility within the entire fragment. The final structures refined by molecular dynamics further support the above results and allow discussion of structural-activity relationships.

Parathyroid hormone (PTH)[1] is a peptide hormone of 84 amino acid residues. It regulates the calcium homeostasis in the serum by stimulation of calcium resorption in the kidney and by enhancing resorption of calcified bone matrix. In addition, it also stimulates bone-forming processes. This pleiotropic function is reflected in the identification of at least two different functional domains within the PTH molecule. One of these is located in the amino-terminal region comprising 27 amino acid residues that binds to the PTH receptor(s) and leads to an increased intercellular cAMP synthesis [for review, see Potts et al. (1982)]. These events appear to be involved in bone catabolic effects (Martz et al., 1983; Herrmann-Erlee et al., 1988; Dewhirst et al., 1990). The other functional domain, whose core is at positions 30-34, stimulates DNA synthesis and subsequent cell proliferation of either chondrocytes (Schlüter et al., 1989) or osteoblasts (Sömjen et al., 1990, 1991) via an as yet unidentified cAMP-independent signal transduction pathway.

A study of whether these functional domains also correspond to structural domains is of obvious interest. Unfortunately detailed structural information on the PTH molecule is not available. Conclusions from dark field electron microscopic investigations of bPTH(1-84) suggest the presence of two globular units located within the amino and carboxyl termini, respectively (Fiskin et al., 1977). Similar models have been derived from theoretical considerations on bPTH(1-84) (Zull et al., 1980) as well as from NMR studies that focused on the pH-dependent shifts of the H2 protons of the three histidine moieties in the bovine PTH fragment (1-34) that implied substantial structure for the hormone in solution (Zull et al., 1987; Smith et al., 1987; Coddington et al., 1989). Other ^{1}H NMR investigations, however, have suggested some regular

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[1] Abbreviations: bPTH, bovine parathyroid hormone; CD, circular dichroism; COSY, correlation spectroscopy; DQF, double-quantum filter; hPTH, human parathyroid hormone; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser and exchange spectroscopy; RMS, root mean square; TFE, trifluoroethanol; TMS, tetramethylsilane; TOCSY, total correlation spectroscopy.

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