Iron Release from Recombinant N-lobe and Mutants of Human Transferrin†

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ABSTRACT: Mutations of kinetically active residues in the recombinant N-lobe of human transferrin may accelerate or retard release of iron from the protein to pyrophosphate, thereby providing means for exploring the individual roles of such residues in the concerted mechanisms of release. Using an established spectrofluorometric method and pyrophosphate as the required iron-sequestering agent, we have compared release from unaltered native transferrin and recombinant N-lobe half-transferrin to release from six N-lobe mutants, R124S, R124K, K206R, H207E, H249Y, and Y95H. Mutation of R124, which serves as a principal anchor for the synergistic carbonate anion ordinarily required for iron binding by transferrin, accelerates release. This effect is most marked at endosomal pH, 5.6, and is also evident at extracellular pH, 7.4, pointing to a critical and perhaps initiating role of carbonate in the release process. Mutation of K206 to arginine, or of H207 to glutamine, each lying in the interdomain cleft of the N-lobe, gives products mimicking the arrangements in lactoferrin. Release of iron from these two mutants, as from lactoferrin, is substantially slower than from unaltered recombinant N-lobe. Interdomain residues not directly involved in iron or anion binding may therefore participate in the control of iron release within the endosome. The H249Y mutant releases iron much more rapidly than its wild-type parent or any other mutant, possibly because of steric effects of the additional phenolic ring in the binding site. No simple explanation is available to account for a stabilizing effect of the Y95H mutation. Chloride (or another simple anion) promotes and is essential for iron release from the C-lobe of human transferrin but exerts a retarding effect on release from the N-lobe in native and mutant transferrins alike. A simple model, entailing binding competition between pyrophosphate used to effect release and chloride, substantially accounts for the negative effect of chloride on the N-lobe.

Transferrin, the iron-binding protein of plasma, functions to transport iron from cells where it is secreted to cells where it is needed. Inability to express adequate amounts of transferrin leads, in man and experimental animals alike, to the apparent paradox of profound iron deficiency anemia and generalized iron overload with resultant early demise (Heilmeyer et al., 1961; Huggenvik et al., 1989). The human transferrin molecule experiences 100−200 cycles of iron binding and release during its lifetime in the circulation (Katz, 1961). Understanding the molecular events in iron release from transferrin is therefore of critical importance in understanding the physiology of the protein and the essential metal it provides to cells. Although many factors are known to influence the release of iron from transferrin, including pH (Morgan, 1979), complex formation with the transferrin receptor (Bali et al., 1991), binding of simple anions (Kretchmar & Raymond, 1988; Egan et al., 1992), and availability and nature of iron-accepting ligands (Harris et al., 1987), little is understood of the molecular events underlying these influences.

The single polypeptide chain of human transferrin consists of 679 amino acid residues arranged in two highly homologous lobes representing the N- and C-terminal halves of the transferrin molecule (MacGillivray et al., 1983). Each lobe is disposed in two domains enclosing a cleft bearing its iron-binding site. The N-lobe has been cloned and expressed in baby hamster kidney cells; site-directed mutagenesis of this lobe has been utilized by Woodworth et al. (1991) in obtaining valuable new insights into structure−function relationships of human transferrin. Cloning and expression of the C-lobe has not yet been reported. To explore molecular mechanisms of iron release, we have used recombinant wild-type and site-mutagenized N-lobe half-transferrins, and monoferric Fe_N-Tf,[1] to examine the roles of particular residues in the release process.

MATERIALS AND METHODS

Materials. All chemicals were reagent grade or the highest quality commercially obtainable and used without further purification. Pyrophosphoric acid was supplied by Aldrich Chemical Co., Inc. Culture media were from Gibco-BRL.

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[1] Abbreviations: Fe_N-TF, native transferrin bearing Fe in the N-lobe site; WT, recombinant wild-type N-lobe half-transferrin; 3,4-LICAMS, 1,5,10-*N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)triazadecane; PP_i, pyrophosphate.

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FIGURE 1: Crystal structure of the N-lobe of human transferrin; coordinates were generously provided by Dr. Harmon Zuccola. Mutated residues are designated by number: Y95, R124, K206, H207, and H249. The For clarity, the N-lobe is shown in the iron-free "open" conformation.

Human transferrin in iron-saturated form was purchased from Boehringer Mannheim Corp. N-Monoferric transferrin was prepared by established methods (Baldwin & de Sousa, 1981; Thompson et al., 1986) and its purity checked by urea gel electrophoresis. Only trace amounts of diferric transferrin, and none of the C-monoferric species, were found.

