A STUDY OF THE ANION BINDING SITE OF TRANSFERRIN

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1. Introduction

Studies of the interaction between carbonate and iron binding by transferrin have centered about attempts to form a specific Fe^{3+}–transferrin complex in the absence of carbonate and carbonate substitutes. Despite intensive experimental effort in our laboratory and others, such a complex has not been formed and verified [1–5]. A complicating factor is the propensity of Fe^{3+} to polymerize and bind in a non-specific fashion to the transferrin molecule [6], however, the continuing negative results clearly indicate that in the absence of suitable anions, transferrin has a low affinity for Fe^{3+}. Another unsolved question is the form of the anion, i.e. carbonate or bicarbonate. We favor carbonate on the basis of potentiometric data [5] and the assumption that the tight binding of the anion is achieved via strong electron withdrawal. This would lead to a decrease of the second acid pK through inductive effects and consequent release of the proton in question.

The nature of the anion binding may be approached through a study of the stability, exchange rates, and physical parameters of Fe^{3+}–transferrin–anion complexes [1, 3]. We report here the preparation of an Fe^{3+}–transferrin–$^{14}CO_3$ complex, free of unbound radioactive carbonate. The exchange of carbonate at the anion binding site follows first order kinetics with a half life of one week. Nitrilotriacetate (NTA) may act as a carbonate substitute [7] and the stability of Fe^{3+}–transferrin–NTA has been shown to be much lower than that of the physiological complex. The results are correlated with the transferrin literature and a new model for the binding of carbonate and iron to transferrin is proposed.

2. Experimental and results

2.1. The preparation of Fe^{3+}–transferrin–$^{14}CO_3$

As an initial step in the study of the exchange rates of anions of Fe^{3+}–transferrin–anion complexes, we examined the behavior of Fe^{3+}–transferrin–$^{14}CO_3$ on gel filtration columns. To a solution of apotransferrin

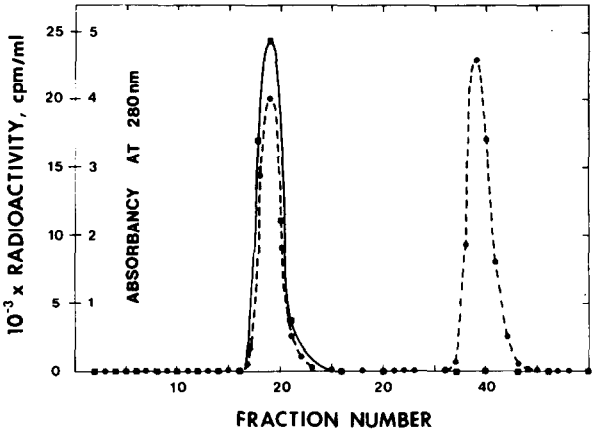

Fig. 1. The elution profile from a Sephadex G-25 column of Fe^{3+}–transferrin–$^{14}CO_3$ prepared by the addition of Fe^{3+}–NTA to apotransferrin in the presence of $NaH^{14}CO_3$. A 2.0 ml aliquot of 3×10^{-4} N apotransferrin was fully saturated with Fe^{3+}–NTA shortly after the addition of 0.5 μCi of $NaH^{14}CO_3$. The preparation was made 0.1 M in $NaClO_4$, allowed to stand 30 min, and then applied to a 2 × 35 cm Sephadex G-25 column equilibrated and eluted with 5 mM Tris-HCl and 20 mM NaCl buffer. Fractions of 2.5 ml were collected and analyzed for absorbancy at 280 nm (——) and radioactivity (– – –). The close correlation of ^{14}C activity with the protein peak is indicative of a tight binding of carbonate in the Fe^{3+}–transferrin–CO_3 complex.

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was added $NaH^{14}CO_3$ (New England Nuclear), followed quickly by a saturating amount of Fe^{3+}–NTA (nitrilotriacetic acid). The solution was made 0.1 M in $NaClO_4$, allowed to stand 30 min, and then applied to a Sephadex G-25 gel filtration column (details in legend). The column was eluted and the fractions monitored for absorbance at 280 nm, and radioactivity using Aquasol solubilizer (New England Nuclear). In fig. 1 is shown the elution profile in terms of these two parameters. It will be seen that a fraction of the labeled carbonate has become firmly attached to the protein and can be initally separated from unbound $NaH^{14}CO_3$. Radioactive bicarbonate added to apotransferrin does not elute with the protein fraction, indicating that all of the tight carbonate binding is linked to iron binding.

If complete freedom from chelating agents is required, it is necessary to make the Fe^{3+}–transferrin–CO_3 complex 10 mM in $NaHCO_3$ and pass the preparation through a gel filtration column equilibrated with 0.1 M $NaClO_4$ [4, 6]. The high bicarbonate and salt concentration appear to destabilize the anion binding site somewhat, and a lower yield of labeled complex is obtained.

