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Receptor-Induced Switch in Site–Site Cooperativity during Iron Release by Transferrin†

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ABSTRACT: Iron removal by PP_i from the N- and C-terminal binding sites of both free and receptor-complexed transferrin, when the partner site remains occupied with kinetically inert Co(III), has been studied at pH 7.4 and 5.6, at 25 °C. At extracellular pH, 7.4, the C-terminal site of free mixed-metal proteins is slightly more labile than its N-terminal counterpart in releasing iron to 0.05 M PP_i. The rate and extent of iron removal are retarded from both sites when transferrins are receptor-bound. At endosomal pH, 5.6, the two sites exhibit greater kinetic heterogeneity in iron release to 0.005 M PP_i. The N-terminal site is 6 times more facile in relinquishing iron than the C-terminal site when mixed-metal transferrins are free. However, the two sites are affected oppositely upon binding to the receptor. Iron release from the C-terminal site of receptor-complexed Co_N–transferrin–Fe_C is 4 times faster than that from receptor-free protein. In contrast, iron removal from the N-terminal site of receptor-complexed Fe_N–transferrin–Co_C is slowed by a factor of 2 compared to that from free protein. These results help explain our previous observation of a receptor-induced switch in site lability during iron removal from diferric transferrin at pH 5.6 (Bali & Aisen, 1991). Site–site cooperative interactions between the two sites of doubly-occupied transferrin during iron release are altered upon binding to receptor at pH 5.6. Iron in the otherwise weaker binding site of the N-terminal lobe is stabilized, while iron in the relatively stable binding site of the C-terminal lobe is labilized.

Transferrin, in providing iron for the needs of iron-dependent cells, is first seized by a specific receptor on the cell surface, then internalized by the cell into an acidified endosome where iron release takes place, and finally returned to the cell surface where it is freed, depleted of iron but otherwise intact, for another cycle of iron transport (Dautry-Varsat et al., 1983; Klausner et al., 1983). Throughout its journey in the cell transferrin remains attached to its receptor. We have recently reported that, in addition to its long-recognized function of capturing diferric transferrin from the circulation for internalization into an endocytotic vesicle, the transferrin receptor also modulates the kinetics of iron release from transferrin (Bali et al., 1991b). At extracellular pH, 7.4, the receptor impedes the release of iron from diferric transferrin, while at endosomal pH, 5.6, it facilitates release to a suitable acceptor of Fe(III).

The effect of receptor is particularly striking at the iron-binding site in the C-terminal lobe of transferrin, since at endosomal pH iron is released with greater facility from this site than from the much weaker binding site in the N-terminal lobe when diferric transferrin is complexed to its receptor (Bali & Aisen, 1991). However, release of iron from monoferric transferrins at pH 5.6 is faster from the N-terminal site, whether the proteins are bound to receptor or free. This seeming inconsistency in the behavior of monoferric and diferric transferrins prompted us to examine whether site–site interactions, when both sites of transferrin are occupied by metal ions, further modulate the kinetics of iron release. Because simultaneous release of iron from each site of diferric transferrin complicates the independent determination of release rates from the two sites of the protein, we have turned for our studies to mixed-metal transferrins, with Fe(III) at one site of the protein and Co(III) at the other. Cobalt(III) is a substitutionally inert species that remains bound to transferrin during the course of iron release, thereby making it possible to examine the effect of occupancy of one site on iron release from the other whether the proteins are free or complexed to receptor.

Materials and Methods

Human serum transferrin was purchased from Boehringer-Mannheim and purified as previously reported (Aisen et al., 1978). Amersham Corporation supplied ^{59}Fe as $^{59}FeCl_3$. Radiolabeled Fe_N–Tf–Co_C[1] and Co_N–Tf–Fe_C were prepared

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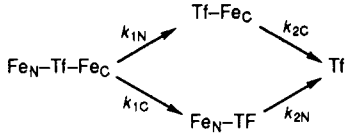
from labeled monoferric transferrins by addition and oxidation of Co(II), (Aisen et al., 1969) and characterized as described (Bali & Harris, 1989). Specific activities were 1906 and 1704 cpm/μg of protein, respectively. Transferrin receptor was isolated by methods used earlier (Turkewitz et al., 1988; Bali et al., 1991b). The complexes of receptor with each form of iron–cobalt transferrin were isolated by gel filtration chromatography as described previously (Bali et al., 1991b).