Recombinant Proteins. The M13mp18 clone containing the DNA sequence for the signal peptide and the first 337 amino acids of human serum transferrin was kindly provided by Prof. R. T. A. MacGillivray (University of British Columbia, Vancouver). Positions of residues selected for mutagenesis are shown in the ribbon diagram of Figure 1, with coordinates of the N-lobe taken from the structure of monoferric transferrin-Fe_C. This representation of the N-lobe is chosen for clarity in depiction, since it is in the iron-free, open conformation. Mutagenesis was performed using the Amersham *in vitro* mutagenesis kit, with mutations confirmed by DNA sequencing. Mutant clones were made blunt-ended, ligated into the expression vector pNUT (Palmiter et al., 1987), and the mutant constructs were transfected into baby hamster kidney cells. Selection with methotrexate was initiated following 24 h in serum-free medium and 24 h in DMEM containing 10% fetal bovine serum. Cells were sequentially passaged into convoluted roller bottles and expression of recombinant proteins (under the control of the metallothionein promoter) induced by addition of zinc sulfate. Cell culture media were removed every 3 days, centrifuged to remove cell debris, and stored at −20 °C. Prior to isolation of recombinant half-transferrins, media were made 100 μM in Fe (as the 1:2 complex with nitrilotriacetate) to ensure saturation of the proteins with iron. Recombinant proteins were then isolated by a two-step procedure involving immunoaffinity chromatography followed by anion-exchange chromatography on a Pharmacia Q-Sepharose HP column. Electrospray mass spectrometry corroborated the nature of the mutations in the recombinant proteins (Crawley, 1993). With one exception, experimentally determined molecular weights were within ±6 mass units of theoretical values. For R124S, the experimental value was 37 093 ± 2.4 compared to a theoretical value of 37 082.

Kinetics of Iron Release. Iron release from native and recombinant proteins was measured by a previously described spectrofluorometric procedure which requires only microgram amounts of protein while providing continuous display of the progress of release (Egan et al., 1993). Best results were obtained with observed rate constants for release in the range of 0.1−0.001 s^{-1}, where neither mixing artifacts nor instrumental drift proved problematic. Because pyrophosphate has been extensively used as an iron-accepting species in earlier studies of iron release from transferrin, and because it is a component of ATP which has been shown to be an important intracellular iron-binding molecule (Pollack, 1994), pyrophosphate was chosen as the required iron-accepting agent in all studies. Progress curves of release were analyzed using the Levenberg−Marquardt nonlinear curve fitting algorithm provided in the SigmaPlot graphics package. Since all of the studies used single-sited proteins, only single-exponential functions were fitted. R^2 values (coefficients of determination) were consistently greater than 0.99. Where shown, error bars represent curve fitting results from measurements made in triplicate. In general, values of k_{obs} in repeated determinations fell within ±8% of the mean.

RESULTS

Studies at pH 7.4. Comparisons of observed iron release rates (k_{obs}) from the N-lobe of native transferrin and recombinant N-lobe half-transferrins at extracellular pH, 7.4, at two different concentrations of chloride, are shown in Figure 2. The results can be broadly classified into three categories: (i) observed release rates which differ little from

