Effect of a Covalently Attached Synergistic Anion on Chelator-Mediated Iron-Release from Ovotransferrin: Additional Evidence for Two Concurrent Pathways†

Christopher T. Bailey,* Cheryl Byrne, Kristi Chrispell, Catherine Molkenbur, Marcella Sackett, Katherine Reid, Kärin McCollum, Denise Vibbard, and Rose Catelli

Department of Biological and Chemical Sciences, Wells College, Aurora, New York 13026

Received June 14, 1996; Revised Manuscript Received November 20, 1996⊗

ABSTRACT: The mechanism by which the iron-transport protein transferrin releases its iron *in vivo* is presently unclear. *In vitro* studies have implicated two concurrent chelator-mediated iron-release pathways: one which is hyperbolic in nature, involving a conformational change in the protein as a rate limiting step, and a second which has been proposed to be first-order in nature and to involve initial release of a synergistic anion. We have examined the effect that an affinity-label analog of the synergistic anion has on chelator-mediated iron-release from this protein. A covalently attached anion would inhibit iron-release via any pathway in which anion release is a prerequisite to iron release. The present investigation examined the effect that the covalently attached anion had on iron-release to pyrophosphate (PP_i) and *N*,*N*-bis(phosphonomethyl)glycine (DPG), two chelators which are believed to utilize both pathways concurrently. Results show that when the affinity-label anion is utilized, strictly hyperbolic data are obtained, with similar observed k_{max} values. This is strong support for the hypothesis of a common, chelator-independent rate-limiting step for the one available pathway. These results also support strongly the hypothesis that synergistic anion removal is a prerequisite step to iron-release via the second pathway.

The transferrins are a class of iron-binding proteins (MW approximately 80 kDa) which include the ovo-, lacto-, and serum transferrins (Aisen, 1989; Harris & Aisen, 1989). X-ray structures for human lactoferrin (Anderson et al., 1987, 1990; Baker et al., 1990), rabbit serum transferrin (Bailey, S., et al., 1988; Sarra et al., 1990), and hen ovotransferrin (Dewan et al., 1993) have shown that the various proteins are compacted into two lobes, each of which binds tightly, but reversibly, a single iron. One of the unique features of the transferrins is that for iron to bind a suitable synergistic anion must also be present (Bates & Wernicke, 1971). Crystallographic results and EPR evidence (Dubach et al., 1991) have shown that the anion, which is strongly indicated to be carbonate, binds directly to the ferric ion in a bidendate fashion. In the absence of carbonate a limited number of other organic anions can also promote binding (Schlabach & Bates, 1975).

Because of the importance of the interaction of small, low molecular weight, iron-chelators with transferrin, much has been done to elicit the exact mechanism of iron release to these chelators. Early *in vitro* studies of iron-release from transferrin to certain chelators discerned a hyperbolic dependence of the rate of iron-release on the chelator concentration (Carrano & Raymond, 1979). This led to the proposal of a mechanism where the rate-limiting step involves a conformational change in the protein between "closed" and "open" forms (Cowart et al., 1982). Under this mechanism the maximal rate of reaction is dependent solely on the rate at which the protein "opens" and would therefore be independent of chelator concentration, [C]. This mechanism has been described by eq 1, where the maximum rate constant, k_{max}, is equal to k'/k''.

$$k_{obs} = \frac{k'[C]}{1 + k''[C]} \quad (1)$$

W. R. Harris et al. (1987; Bali et al., 1989, 1991) have shown that a plot of the observed rate constants for release of iron to pyrophosphate or certain other chelators does not exhibit strictly hyperbolic dependence on chelator concentration but includes a second, less steep, almost linear slope at higher concentrations. These researchers modified eq 1 to include a term which is first-order in chelator (eq 2) and used these results to propose that there are in fact two iron-release mechanisms operating in transferrin: the saturation pathway discussed above, and one that is first-order in chelator.

$$k_{obs} = \frac{k'[C]}{1 + k''[C]} + k'''[C] \quad (2)$$

Some chelators, for example AHA[1] (Cowart et al., 1982) and 3,4-LICAMS (Kretchmar & Raymond, 1986), which appear to exhibit strictly saturation kinetics were proposed by Harris to utilize only the conformational change pathway

† This work was supported by a William and Flora Hewett Foundation Grant of Research Corporation (C-2612). Partial support for instrumentation used in this work was provided by the National Science Foundation's Instrumentation and Laboratory Improvement Program through Grant No. USE-8851384.

* To whom correspondence should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1997.