Iron removal from iron–cobalt transferrins and their complexes with receptor by 0.005 M PP_i was studied in 0.05 M MES/0.1 M NaCl/0.01 M CHAPS, pH 5.6, at 25 °C, using PEG precipitation to separate iron bound to protein from that released by protein and bound to PP_i (Bali et al., 1991b). Relative amounts of each were assessed by radioactivity of the ^{59}Fe(III) label. Release at pH 7.4 to 0.05 M PP_i was followed in 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS. These conditions were chosen to obtain measurable initial release rates. The concentration of transferrin in all experiments was 0.2 μM. Data were normalized to a reaction coordinate, the percent of iron released, and fit to a truncated pseudo-first-order rate expression as already described (Bali & Aisen, 1991). This phenomenological kinetic analysis yielded two descriptive parameters: k, the apparent pseudo-first-order rate constant, and B, the truncation point. A lower value of B reflects a more nearly complete release of iron from a binding site. Reported values are the averages for precipitate and supernatant for two separate runs.

Results

Iron Removal from Diferric Transferrin. A two-sited protein like transferrin requires four microscopic constants to characterize the removal of two specifically-bound Fe(III) ions, as shown in Scheme I.

Scheme I



The subscript N or C to each rate constant denotes the site of the bound iron and 1 or 2 refers to first or second ferric ion released from the protein. Since monoferric transferrins of reasonable purity can be prepared, the rate constants k_{2N} and k_{2C} for iron release from one site when other site is empty have been measured for a variety of iron chelates at pH 7.4 (Bali & Harris, 1989; Bali et al., 1991a). We have recently reported these as well as k'_{2N} and k'_{2C} (primed phenomenological rate constants are intended to distinguish free from receptor-bound transferrins) for iron removal by 0.005 M PP_i at pH 5.6 (Bali & Aisen, 1991). In order to determine k_{1N} and k_{1C}, the rate constant for iron removal from one site when the partner site remains occupied with Co(III) ion, we have now employed Fe$_N$–Tf–Co$_N$ and Co$_C$–Tf–Fe$_N$ transferrins. These mixed-metal transferrins have been shown to mimic the diferric transferrin closely (Bali & Harris, 1990). This has made it possible to analyze cooperative interactions between the two sites of transferrin during iron removal from either site and the effect of receptor upon these interactions.

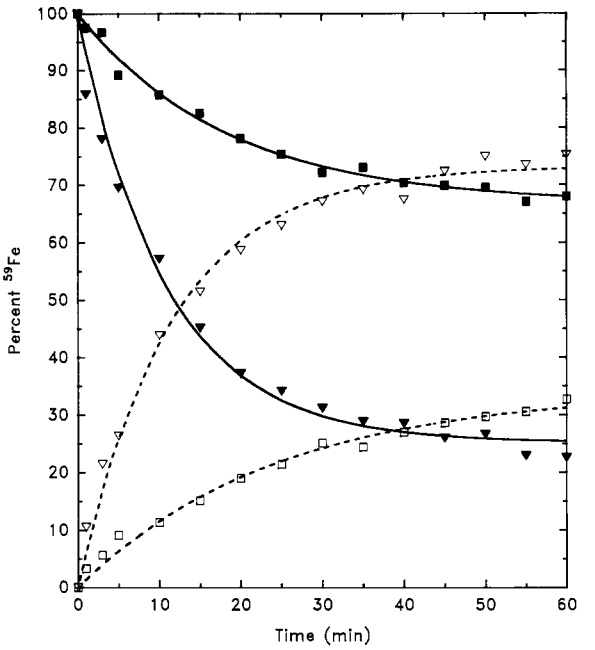


FIGURE 1: Plots of normalized reaction coordinate (percent of ^{59}Fe) as a function of time for the removal of iron from Co$_N$–Tf–Fe$_C$ (▽, ▼) and [TfR–(Co$_N$TfFe$_C$)$_2$] (□, ■) by 0.05 M PP_i at pH 7.4 in 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS. Key: activity in PEG precipitates (▼, ■, —); activity in PEG supernatants (▽, □, – –). Symbols are experimental data points and lines are calculated curve fits from eq 1 using the parameters listed in Table I.