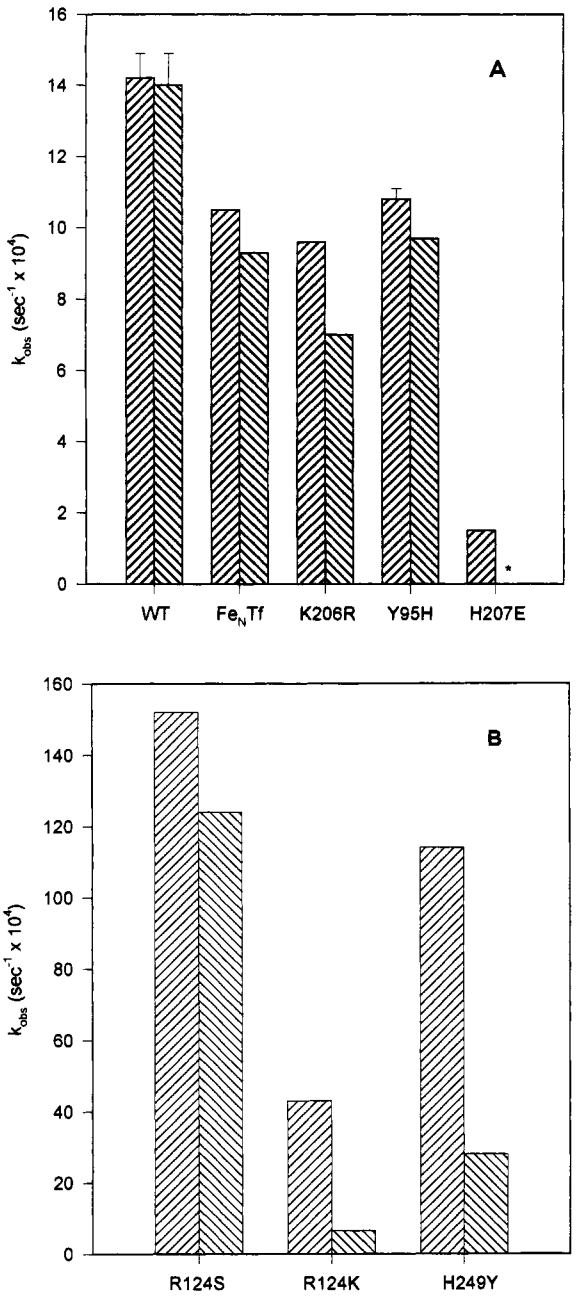


FIGURE 2: (A) k_{obs} for iron release from Fe_N-Tf and mutants to 100 mM pyrophosphate at pH 7.4, 50 mM HEPES. (*) Release too slow to measure spectrofluorometrically. (B) k_{obs} at pH 7.4 for iron release from selected mutants to 1 mM (R124S and R124K) or 0.05 mM (H249Y) pyrophosphate, 50 mM HEPES. Adjacent bars display the effects of 100 mM (///) and 600 mM chloride (\\\), respectively.

rates observed in Fe_N-Tf or wild-type recombinant N-lobe; (ii) observed rates which are much slower than the reference proteins; and (iii) observed rates which are much faster. In wild-type and mutant proteins a retarding effect of chloride on iron release is evident, in contrast to the accelerating effect of chloride on iron release from the C-lobe of intact transferrin (Kretchmar & Raymond, 1988; Egan et al., 1992) or a proteolytic single-sited fragment (data not shown). Mutation of Lys 206 to arginine, to mimic the arrangement in lactoferrin, results in some slowing of release at pH 7.4, particularly when compared to wild-type recombinant N-lobe. Again in mimicry of lactoferrin, conversion to glutamate of His 207, which lies adjacent to Lys 206 in the interdomain cleft of the N-lobe but appears more accessible to solvent (Figure 1), leads to a substantial slowing of release.

Table 1: Iron Release from N-Lobe Recombinants of Human Transferrin (pH 7.4)

protein	[PP_i] (mM)	[Cl^-] (mM)	k_{obs} ($s^{-1} \times 10^4$)
wild type	100	100	142
wild type	100	600	140
	Slow Releasers		
K206R	100	100	9.6
K206R	100	600	7.0
Y95H	100	100	10.8
Y95H	100	600	9.7
H207E	100	100	1.5
H207E	100	600	*a*
	Fast Releasers		
R124S	1	100	152
R124S	1	600	124
R124K	1	100	43
R124K	1	600	6.5
H294Y	1	100	114
H249Y	1	600	28

[a] Too slow to measure spectroflurometrically.

Increased iron-binding affinity of the H207E mutant has been reported (Woodworth et al., 1991); the present findings are consistent with this observation.

Arg 124 serves as the principal anchor, reinforced by an elaborate network of hydrogen bonds (Anderson et al., 1989), for the synergistic carbonate anion required for iron binding by all transferrins. Not surprisingly, therefore, mutation of Arg 124 to serine or to lysine generates a protein much more facile in releasing iron at pH 7.4 than the N-lobe of transferrin. In order to quantify release rates, it was therefore necessary to reduce the concentration of pyrophosphate from 100 to 1 mM, with results shown in Figure 2B.

Transferrin belongs to the class of iron-binding proteins with tyrosyl phenolate ligands (Que, 1983). In this class the tyrosine serves to anchor iron strongly as well as to provide visible charge transfer absorption bands which account for the relatively intense colors of the proteins. In the N-lobe of human transferrin two tyrosyl phenolate oxygens of Tyr 95 and Tyr 188, respectively, serve as iron donor atoms, with Asp 63 and His 249 completing the ensemble of protein ligands. Iron−ligand distances are shortest, and bond strengths are presumably greatest, for the tyrosyl ligands. Accordingly, we measured release rates of iron from a Y95H mutant. The mutant behaved nearly the same as the wild-type N-lobe (Figure 2A). A "reciprocal" mutant in which a histidine has been changed to a tyrosine, H249Y, showed much more facile release, however (Figure 2B), when a stabilizing effect might have been anticipated. Indeed, iron binding by the H249Y mutant was the least stable of the mutants we have studied, so that to obtain measurable release rates required lowering the pyrophosphate concentration to 0.05 mM. A summary of studies at pH 7.4 is presented in Table 1.