[1] Abbreviations: AHA, acetohydroxamic acid; DPG, *N*,*N*-bis-(phosphonomethyl)glycine; HEPES, *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]; KISAB, kinetically significant anion binding site; 3,4-LICAMS, *N*,*N*′,*N*″-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane; MES, 2-(*N*-morpholino)ethanesulfonic acid; NTA, nitrilotriacetic acid; PP_i, pyrophosphate.

S0006-2960(96)01430-4 CCC: $14.00

($k''' = 0$). Others, such as NTA (Bali et al., 1991), which appear to exhibit strictly first-order kinetics, were proposed to utilize only the first-order pathway ($k' = k'' = 0$). PP_i (Cowart et al., 1986) and DPG (Harris et al., 1987) each require significant nonzero values for k', k'', and k''' in eq 2 to completely describe their kinetic behavior; these results were used to propose that these chelators utilize both the saturation and first-order pathways concurrently.

Kretchmar and Raymond (1988) have shown that binding of ion to the protein is an absolute prerequisite for iron release. Bertini et al. (1988), have hypothesized that the second term in eq 2 results from the interaction of the chelator, or some other nonchelating lyotropic anion, with positively charged allosteric sites, termed "kinetically significant anion binding sites" (KISAB) (Marques et al., 1990, 1991), which affect the thermodynamic and kinetic properties of the protein. The requirement of KISAB occupation by either salt or chelator is central to the mechanistic scheme presented by Egan et al. (1992).

One potential mechanistic difference between the two pathways has been put forth by Harris (1987), who suggested that substitution of the synergistic anion by the chelator might be involved in what he describes as the first-order pathway. If anion release is a prerequisite to iron-release via this pathway, but not via the hyperbolic pathway, then discrimination between the two pathways might be obtained by preventing loss of the synergistic anion.

In previous studies (Bailey, C. T., et al., 1988), hydroxypyruvate was shown to be effective as an affinity label analog for the synergistic anion in *ovo*transferrin. Hydroxypyruvate was shown to behave synergistically, mediating the uptake of iron, and also to react covalently with a *lysine* in the protein. Nadeau et al. (1996), have reported on the possible sites of this modification. The stability of the affinity labeled protein was such that the bound iron could be removed and then, in the absence of other potential synergistic anions (e.g., carbonate), successfully reconstituted into the protein; iron does not bind to ovotransferrin in the absence of a suitable anion. Control experiments showed that protein treated exactly like the affinity-label (including exposure to sodium borohydride), with the exception that carbonate and not a potential affinity-label was utilized, behaved exactly as regularly prepared carbonate-bound protein.

We report here the effect that a covalently attached synergistic anion has on the observed rates of chelator-mediated iron release from ovotransferrin to two chelators, DPG and PP_i, which have previously been described as utilizing both the saturative and first-order pathways concurrently.

MATERIALS AND METHODS

Preparation of Three Protein−Iron−Anion Complexes. Ovotransferrin was obtained from Sigma Chemical Company. Carbonate-bound diferric ovotransferrin was prepared according to established procedures (Schlabach & Bates, 1975), with final dialysis against several changes of HEPES buffer (10 mM HEPES, 0.15 M NaCl, pH 7.4) and ultimate dilution to approximately 50 μM. Ferrated ovotransferrin with hydroxypyruvate or pyruvic acid as the anion was prepared as follows: carbonate-free solutions of apo-ovotransferrin were prepared by dissolving the protein in MES buffer (30 mM MES and 0.15 M NaCl, pH 6.1), titrating to pH 4−4.5 with 0.1 M HCl, and allowing ascarite-filtered nitrogen to pass over the solution with gentle stirring for 1 h. After this degassing procedure, one part iron and an excess of four parts anion (both per binding site) were added. The pH was then raised to 6.5 by addition of aliquots of carbonate-free ammonia gas. In the case of the affinity-label, hydroxypyruvate, after reaction for approximately 1 h, the solution was titrated to pH 8 and an excess of sodium borohydride was added to reduce the imine bond of the Schiff base. Both protein preparations were dialyzed against several changes of HEPES buffer, titrated to pH 7.4, and finally diluted to approximately 50 μM with the buffer.

Chelators. The chelators utilized, *N*,*N*-bis(phosphonomethyl)glycine (DPG) and pyrophosphate (PP_i), are available commercially and all have been utilized elsewhere to mediate iron-release from serum transferrin. Bulk solutions were prepared by dissolving the respective solid in HEPES buffer, titrating the solution to pH 7.4, and then diluting to the required concentration.