2.2. *Carbonate exchange rates*

The rate of exchange of the carbonate of Fe^{3+}–transferrin–$^{14}CO_3$ was examined by dialysis against air equilibrated buffer in closed equilibtrium dialysis cells. A fresh Fe^{3+}–transferrin$^{14}CO_3$ preparation in air equilibrated buffer was placed on one side of the dialysis membrane and buffer in the other compartment. The cells were shaken continuously and aliquots taken for liquid scintillation counting at about 20 hr intervals. The amount of radioactivity bound to the protein was determined by subtracting the radioactivity in the buffer aliquot from that in the aliquot from the protein compartment. Since the dialysis of the unbound $NaHCO_3$ is rapid compared to the 20 hr interval, and since a large reserve of CO_2 is present in the solutions, the technique should be reasonably accurate. The results are plotted in fig. 2 in first order form. The exchange of carbonate follows first order kinetics with a half life of about one week. This value may vary with conditions.

As a control $NaHCO_3$ was added to apotransferrin and the dialysis experiment run in the same fashion.

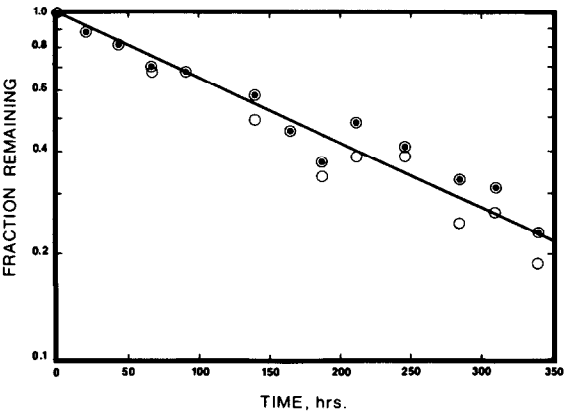

Fig. 2. A first order presentation of the exchange of the radioactive label of Fe^{3+}–transferrin–$^{14}CO_3$ with the unlabeled CO_2 of air equilibrated buffer (5 mM Tris-HCl, 20 mM NaCl at pH 7.45). The exchange rate was studied in dialysis cells as described in the text. The symbols represent two experiments.

A rapid equilibration of the $NaH^{14}CO_3$ occurred and little, if any, evidence was obtained for the binding of carbonate by apotransferrin. It is apparent that carbonate is bound only weakly to apotransferrin, and that the very tight binding of the carbonate of Fe^{3+}–transferrin–CO_3 is almost entirely linked to the affinity of the protein for the metal.

2.3. *A comparison of the affinity of Fe^{3+}–transferrin–CO_3 and Fe^{3+}–transferrin–NTA for iron*

Ideally, the interaction between carbonate and iron binding would be examined by determining the affinity of the protein for iron in the presence and absence of CO_2. Direct competition between transferrin and chelating agents in the absence of CO_2 or substitutes has been complicated so far by the tendency of iron complexes to polymerize at neutral pH and the lack of suitable spectrophotometric properties assignable to a CO_2-free Fe^{3+}–transferrin complex. We have chosen, therefore, to examine the iron binding affinity of transferrin with carbonate substitutes at the anion binding site. Fe^{3+}–transferrin–NTA was prepared as described previously and verified by the spectral change that occurs upon the addition of $NaHCO_3$ [7]. A solution of CO_2-free citrate was prepared and adjusted to pH 7.45 by passing a stream of ammonia over the surface. To a 1 ml aliquot of Fe^{3+}–trans-

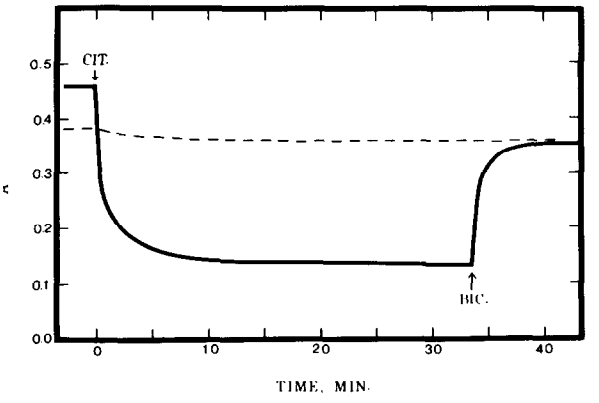

Fig. 3. A spectrophotometric study of the reaction of 10^{-2} M citrate with Fe^{3+}–transferrin–CO_3 at 1.5×10^{-4} N under CO_2-free conditions (——). The reaction was carried out in capped cells containing a nitrogen atmosphere and was monitored using a Cary Model 15 spectrophotometer. The citrate was added at time zero as designated by the "CIT" on the drawing. After the reaction had neared completion, a small amount of solid $NaHCO_3$ was added, at the point designated "BIC". As a control a Fe^{3+}–transferrin–NTA aliquot was equilibrated 36 hr with atmospheric CO_2 and then reacted with citrate (– – –). The final pH of all reaction mixtures was between 7.2 and 7.5. Not that the molar absorptivity of the Fe^{3+}–transferrin–NTA complex is higher (3.0×10^3 ℓ eq^{-1} cm^{-1}) than that of the cabonate complex (2.5×10^3 ℓ eq^{-1} cm^{-1}).