Data Analysis. Co(III) is a substitutionally inert low-spin d^6 ion with a filled t_{2g} subshell so that no detectable amount of it is lost from Fe$_N$–Tf–Co$_C$ and Co$_N$–Tf–Fe$_C$ during iron removal by 0.05 M PP_i at pH 7.4 or by 0.005 M PP_i at pH 5.6. The analysis of iron removal reactions from these proteins can therefore be treated by simple pseudo-first-order kinetics. Because reactions do not generally proceed to completion, kinetics are phenomenologically treated by the truncated first-order rate expression given below:

$$R_t = (100 - B)\exp(-kt) + B \qquad (1)$$

where R_t = percent of iron removed at time t, 100 = percent of iron at time zero, and (100 – B) = total percent of iron removed in the time for which reactions were followed, i.e., the extent of reaction at the end of this time. Plots of R_t as a function of time for iron removal by 0.05 M PP_i at pH 7.4 from Co$_N$–Tf–Fe$_C$ and [TfR–(Co$_N$TfFe$_C$)$_2$] are shown in Figure 1, and those for Fe$_N$–Tf–Co$_C$ and [TfR–(Fe$_N$TfCo$_C$)$_2$] are presented in Figure 2. Corresponding plots for iron removal by 0.005 M PP_i at pH 5.6 are given in Figures 3 and 4, respectively. The phenomenological parameters k and B, calculated by non-linear least-squares fitting of eq 1 to experimental data points using the Marquardt–Levenberg algorithm (SigmaPlot V. 4.0, Jandel Scientific), are listed in Table I. For evaluation of site–site interactions, the corresponding parameters for monoferric transferrins from a previous study (Bali & Aisen, 1991) are also used for this table. In some cases these values differ slightly from those of the previous study because we have carried out additional experiments, presenting the average values obtained along with

[1] Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PEG, poly(ethylene glycol); PAGE, polyacrylamide gel electrophoresis; PP_i, pyrophosphate; Tf, human serum apotransferrin; Fe$_N$–Tf–Fe$_C$, diferric transferrin; Fe$_N$–Tf, N-terminal monoferric transferrin; Tf–Fe$_C$, C-terminal monoferric transferrin; Fe$_N$–Tf–Co$_C$, mixed-metal Fe–Co transferrin (Fe in N-terminal site and Co in C-terminal site); Co$_N$–Tf–Fe$_C$, mixed-metal Co–Fe transferrin (Co in N-terminal site and Fe in C-terminal site); TfR, transferrin receptor; [TfR–(Fe$_N$TfFe$_C$)$_2$], complex of receptor and diferric transferrin; [TfR-(Fe$_N$Tf)$_2$], complex of receptor and N-terminal monoferric transferrin; [TfR-(TfFe$_C$)$_2$], complex of receptor and C-terminal monoferric transferrin; [TfR–(Fe$_N$TfCo$_C$)$_2$], complex of receptor and Fe–Co transferrin; TfR-(Co$_N$TfFe$_C$)$_2$], complex of receptor and Co–Fe transferrin.

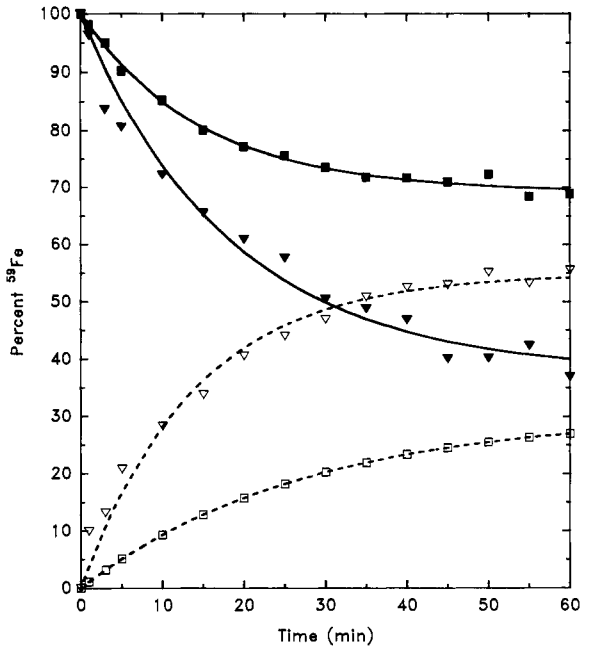


FIGURE 2: Plots of normalized reaction coordinate (percent of ^{59}Fe) as a function of time for the removal of iron from Fe_N–Tf–Co_C and [TfR–(Fe_NTfCo$_C$)$_2$] by 0.05 M PP_i at pH 7.4 in 0.05 M HEPES/0.1 M NaCl/0.01 M CHAPS. Symbols and lines are as in Figure 1.