Chelator-Mediated Iron Release Experiments. Kinetic experiments were performed in a constant temperature cuvette maintained at 25 °C by a circulating water bath. Appropriate aliquots of chelator and buffer were added to 1.000 mL of one of the three respective protein−iron−anion complexes at pH 7.4, and the progress of iron-release followed on a Perkin-Elmer Lambda 6 computer-controlled UV/vis spectrophotometer. The absorption data was collected approximately 15 s after mixing and then every 30 s for up to 600 min. The resulting data were fit to eq 3 using

$$A_t = ((A_o - A_\infty)/2)\,(e^{-k_a t} + e^{-k_b t}) + A_\infty \qquad (3)$$

the general nonlinear least-squares program NL-REGR (Ebert et al., 1989) and a four-parameter (A_o, A_∞, k_a, k_b) fitting procedure. A_o, A_∞, and A_t are the absorbances at the observation wavelength (460 nm) at time zero, infinity, and the data acquisition time, respectively. Because only the early portions of the reaction were examined, the value of $k_{obs} = (k_a + k_b)/2$. The data obtained in investigating the effect of chelator concentration on k_{obs} was fit to the appropriate equations (*vide infra*) also utilizing NL-REGR.

RESULTS

The ability of the chelators DPG and PP_i to mediate iron release from the three protein−iron−anion complexes was initially examined at single chelator concentrations. Identical parallel iron-release experiments were performed using ovotransferrin with carbonate (the native anion), hydroxypyruvate (the affinity label), and pyruvic acid as the synergistic anion. Pyruvic acid, which is structurally quite similar to the affinity-label anion, behaves as a synergistic anion, but does *not* react covalently with the protein (Bailey, C. T., et al., 1988). It was found that each of the chelators successfully mediated iron release from both carbonate and pyruvic acid bound ovotransferrin. This would indicate that simply replacing the carbonate with an alternate synergistic anion (i.e., pyruvic acid) did not affect the ability of these chelators to mediate iron-release.

Iron-release studies were performed with DPG and PP_i over a range of concentrations against both the carbonate-bound and the affinity-labeled proteins. Plots of k_{obs} vs

Table 1: Kinetic Constants for Iron Removal by Two Chelators from Affinity-Labeled and Carbonate-Bound Ovotransferrin[a]

	affinity-labeled ovotransferrin		carbonate-bound ovotransferrin	
	PP_i	DPG	PP_i	DPG
k' (min^{-1} M^{-1})	3.49 ± 0.15	1.29 ± 0.035	2.73 ± 0.14	2.95 ± 0.15
k'' (M^{-1})	158 ± 8.5	55.0 ± 1.8	136 ± 11	315 ± 9.1
k''' (min^{-1} M^{-1})			0.962 ± 0.027	0.0762 ± 0.00063

[a] Constants correspond to those in eq 2.

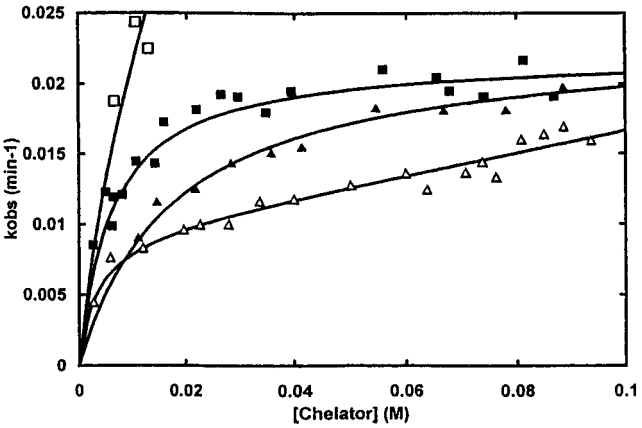

FIGURE 1: Comparisons of the pseudo-first-order rate constants for the removal of ferric ion from carbonate-bound ovotransferrin (open symbols) and affinity-labeled ovotransferrin (filled symbols) by PP_i (□, ■) and DPG (△, ▲). Both proteins were in 10 mM HEPES, pH 7.4; all experiments were performed at 25 °C. Traces are calculated fits based on eq 2 and the parameters listed in Table 1.

concentration for the release of iron from the native protein to the two respective chelators were quite similar to previously published plots utilizing human serum transferrin [see, e.g., Harris et al. (1987) and Egan et al. (1992)]. These plots exhibited predominately hyperbolic characteristics at low ligand concentration, with apparently first-order characteristics predominating at higher concentrations. When similar chelator-mediated iron-release studies were performed with the affinity-label protein, very different results were obtained (Figure 1). For both chelators, strictly hyperbolic data was obtained. Although the traces for the two chelators are different, their calculated k_{max} values are very similar.