ferrin–NTA was added 0.1 ml of the 0.1 M citrate solution under a nitrogen atmosphere. The reaction was followed at 470 nm and is presented in fig. 3. There is a rapid drop in absorbance which reaches equilibrium after about an half hr. We ascribe this absorbance change to a removal of Fe^{3+} from the protein. Parallel CO_2-free ultrafiltration experiments were not quantitative (due to polymerization of ferric citrate) but indicated that the iron had been released by transferrin. Upon the addition of $NaHCO_3$ the 470 nm peak is regenerated and ultrafilterable iron is greatly diminished. The transferrin has regained the iron and the stronger Fe^{3+}–transferrin–CO_3 complex has formed. Note the difference in the molar absorptivity of the two Fe^{3+}–transferrin–anion complexes as described in the legend.

As a control experiment, aliquots of Fe^{3+}–transferrin–NTA were first equilibrated with atmospheric CO_2 and then reacted with citrate. This reaction is shown as the dashed line of fig. 3. The citrate removed ten times the amount of iron from the Fe^{3+}–transferrin–NTA complex as from the Fe^{3+}–transferrin–CO_3 complex, indicating a significant decrease in affinity of transferrin for iron when NTA occupies the anion binding site.

3. Discussion

The results presented here emphasize a very strong binding of carbonate to iron transferrin, a very weak binding of carbonate to apotransferrin, and the consequential strong interaction between the carbonate and iron of Fe^{3+}–transferrin–CO_3. Three models must be considered for the structural relationship of carbonate and iron binding by transferrin. These are analogous to the enzyme, metal, substrate models discussed in detail by Mildvan [8], and include carbonate binding to: i) only the metal; ii) only the protein; and iii) both the metal and the protein. Aisen et al. [3] have presented a convincing argument against model 1, above, and suggest that some direct interaction of carbonate with the protein is operative. The data presented here indicating an exchange rate on the order of a week is in full agreement with their arguments.

We have searched for possible schemes that would allow for both a very high stabilization of iron binding by carbonate, and an unstable binding site in the absence of carbonate. In fig. 4 is presented a hypothetical structure that has some attractive features. The scheme depicts a carbonate anion interacting with positive charges on the protein via two of its oxygens. The third oxygen is coordinated to the iron. Resonance

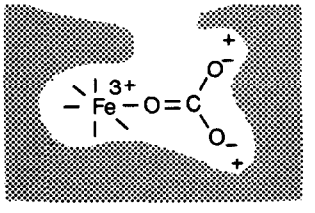

Fig. 4. A hypothetical scheme for the nature of carbonate binding in Fe^{3+}–transferrin–CO_3^{2-}. The carbonate is shown as an intrinsic portion of the metal binding site. The scheme is based on the observed strong interaction between iron and carbonate binding, and is consistent with a variety of experimental observations. Further explanation is given in the text.

stabilization of the carbonate would, of course, be expected. In the absence of the carbonate, charge repulsion between the ferric ion and the positive charges on the protein would be anticipated. The binding would be further destabilized by the loss of one of the iron ligands. The net result would be a far less stable Fe^{3+} coordination site.

Using space filling models we have constructed such a site with tyrosyl and histidyl ligands directly bound to the iron, and carbonate bound simultaneously to two lysyl residues and the iron. The site is quite free from steric hinderance when carbonate or oxalate are used as the anions. However, NTA is bulkier and causes some strain in the site.

While this scheme is highly speculative, it is presented here since it is amenable to experimentation and it is in agreement with a large number of observations. These observations include: i) the demonstrated high degree of interaction between carbonate and iron binding by transferrin; ii) the very slow exchange rate of bound carbonate; iii) the fact that all carbonate substitutes contain at least three oxygens and are capable of carrying two negative charges; iv) the observation that the reactivity of apotransferrin depends on carbonate concentration [7, 9, 10] and the suggestion, therefore, that carbonate binding preceeds Fe^{2+} [10, 11] and Fe^{3+} binding; v) the lack of a demonstrable, stable CO_2-free Fe^{3+}–transferrin complex; and vi) the observation by Aasa and Aisen [12] with electron paramagnetic resonance techniques that 2 or 3 additional nitrogen nuclei appear in the binding site of Cu^{2+}–transferrin upon the removal of carbonate.

Acknowledgements

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