Studies at pH 5.6. In cells of erythroid lineage, iron is released from transferrin in an acidified endosome where the pH has been reported to be in the range of 5−6 (vanRenswoude et al., 1982; Watkins et al., 1992). We have therefore chosen to work at pH 5.6−5.7, as in earlier studies from this laboratory (Bali et al., 1991; Egan et al., 1993). Iron is released more than twice as rapidly from the N-lobe of native transferrin as from the recombinant N-lobe half-transferrin (Figure 3) at this pH. Modulation of iron release by stabilizing interlobe interactions has been noted in

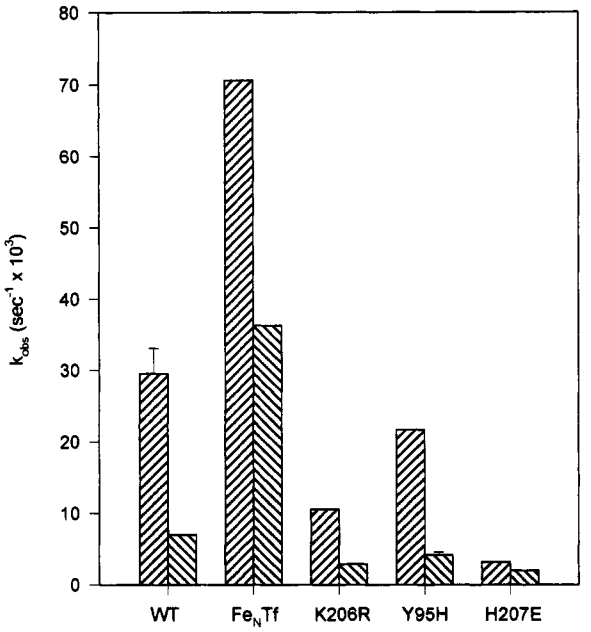


FIGURE 3: k_{obs} for iron release from Fe_N-Tf and mutants to 50 μM pyrophosphate, 100 mM MES, pH 5.6. Adjacent bars display the effects of 100 mM (///) and 600 mM chloride (\\\), respectively.

Table 2: Iron Release from N-Lobe Recombinants of Human Transferrin to 50 μM PP_i at pH 5.6

protein	$[Cl^-]$ (mM)	k_{obs} ($s^{-1} \times 10^3$)
wild type	100	30
wild type	600	7.0
K206R	100	11
K206R	600	2.9
Y95H	100	22
Y95H	600	4.2
H207E	100	3.2
H207E	600	2.0

comparisons of recombinant human lactoferrin N-lobe with native lactoferrin (Day et al., 1992), while in ovotransferrin at pH 7.5 such interactions produce opposite effects on the two lobes (Lin et al., 1991). Our measurements suggest that interlobe interactions destabilize iron binding to the N-lobe of transferrin at endosomal pH.

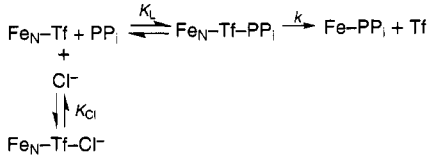
In extension to what is observed at pH 7.4, iron release at pH 5.6 from the K206R and Y95H mutant is in each case appreciably slower than from wild-type recombinant N-lobe. Observed release rates from the H207E mutant at pH 5.6 and 0.1 or 0.6 M chloride are even more dramatically slowed at the low pH, where they are no more than one-fourth to one-tenth those of recombinant wild-type or native N-lobe.

What holds true for the effect at pH 7.4 of mutating Arg124 is even more evident at pH 5.6, where release rates from R124S and R124K alike are too fast to measure with the spectrofluorometric method even with micromolar concentrations of pyrophosphate as iron-sequestering reagent. A similar problem occurs with H249Y, the most facile iron-releasing mutant we have studied. A summary of results at pH 5.6 is presented in Table 2.

The Negative Chloride Effect. At pH 5.6, as at pH 7.4, increasing chloride concentration depresses release rates in recombinant and native proteins. To investigate further the mechanism of this negative response of release, we turned to detailed studies of the effects of chloride and pyrophosphate concentration on iron release from recombinant R124K at pH 7.4 and wild type N-lobe at pH 5.6. Each was chosen to permit working over a wide range of chloride or ligand concentrations. Release from wild type N-lobe is too slow at pH 7.4 for reliable measurements unless high concentrations of pyrophosphate are used, resulting in a confoundingly high ionic strength in the working buffer. Conversely, mutants releasing iron in facile manner at pH 7.4 release iron too rapidly at pH 5.6. It was not possible to find one preparation suitable for studies at both pH values. Buffer concentrations were set at 50−100 mM, to provide for a reasonably stable pH during the course of an experiment without providing an unnecessarily high background ionic strength.