DISCUSSION

Harris has proposed a two-pathway mechanism (exhibiting hyperbolic and first-order kinetics, respectively) for chelator-mediated iron-release from transferrin and has described this mechanism with eq 2. The first term in this equation would describe iron-release via the hyperbolic pathway, the second term via the first-order pathway. Harris also suggested that one potential mechanistic difference between the two pathways is the point at which the protein's synergistic anion is released. A covalently-attached synergistic anion could not be removed from the protein and should, therefore, prohibit utilization of any pathway in which anion release is a prerequisite to iron release. Quantitative studies of chelator-mediated iron-release from affinity-labeled ovotransferrin utilizing DPG and PP_i, two chelators which have been proposed to utilize both pathways concurrently, exhibited purely hyperbolic kinetics. These results have led us to conclude that the covalently attached affinity-label does not affect the ability of chelators to mediate iron-release via the saturative (hyperbolic) pathway, but does prohibit utilization of the first-order pathway. These results give strong support for the hypothesis that synergistic anion removal is a prerequisite step to iron release via the first-order pathway. Bertini (1988) has suggested that the second term of eq 2 results from the interaction of the iron-chelator with the KISAB to affect the thermodynamic and kinetic properties of the protein. Our results may imply that the purpose of the chelator's presence in the KISAB is to dislodge the synergistic anion—and possibly replace it—prior to iron-release. Such an action would be prevented by a covalently attached anion.

Nonlinear least-squares analysis of the respective kinetic data by the first term of eq 2 for iron-release from the affinity-labeled protein to DPG and PP_i yielded the values for k' and k'' as shown in Table 1 and the corresponding two traces (solid symbols) in Figure 1. Both plots yield very similar k_{max} values, 0.0234 and 0.0220 min^{-1}, for DPG and PP_i, respectively. These data are consistent with a single common, chelator independent, rate-limiting step for the saturation pathway.

There are potential problems when eq 2 is utilized to describe chelator-mediated iron-release from the native protein, including inconsistent maximum rate constants obtained for the saturation pathway. It would be expected that these k_{max} values, which are limited by the conformational change in the protein, should be consistent regardless of the chelator utilized. Even for those chelators which are proposed to utilize both pathways, the maximum rate of release *by the saturation pathway* should still be limited by the conformational change in the protein regardless of the fact that another pathway is available. However, reported values for a variety of chelators (Harris et al., 1987) vary over an order of magnitude. Our results indicate that when only the hyperbolic pathway is available consistent k_{max} values can be obtained. Because the *values* of k_{max} and k_m (expressed as k'/k'' and $1/k''$, respectively, in eq 2) obtained with the affinity-labeled protein could presumably be considered intrinsic properties of the hyperbolic pathway itself, these values might be expected to remain constant even when both pathways are available (i.e., in the absence of the affinity-label). Thus, nonlinear least-squares analysis of the respective data for DPG and PP_i-mediated iron-release from carbonate-bound ovotransferrin was performed utilizing eq 2 with the respective values of k' and k'' (as obtained in the affinity-labeled experiments above) defined as *constants*. In the case of PP_i only a fair fit for k'' could be obtained, and in the case of DPG a value for k''' could NOT be obtained. Further analysis of the kinetic data for iron-release from native ovotransferrin was performed with eq 2 but allowing for the calculation of all three kinetic constants. This analysis yielded the values for k', k'', and k''' as shown in Table 1 and the respective two traces (open symbols) in Figure 1. The k_{max} values for DPG and PP_i determined by this method (0.00930 and 0.0200 min^{-1}, respectively) are not uniform but are similar to those previously presented by Harris (1987)

to describe iron-release by these chelators from human serum transferrin.

We see two ways in which these results could be interpreted. First, the presence of the affinity label may, in addition to inhibiting totally the first-order pathway, also change the kinetics of the available hyperbolic pathway. That alteration of the protein should lead to modification of the kinetic behavior of both pathways would not be beyond expectation. The fact that the k_{max} values obtained with the affinity-labeled protein are consistent may be only coincidental. Second, the k_{max} values for the hyperbolic may not be significantly affected by the affinity label. Such an interpretation would lead to the conclusion that eq 2 does not sufficiently describe the mechanism of iron-release from transferrin. Equation 2 implies an additive relationship between the two pathways, and although this might be evidenced by the results with PP_i, it is certainly not supported by the DPG data. For this chelator, the observed rate of iron-release is faster when only the hyperbolic pathway is utilized than when both pathways are available. The implication of this second interpretation would be the existence of an inhibitory relationship between the two pathways.