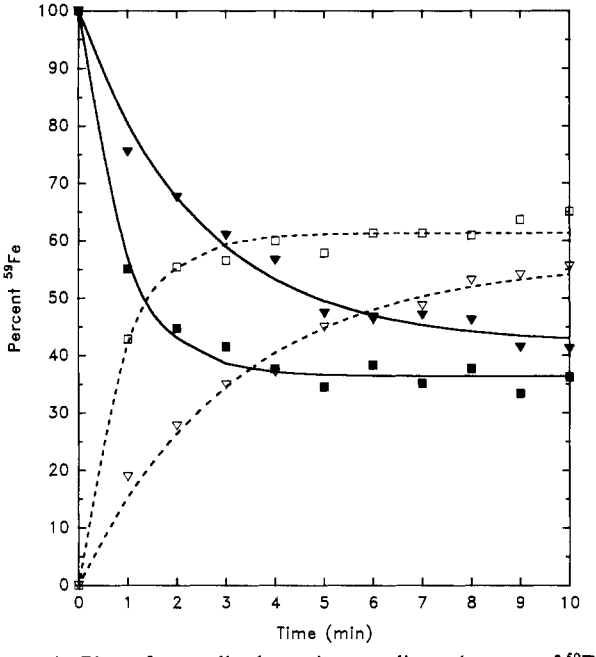


FIGURE 3: Plots of normalized reaction coordinate (percent of ^{59}Fe) as a function of time for the removal of iron from Co_N–Tf–Fe_C and [TfR–(Co_NTfFe$_C$)$_2$] by 0.005 M PP_i at pH 5.6 in 0.05 M MES/0.1 M NaCl/0.01 M CHAPS. Symbols and lines are as in Figure 1.

the standard errors of the measurements.

Iron Removal from Mixed Metal Fe–Co Transferrins at Extracellular pH. At pH 7.4, the C-terminal site of free Co_N–Tf–Fe_C is slightly more labile than the N-terminal site of free Fe_N–Tf–Co_C (compare k_{1C} = 0.085 min^{-1} to k_{1N} = 0.064 min^{-1} and B_{1C} = 26% to B_{1N} = 42%) for iron removal by 0.05 M PP_i. As observed previously with diferric transferrin (Bali et al., 1991b) as well as both forms of monoferric transferrins (Bali & Aisen, 1991), receptor binding to transferrin retards both the rate and extent of iron removal reaction from both sites. The C-terminal site is then slightly less reactive than its N-terminal counterpart (compare k'_{1C} = 0.042 min^{-1} to k'_{1N} = 0.052 min^{-1} and B'_{1C} = 66% to B'_{1N} = 69%).

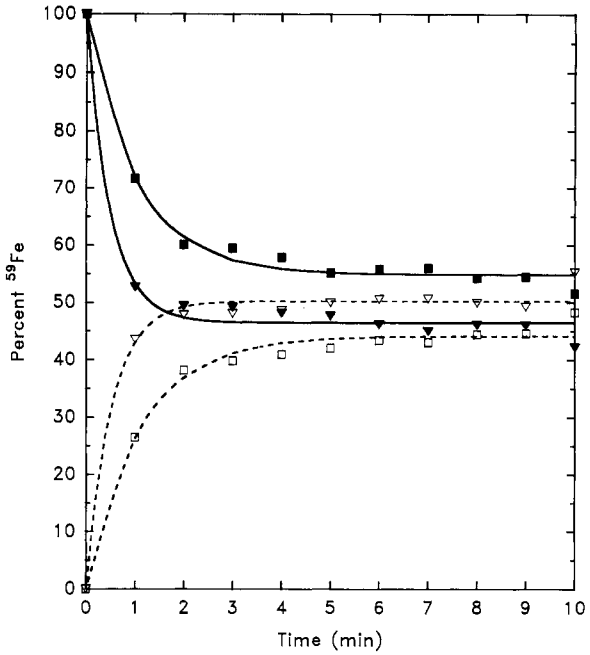


FIGURE 4: Plots of normalized reaction coordinate (percent of ^{59}Fe) as a function of time for the removal of iron from Fe_N–Tf–Co_C and [TfR–(Fe_NTfCo$_C$)$_2$] by 0.005 M PP_i at pH 5.6 in 0.05 M MES/0.1 M NaCl/0.01 M CHAPS. Symbols and lines are as in Figure 1.