The effect of increasing concentrations of chloride on the course of iron release from R124K to pyrophosphate at pH 7.4 is shown in Figure 4A. Although release rates are much faster at 5 mM pyrophosphate than at 1 mM pyrophosphate, as expected, the effect of chloride is similar in both cases: release is progressively slowed as chloride concentration increases. This negative effect is also observed with wild-type recombinant N-lobe at pH 5.6 (Figure 4B). Increasing chloride concentration from 10 to 1000 mM slows k_{obs} by close to a factor of 10.

This negative chloride effect can be approximately modeled by assuming competition between pyrophosphate and chloride for binding to a site sufficiently close to the iron so that release of the metal can be effected by ligand exchange reactions between iron accepting anion and transferrin, as in the following scheme:



where binding and release of pyrophosphate and chloride are assumed to be rapid and therefore representable by equilibrium constants. These assumptions lead to an apparent first-order rate constant:

$$k_{obs} = \frac{K_L k[PP_i]}{1 + K_{Cl}[Cl^-] + k[PP_i]} \quad (1)$$

An expression for k_{obs} of the same form as eq 1 can be derived if a steady state in [Fe_N-Tf-PP_i] rather than an equilibrium in binding of PP_i is assumed, i.e., if k' is rapid compared to the forward rate constant for binding of PP_i to Fe-Tf. Our studies cannot distinguish between these possibilities, and we have chosen to assume the preequilibrium rather than steady-state mechanism.

Equation 1, which resembles that for competitive inhibition in a Michaelis−Menten process, predicts saturation kinetics as [PP_i] is increased and a decreasing rate of iron release with increasing [Cl^-]. Tests of eq 1 for iron release from R124K (pH 7.4) and from wild-type recombinant N-lobe (pH 5.7) are shown in Figure 4, where the dotted curves are least-squares fits of k_{obs} as a function of [PP_i] with the equilibrium parameters K_L and K_{Cl}, and the kinetic parameter k are freely adjustable. For the experimental conditions used, the gradient of k_{obs} is much steeper in the direction of k, with K_L and K_{Cl} strongly interdependent, so that the fits are sensitive functions only of the kinetic parameter. It is not possible, therefore, to obtain unequivocal values for K_{Cl} and K_L from our data. Values of k for R124K were found to be 0.029

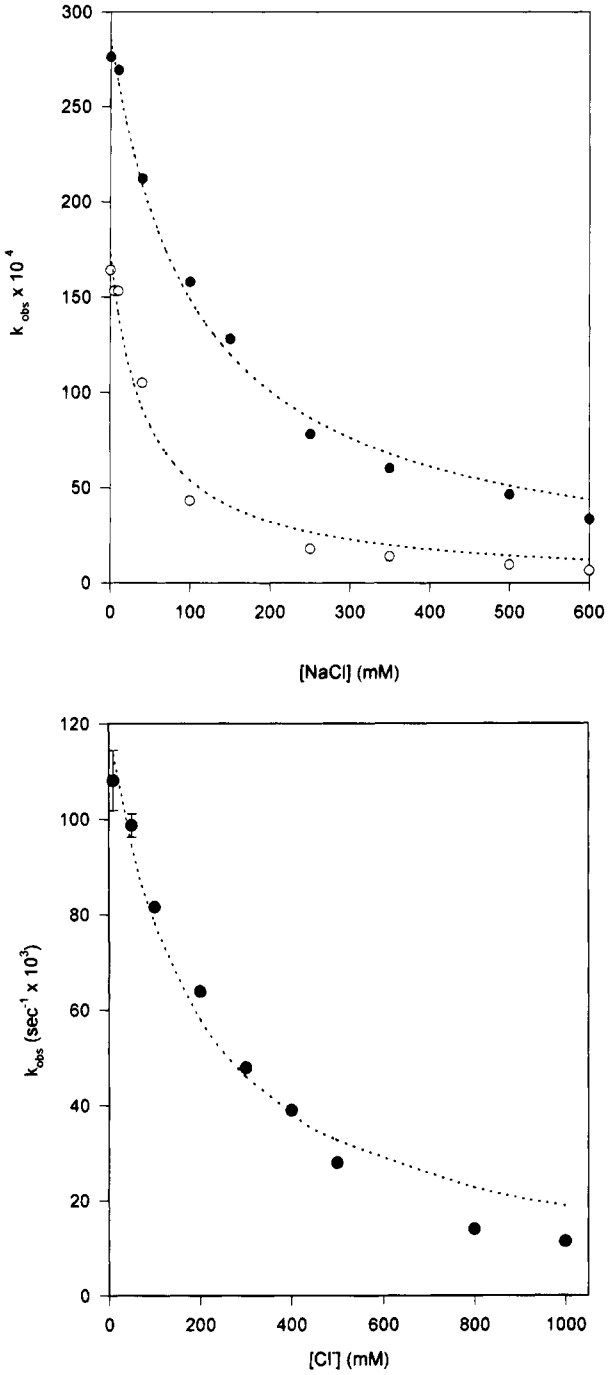


FIGURE 4: Retarding effect of chloride on iron release. (A) R124K at pH 7.4, 50 mM HEPES: (●) 5 mM pyrophosphate; (○) 1 mM pyrophosphate. (B) Wild-type N-lobe at pH 5.6, 100 mM MES, 125 μM pyrophosphate. Dashed curves represent least-squares fits to eq 1.