We believe that the affinity-labeled synergistic anion provides us with a unique tool for examining the mechanism of chelator-mediated iron-release from transferrin. Our results utilizing PP_i and DPG provide additional evidence for the presence of two concurrent pathways for iron release. One of these pathways is hyperbolic in nature and, when it is the only pathway available, has a maximum rate of iron-release which appears to be independent of chelator utilized. This would be consistent with a pathway involving a conformational change in the protein as a rate-limiting step. An alternate second pathway involves release of the protein's synergistic anion as a prerequisite to iron release.

ACKNOWLEDGMENT

The authors thank Dr. Wesley Harris, University of Missouri, Saint Louis, MO, for many useful suggestions concerning this manuscript.

REFERENCES

Aisen, P. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., Ed.) pp 353−371, VCH Publishers, New York.

Anderson, B. F., Baker, H. M., Dodson, E. J., Norris, G. E., Rumball, S. V., Waters, J. M., & Baker, E. N. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1769−1773.

Anderson, B. F., Baker, H. M., Norris, G. E., Rumball, S. V., & Baker, E. N. (1990) *Nature 344*, 784−787.

Bailey, C. T., Patch, M. G., & Carrano, C. J. (1988) *Biochemistry 27*, 6276−6282.

Bailey, S., Evans, R. W., Garratt, R. C., Gorinsky, B., Hasnain, S., Horsburgh, C., Jhoti, H., Lindley, P. F., Mydin, A., Sarra, R., & Watson, J. L. (1988) *Biochemistry 27*, 5804−5812.

Baker, E., Anderson, B. F., Baker, H. M., Haridas, M., Norris, G. E., Rumball, S. V., & Smith, C. A. (1990) *Pure Appl. Chem. 62*, 1067−1070.

Bali, P. K., & Harris, W. R. (1989) *J. Am. Chem. Soc. 111*, 44576−4461.

Bali, P. K., Harris, W. R., & Nesset-Tollfson, D. (1991) *Inorg. Chem. 30*, 502−508.

Bates, G. W., & Wernicke, J. (1971) *J. Biol. Chem. 246*, 3679−3685.

Bertini, I., Hirose, J., Luchinat, C., Messori, L., Piccioli, M., & Scozzafava, A. (1988) *Inorg. Chem. 27*, 2405−2409.

Carrano, C. J., & Raymond, K. N. (1979) *J. Am. Chem. Soc. 101*, 5401−5404.

Cowart, R. E., Kojima, N., & Bates, G. W. (1982) *J. Biol. Chem. 257*, 7560−7565.

Cowart, R. E., Swope, S., Loh, T. T., Chasteen, N. D., & Bates, G. W. (1986) *J. Biol. Chem. 261*, 4607−4614.

Dewan, J. C., Mikami, B., Hirose, M., & Sacchettini, J. C. (1993) *Biochemistry 32*, 11963−11968.

Dubach, J., Gaffney, B. G., More, K., Eaton, G. R., & Eaton, S. S. (1991) *Biophys. J. 59*, 1091−1100.

Ebert, K., Ederer, H., & Isenhour, T. L. (1989) in *Computer Applications in Chemistry*, pp 360−365, VCH Publishers, New York.

Egan, T. J., Ross, D. C., Purves, L. R., & Adams, P. A. (1992) *Inorg. Chem. 31*, 1994−1998.

Harris, D. C., & Aisen, P. (1989) in *Iron Carriers and Iron Proteins* (Loehr, T. M., Ed.) pp 241−351, VCH Publishers, New York.

Harris, W. R., Rezvani, A. B., & Bali, P. K. (1987) *Inorg. Chem. 26*, 2711−2716.

Kretchmar, S. A., & Raymond, K. N. (1986) *J. Am. Chem. Soc. 108*, 6212−6218.

Kretchmar, S. A., & Raymond, K. N. (1988) *Inorg. Chem. 27*, 1436−1441.

Marques, H. M., Egan, T. J., & Pattrick, G. (1990) *S. Afr. J. Sci. 86*, 21−24.

Marques, H. M., Watson, D. L., & Egan, T. J. (1991) *Inorg. Chem. 30*, 3758−3762.

Nadeau, O. W., Falick, A. M., & Woodworth, R. C. (1996) *Biochemistry 35*, 14294−14303.

Sarra, R., Garratt, R., Gorinsky, B., Jhoti, H., & Lindley, P. (1990) *Acta Crystallogr. B46*, 763−771.

Schlabach, M. R., & Bates, G. W. (1975) *J. Biol. Chem. 250*, 2182−2188.

BI961430Y