Table I: Parameters Characterizing Removal of Fe(III) by PP_i from Transferrins and Their Complexes with Transferrin Receptor

transferrin species	pH	[PP_i] (mM)	k (min^{-1})	B (%)
Fe_N–Tf[a]	7.4	50	k_{2N}, 0.063 ± 0.002	B_{2N}, 40 ± 1
Tf–Fe_C[a]	7.4	50	k_{2C}, 0.070 ± 0.008	B_{2C}, 17 ● 1
Fe_N–Tf–Co_C	7.4	50	k_{1N}, 0.064 ± 0.004	B_{1N}, 42 ± 2
Co_N–Tf–Fe_C	7.4	50	k_{1C}, 0.085 ± 0.005	B_{1C}, 26 ± 1
[TfR–(Fe_NTf)$_2$][a]	7.4	50	k'_{2N}, 0.063 ± 0.009	B'_{2N}, 52 ± 4
[TfR–(TfFe$_C$)$_2$][a]	7.4	50	k'_{2C}, 0.064 ± 0.007	B'_{2C}, 50 ± 1
[TfR–(Fe_NTfCo$_C$)$_2$]	7.4	50	k'_{1N}, 0.052 ± 0.006	B'_{1N}, 69 ± 1
[TfR–(Co_NTfFe$_C$)$_2$]	7.4	50	k'_{1C}, 0.042 ± 0.004	B'_{1C}, 66 ± 1
Fe_N–Tf[a]	5.6	5	k_{2N}, 0.91 ± 0.04	B_{2N}, 43 ± 1
Tf–Fe_C[a]	5.6	5	k_{2C}, 0.24 ± 0.04	B_{2C}, 49 ± 3
Fe_N–Tf–Co_C	5.6	5	k_{1N}, 1.95 ± 0.03	B_{1N}, 48 ± 1
Co_N–Tf–Fe_C	5.6	5	k_{1C}, 0.30 ± 0.04	B_{1C}, 42 ± 1
[TfR–(Fe_NTf)$_2$][a]	5.6	5	k'_{2N}, 0.94 ± 0.09	B'_{2N}, 41 ± 1
[TfR–(TfFe$_C$)$_2$][a]	5.6	5	k'_{2C}, 0.50 ± 0.06	B'_{2C}, 30 ± 1
[TfR–(Fe_NTfCo$_C$)$_2$]	5.6	5	k'_{1N}, 0.92 ± 0.01	B'_{1N}, 55 ± 1
[TfR–(Co_NTfFe$_C$)$_2$]	5.6	5	k'_{1C}, 1.22 ± 0.05	B'_{1C}, 41 ± 1

[a] The parameters corresponding to these transferrin species are derived in part from Bali and Aisen (1991).

Iron Removal from Mixed Metal Fe–Co Transferrins at Endosomal pH. The two sites exhibit a greater degree of kinetic heterogeneity for iron removal by 0.005 M PP_i at pH 5.6. Although the extent of the reaction is nearly the same at each site, initial release from the N-terminal site is more than 6 times faster than from the C-terminal site of doubly-occupied transferrin (compare k_{1N} = 1.95 min^{-1} to k_{1C} = 0.30 min^{-1} and B_{1N} = 48% to B_{1C} = 42%). Receptor binding at this pH, however, has opposite effects on the two sites. The rate of iron removal from the C-terminal site is accelerated 4 times (compare k_{1C} = 0.30 min^{-1} to k'_{1C} = 1.22 min^{-1}). In contrast, the rate of iron release from the N-terminal site in

receptor-bound transferrin is retarded to half of that for free transferrin (compare k_{1N} = 1.95 min^{-1} to k'_{1N} = 0.92 min^{-1}), while the extent of reaction is only slightly reduced, from 48% for free to 55% for receptor-bound transferrin.