and 0.024 s^{-1} for release to 1 and 5 mM pyrophosphate, respectively. The agreement in estimates of kinetic parameters, although not perfect, is reasonably satisfactory in suggesting that the competitive inhibition model offers a fair description of the mechanism of the chloride effect. For the wild-type recombinant N-lobe at pH 5.6 the fitted value of the kinetic parameter is 0.15 s^{-1}, in keeping with the much faster release at this pH.

The inhibitory effect of chloride on release kinetics is evident for the recombinant wild type N-lobe at pH 5.6, as well as for the R124K mutant at pH 7.4. In the former case the concentration of pyrophosphate needed to obtain satisfactory data over a reasonably wide range of chloride concentrations was only 0.125 μM (Figure 4B), which makes a negligible contribution to ionic strength (0.00012). Reliably measurable release rates at pH 7.4 for the wild-type protein required a pyrophosphate concentration of 100 mM, and hence a contribution of pyrophosphate to ionic strength of 0.63, which was deemed too great for assessment of the chloride effect. At this pH, however, satisfactory data could be obtained with the R124K, R124S and H249Y mutants at pyrophosphate concentrations of 1 mM or less (Figure 2B), with minor contributions of pyrophosphate to ionic strength (0.0063).

Equation 1 also predicts a saturation effect with increasing concentrations of pyrophosphate. This prediction is tested in Figure 5. Although the fits to the kinetic model are reasonably good at lower concentrations of pyrophosphate, they are somewhat less satisfactory at higher concentrations. Saturation is noted experimentally for R124K at pH 7.4 (Figure 5A) and for wild-type N-lobe at pH 5.6 (Figure 5B), but in each case the curve fitting procedure calls for saturation at higher concentrations of ligand than are experimentally observed. The deviations of experimental data points from the fitted curve of k_{obs} as a function of pyrophosphate concentration may indicate, therefore, that the mechanism of iron release is more complex than indicated in the simple competitive scheme. Nevertheless, the general agreements between fitted curves and experimental data support a model of competitive inhibition between chloride and ligand.

DISCUSSION

Iron Release at pH 7.4. Iron release from lactoferrin at pH 7.4 is much more sluggish than release from transferrin, at least in part because of stabilization of the closed configuration in lactoferrin by salt bridging between Glu 211 (corresponding to His 207 in transferrin) and Arg 89 (corresponding to Gln 92), with an O−N distance of 2.3 Å (Anderson et al., 1989). No corresponding bonding is evident in the structure of the N-lobe of human transferrin. The arrangement in lactoferrin is mimicked in the H207E mutant, from which iron release to a fixed concentration of pyrophosphate is nearly an order of magnitude slower than from recombinant N-lobe or the N-site of native transferrin (Figure 2A). A possible explanation is that conversion of His 207 to the more hydrophilic and negatively charged glutamate results in hydrogen-bonding with Gln 92, thereby favoring the closed conformation of the two domains in transferrin and so impeding release.

Further insight into mechanisms of iron release from transferrin is obtained from studies of the R124K mutant, in which the arginine anchoring the synergistic carbonate ligand is altered to a lysine. In order to achieve measurable release rates from both Arg-124 mutants, it was necessary to reduce the pyrophosphate concentration from 100 to 1 mM. Although lysine may be positively charged at pH 7.4, its side chain extends less than that of arginine, and steric effects as well as charge interactions are likely involved in the carbonate-protein bond. It is not surprising, therefore, that the R124K mutant is much more facile in releasing iron than the native structure in Fe_N-Tf or the wild-type recombinant protein. The side chain of serine is not charged, and the strength of any bond it forms with carbonate is likely to be much weaker than that formed by arginine or lysine. Release