We have previously observed acceleration of the rate of iron release (k_{2C} = 0.24 min^{-1} versus k'_{2C} = 0.50 min^{-1}) from C-terminal monoferric transferrin upon binding to the receptor at pH 5.6, but neither the rate (k_{2N} = 0.91 min^{-1} versus k'_{2N} = 0.94 min^{-1}) nor the extent of reaction from the N-terminal monoferric transferrin was affected appreciably upon receptor binding (Bali & Aisen, 1991). Perplexingly, receptor-bound N-terminal monoferric transferrin relinquished iron about 2 times faster than receptor-bound C-terminal transferrin (k'_{2N} = 0.94 min^{-1} versus k'_{2C} = 0.50 min^{-1}), but urea/PAGE analysis indicated that the C-terminal site is more labile at pH 5.6 when diferric transferrin is receptor-bound, while the N-terminal site is more labile when diferric transferrin is free (Bali & Aisen, 1991). The receptor-mediated inhibition of iron release from the N-terminal site of Fe–Co transferrin, observed in the present study, helps reconcile those apparently discordant results.

Discussion

Mixed-Metal Co(III)–Fe(III) Transferrins. Incorporation of substitutionally inert Co(III) in either site of transferrin affords a means of studying the rate of iron removal from one site when its partner site is occupied. Thus, it becomes possible to measure the k_1's of Scheme I for free and receptor-bound transferrin. We have earlier reported that receptor binding modulates the overall kinetics of iron release from diferric transferrin (Bali et al., 1991) and exerts a particularly marked effect on k_2 in C-terminal monoferric transferrin (Bali & Aisen, 1991). Now, we report the effects of receptor binding on site–site cooperativities in doubly-occupied transferrins, for which values of k_1 and k_2 are both needed.

Cooperative Interactions between the Two Sites of Transferrin. If the sites function indentically in iron release, each k_C should be the same as the corresponding k_N, which has long been known to be untrue (Princiotto & Zapolski, 1977; Baldwin & de Sousa, 1981). If the sites are noninteracting, then according to Scheme I, k_{1C} should be equal to k_{2C} and k_{1N} should be equal to k_{2N} (Table I); again, that is not true. Thus, the state of one site, whether vacant or occupied, affects the kinetics of iron release from the other site. This amounts to a cooperative effect in which the presence or absence of ferric ion in the cleft between the two domains of one transferrin lobe is communicated to the metal-binding region in the other lobe. Taking the cooperativity factor as the ratio of k_{1i} to k_{2i} (i = C or N), then values greater than unity would mean a positive cooperativity for the site with the greater value of k when the partner site is occupied (Aisen et al., 1978; Williams et al., 1982). A positive cooperativity, thus, is the acceleration in the rate of iron release from one site caused by occupancy of the other site by a metal ion. With the site-preference factor defined as the ratio of k_{jC} to k_{jN} (j = 1 or 2), a value other than unity implies more facile release from the site with the larger k.

Extracellular pH. For iron removal from free transferrins at pH 7.4, the cooperativity factor, k_{1C}/k_{2C}, is 1.2 at the C-terminal site, pointing to a slight positive cooperativity for that site. No cooperativity is observed at the N-terminal site, since k_{1N}/k_{2N} is unity. The site-preference factors, k_{1C}/k_{1N} and k_{2C}/k_{2N}, are 1.3 and 1.1, respectively. Thus, the C-terminal site is slightly preferred for iron removal at pH 7.4. A positive cooperativity at the C-terminal site and no cooperativity at the N-terminal site during iron removal from diferric transferrin by PP_i (Bali & Harris, 1989) and nitrilotris(methylenephosphonic acid) (Bali et al., 1991a) has previously been noted at pH 7.4. Receptor binding to the transferrins at pH 7.4 results in a negative cooperativity of 0.66 and 0.82 at C- and N-terminal sites, respectively (Table II), indicating that the receptor obstructs the loss of iron from both sites of transferrin, the effect being more marked at the C-terminal site.