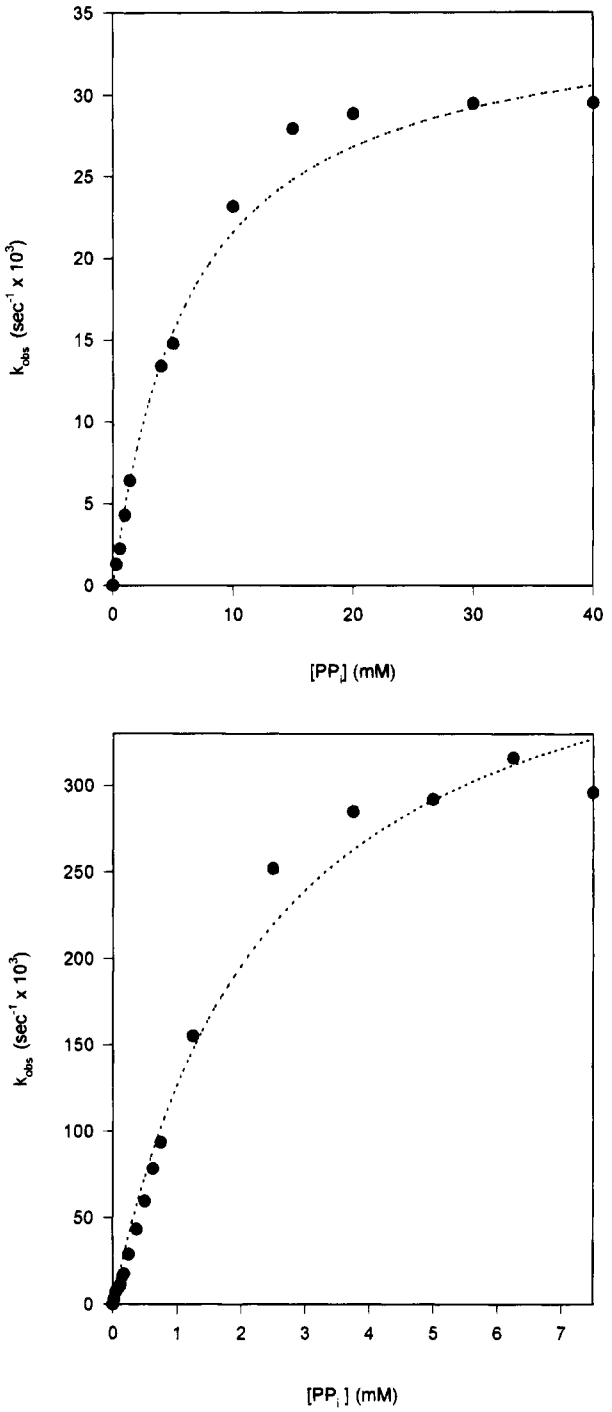


FIGURE 5: Dependence of k_{obs} on [PP_i] for iron release. (A) R124K, 50 mM HEPES, 100 mM chloride, pH 7.4. (B) Wild-type N-lobe, 100 mM MES, 600 mM chloride, pH 5.7; the high concentration of chloride was needed to provide a range of [PP_i] throughout which reliable measurements of k_{obs} could be obtained. Dashed curves represent fits to eq 1.

of iron from R124S is thus even faster than release from R124K. The greater facility with which the arginine mutants release iron is consistent with the hypothesis that release of carbonate is a critical and perhaps initiating event in release of iron (Aisen & Leibman, 1973). In both R124 mutants the effect of increasing chloride concentration is to retard iron release to pyrophosphate, as it does in unaltered N-lobes, thus excluding R124 as a kinetically active chloride binding site.

Replacing the hinge ligand His 249 with a tyrosine results in three bulky phenolic groups at the iron-binding site. In the native structures of transferrin N-lobes the binding sites are filled by the protein ligands and the synergistic carbonate anion, allowing no room for even a water molecule in the coordination sphere (Baker, 1994). A reasonable inference, therefore, is that replacement of the histidine imidazole ring (ligand N−Cα distance ≈ 4.3 Å) with the larger phenolate ring of tyrosine (ligand O−Cα distance ≈ 6.5 Å) results in steric strain of the binding ligands, thereby substantially weakening the iron-protein bond.

Iron Release at pH 5.6. In a 2.3 Å resolution structure of the iron-bearing N-lobe of hen ovotransferrin crystallized at pH 5.9, a Lys 209−Lys 301 pair with NZ atoms separated by 2.3 Å has been identified (Dewan et al., 1993). In the corresponding pair in human transferrin, Lys 206−Lys 296, the separation distance is 2.61 Å. Since these lysines are located on opposite domains, their joint protonation would force the two domains apart by electrostatic repulsion, thereby exposing bound iron and facilitating its release. Homologous dilysine pairs are found in other serum transferrins, but in human and mouse lactoferrin one of the lysines is replaced by an arginine. It was therefore of interest to investigate the kinetics of iron release from a transferrin mutant constructed with the Lys-Arg arrangement in lactoferrin, K206R. Iron release to a fixed concentration of pyrophosphate occurs nearly an order of magnitude more slowly from K206R than from recombinant N-lobe (Figure 3), in support of the hypothesis of Dewan et al. (1993) that the dilysine pair functions in triggering iron release. At pH 5.6 both lysines are likely to be protonated, charged, and mutually repulsive, thus accounting (at least in part) for the increased facility of release from transferrin at this pH compared to pH 7.4. Although mutation of Lys 206 to arginine preserves charge, the Coulombic repulsion, with a force varying as the square of the charge separation, could be substantially less in the mutant because of the greater length of the arginine side chain and hence the greater separation of charges. If so, the ease with which the two domains separate would be correspondingly reduced in the mutant and iron release correspondingly slowed. Our present result also accords with observations by Woodworth et al. (1991) on a mutant with an uncharged glutamine replacing a lysine, K206Q. Iron release from K206Q requires much longer times than release from wild-type N-lobe or a pH of 4 and the presence of deferoxamine. We have no ready explanation for the retarded release from Y95H at acidic pH. Possibly, the tyrosine ligand in the native structure is more readily protonated than is its replacement histidine.

A very recent crystallographic study of the H207E mutant points to a possible mechanism for the stabilizing effect of the mutation (Woodworth et al., 1995). As pH is lowered, repulsion between positively charged Lys 206 and His 207 in the native structure may also promote release, while in the H207E mutant the carboxyl group would bear zero or negative charge, thereby abolishing destabilizing electrostatic effects.

Rapidity of release from Arg-124 mutants at pH 5.6, more than an order of magnitude faster than from nonmutagenized N-lobe, again is consistent with a critical role of the synergistic carbonate ligand in stabilizing the transferrin iron−protein bond. In the absence of the anchoring Arg 124, the carbonate is held in place only by hydrogen bonding to the N-terminus of helix (5) and Thr 120 (Anderson et al., 1989). Protonation of the carbonate at low pH would then result in decreased electrostatic attraction to bound Fe(III)

and hence increased accessibility of the iron to chelating agents or to hydrolysis.

The Negative Chloride Effect. A detailed investigation by Harris et al. (1987) of iron release from transferrin to pyrophosphate and phosphonates showed saturation-linear behavior, rather than the expected simple saturation, of k_{obs} as a function of ligand concentration. A remarkable finding by Kretchmar and Raymond (1988) was that the rate of release of iron from either lobe of transferrin to the synthetic iron chelator 3,4-LICAMS varies nearly linearly with ionic strength, extrapolating to zero as ionic strength goes to zero. Studies of Egan and associates then produced a model accounting for these results, in which release requied that a kinetically significant anion binding (KISAB) site of transferrin must be occupied before release of iron to pyrophosphate or phosphate could occur (Egan et al., 1992, 1993; Marques et al., 1995). The anion-binding requirement can be satisfied by chelating anions such as pyrophosphate or by simple anions such as chloride. There is therefore general agreement in the literature that chloride accelerates release from the C-lobe, and that the mechanism of the chloride effect is explained by postulating a KISAB site which must be occupied for release to occur.

Our studies of iron release from the N-site of intact transferrin or N-lobe mutants, or single-sited proteolytic fragments representing the N-lobe (data not shown), do not conform to this model, however. Rather, the effect of chloride is in each case to inhibit iron release to pyrophosphate. Previous reports from several laboratories on the effects of chloride on iron release from the N-lobe of human transferrin have been discordant. A retarding effect has been found with pyrophosphate as iron-sequestering species (Williams et al., 1982), little influence with EDTA (Baldwin & de Sousa, 1981), and an accelerating action with the catechol-based synthetic iron-binder 3,4-LICAMS (Kretchmar & Raymond, 1988). In the last instance, as already indicated, an absolute dependence of iron release on ionic strength was observed. A dependence of the chloride effect on pyrophosphate concentration has also been noted, with chloride retarding release at 5 mM PP_i but accelerating release at 100 mM PP_i. We propose that in the N-lobe chloride competes with pyrophosphate for binding to a site where the chelator accepts iron by ligand exchange reactions with the protein. The existence of such a site is suggested by EPR studies showing perturbation by pyrophosphate of the $g' = 4.3$ signal of specifically bound ferric iron (Foljatar & Chasteen, 1982). The simple scheme leading to eq 1, tested in Figures 4 and 5, accords with this model, although alternative or additional mechanisms are not excluded. It also seems likely that the negative chloride effect depends on the ligand used to effect iron release, hence its absence when 3,4-LICAMS is used to sequester released iron.

Differences between N- and C-lobes further suggest that at least three classes of anion-binding sites govern the binding and release of iron from transferrin. The first of these, the synergistic carbonate-binding site, must be occupied in each lobe for iron to bind at all to that lobe. Occupancy of the second or KISAB site is required for iron release from the C-lobe, presumably by promoting the "open" conformation from which release is facile. The third site, in the N-lobe, is the locus where chloride competes with iron-sequestering pyrophosphate. Mechanisms of iron release from the N- and C-lobe sites would therefore appear to be different and more complex than once believed.

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