Table II: Cooperativity and Site-Preference Factors for Iron Removal from Transferrin by PP_i at Extracellular and Endosomal pH

factor	pH 7.4	pH 5.6
Free Transferrin		
cooperativity		
k_{1C}/k_{2C}	1.2	1.3
k_{1N}/k_{2N}	1.0	2.1
site preference		
k_{1C}/k_{1N}	1.3	0.15
k_{2C}/k_{2N}	1.1	0.26
Receptor-Bound Transferrin		
cooperativity		
k'_{1C}/k'_{2C}	0.66	2.4
k'_{1N}/k'_{2N}	0.82	0.98
site preference		
k'_{1C}/k'_{1N}	0.81	1.3
k'_{2C}/k'_{2N}	1.0	0.53

Endosomal pH. At pH 5.6, the cooperativity factor (k_{1N}/k_{2N} = 2.1) is appreciable at the N-terminal site but only marginal at the C-terminal site (k_{1C}/k_{2C} = 1.3) for iron removal from free Fe–Co transferrins by 0.005 M PP_i. Thus, occupancy of the C-terminal site by a metal ion considerably enhances the initial rate of iron release from the N-terminal site, whereas loading of the N-terminal site exerts relatively little effect on the C-terminal site, the converse of what is found at pH 7.4. At pH 5.6, the site-preference factors k_{1C}/k_{1N} and k_{2C}/k_{2N} of 0.15 and 0.26 for loss of the first and second ferric ion, respectively, are substantially in favor of depletion of iron from the N-terminal site of free mixed-metal protein.

Receptor-Induced Switch in Site Lability. The receptor modulates the kinetics of iron removal from diferric transferrin at pH 5.6, the pH of the endosome, by switching cooperativity and site-preference factors. Receptor binding changes the positive cooperativity of 2.1 for the N-terminal site of free transferrin to virtual noncooperativity, k'_{1N}/k'_{2N} = 0.98, in receptor-bound transferrin. More strikingly, the slight positive cooperativity of 1.3 at the C-terminal site of free transferrin is changed to a substantial positive cooperativity, k'_{1C}/k'_{2C} = 2.4, in receptor-bound transferrin. This is accompanied by a change in site-preference factor (k_{1C}/k_{1N} = 0.15) substantially favoring depletion of the N-terminal site in free transferrin to one (k'_{1C}/k'_{1N} = 1.3) promoting iron release from the C-terminal site when transferrin is receptor-bound. Thus, the overall effect of binding of diferric transferrin to its receptor at pH 5.6 is stabilization of iron in the otherwise reactive N-terminal site and labilization of iron in the relatively stable C-terminal site.

Our earlier study, based on urea gel electrophoresis of diferric transferrin (Bali & Aisen, 1991), also showed faster release of iron from the stronger binding site of the C-terminal lobe. However, the effect of receptor appeared much more dramatic in that study than in the present investigations with mixed-metal transferrins. The electrophoresis studies were carried out in 0.002 M PP_i, while the present studies were done in 0.005 M PP_i; the difference in [PP_i] is necessitated by the time constraints of the two types of study. Observed rate constants for iron removal may not scale simply with [PP_i]

for the two sites (Bali & Harris, 1989), possibly accounting for the apparent difference in our two studies.

Whether our observations with detergent-solubilized receptor apply to events in the endosome is not yet clear, although the effects of receptor are evident when it is membrane-bound in the intact cell (Sipe & Murphy, 1991). The physiological benefit, if any, of favoring the acid-stable site over the acid-labile site as a source of iron for cellular needs also remains uncertain.

Singly- and Doubly-Occupied Transferrins. The effects of receptor binding on iron release are more marked upon doubly-occupied than upon singly-occupied transferrins, particularly at pH 5.6. Possibly, therefore, the binding of transferrin to its receptor is strengthened when both sites are filled and therefore presumably in the "closed" conformation (Anderson et al., 1990). Earlier studies, indicating that monoferric transferrins bind to surface receptors of the rabbit reticulocyte with affinities intermediate to those of apotransferrin and diferric transferrin (Young et al., 1984), are consistent with metal-induced conformational changes. Whether this sequence of binding affinities also is obtained in transferrin interactions with isolated receptor remains to be determined.

We have previously proposed that, at endosomal pH, binding of transferrin to its receptor opens the binding cleft in the C-terminal lobe, thereby facilitating release of iron from this lobe (Bali & Aisen, 1991). Now, it appears that the receptor also influences the conformational state of the N-terminal lobe, at least in the doubly-occupied protein, but in opposite fashion to that of the C-terminal lobe so that iron release is slowed.

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