Calorimetric Studies of the Binding of Ferric Ions to Human Serum Transferrin†

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ABSTRACT: The binding of ferric ions, chelated with nitrilotriacetate, to human serum transferrin (hTF) has been studied using ultrasensitive titration calorimetry. Studies were done in both the presence and the absence of the synergistic bicarbonate anion. It was found that the C-site of hTF is capable of weakly binding bicarbonate (K of 250 M^{-1}, ΔH of −8 kcal) at the binding site even before ferric ion is added, although this does not happen to the same extent at the N-site. When preinsertion of the bicarbonate ion occurs, then ferric ion can subsequently bind very quickly to the C-site. Although the chelated ferric ion can bind weakly to the N-site in a fast reaction, the insertion of the bicarbonate ion occurs subsequently in a slow endothermic reaction. Binding of ferric ion to both sites is quickly reversible in the absence of bicarbonate but becomes kinetically controlled for long periods of time once bicarbonate has inserted into the metal-binding site due to the long time required for release of ferric ion. Estimates of the heats of binding to each site, apparent binding constants, and heat capacities of binding are made for different sets of solution conditions. Results from this study are compared to earlier results with ovotransferrin (Lin, L.-N., Mason, A. B., Woodworth, R. C., & Brandts, J. F. (1991) *Biochemistry 30*, 11660–11669), with major differences and some similarities noted.

The transferrins are a group of proteins with the property of strongly binding metal ions and synergistic anions (Harris & Aisen, 1989; Chasteen & Woodworth, 1990). Extensive studies on human serum transferrin (hTF)[1] and hen egg white ovotransferrin (OTF) have shown many similarities in their structure and function. Both are glycoproteins with a single polypeptide chain of molecular mass 80 kDa. There is a 50% homology in amino acid sequence between the two proteins (Jeltsch & Chambon, 1982; Williams et al., 1982; MacGillivray et al., 1983). X-ray crystallographic studies (Bailey et al., 1988; Anderson et al., 1989, 1990; Baker et al., 1991) indicate that both proteins are composed of two homologous lobes (domains) of about equal size, connected by a short bridging peptide. There is ca. 40% homology between the N-terminal and C-terminal lobes in both proteins. Each lobe consists of two dissimilar subdomains, with the binding site located in a deep cleft at the interface between the two subdomains. In all transferrins for which X-ray data are available the ferric ion is octahedrally coordinated to two tyrosines, one histidine, one aspartic acid, and two oxygens from the synergistic carbonate anion (Anderson et al., 1989; Shongwe et al., 1992).

We have recently employed isothermal titration calorimetry for investigating the binding of ferric ion to the N- and C-sites of OTF and for estimating the energetics of domain–domain interactions and their effect on the binding of ferric ions (Lin et al., 1991). The binding of ferric ion (in the presence of the chelator NTA) occurs in two kinetic steps at both sites in OTF. Very quickly after injection, the ferric ion with attached chelator (Fe–NTA) binds weakly and reversibly by a process we call *contact binding*. In a much slower process occurring in the time range of minutes, bicarbonate ions insert into the binding site, replacing the chelator NTA. Once bicarbonate insertion is complete, binding to either site becomes considerably stronger, and subsequent dissociation of the ferric ion requires many days. The results showed that the energetics and kinetics for binding ferric ion to the N-site are quite different from those for binding to the C-site, despite the great similarity in structure between the two lobes. The strong preference for the first ferric ion to bind to the N-site of OTF is mainly due to the N-site's stronger contact binding and the faster bicarbonate insertion and probably has nothing to do with the true thermodynamics of binding, which require a longer time to achieve. In fact, DSC data (to be published) suggest that the N-site may have a substantially smaller binding constant than the C-site even though it is the first to saturate.

In view of the close similarity in their composition and structure, it is of interest to investigate ferric ion binding to hTF for comparison to OTF. In this study, we report on calorimetric titrations of apo-hTF and its recombinant N-terminal half-molecule with Fe–NTA, which were carried out in a manner similar to the earlier studies on OTF. The results show that the thermodynamics and kinetics of binding are quite different for the two sites of hTF and are also different from those for the corresponding sites in OTF. We believe that the overwhelming preference of Fe–NTA to bind first to the C-site of apo-hTF arises in large part because there is a strong tendency for bicarbonate anion to preinsert at the C-site of apo-hTF before addition of Fe–NTA, thereby making it faster kinetically for the C-site to achieve its final state once the Fe–NTA is added. Even when the bicarbonate concentration is too low for preinsertion, it inserts quite quickly subsequent to Fe–NTA binding. The insertion of bicarbonate at the N-site occurs predominantly after ion binding, and the insertion process is much slower.

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[1] Abbreviations: hTF, human serum transferrin; hTF/2N, recombinant N-terminal half-molecule of human transferrin, ending at Asp 337; NTA, nitrilotriacetate; Fe–NTA, solution consisting of Fe^{3+} and NTA in a 1:2 molar ratio; OTF, hen ovotransferrin.

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MATERIALS AND METHODS

Materials. Human serum transferrin (hTF) used in this study was obtained from Sigma Chemical Co. (stated purity 98%), or Calbiochem Co. (stated purity 100%) or was prepared essentially as described earlier (Penhallow et al., 1986) except that the sample was equilibrated with 5 mM Tris-HCl, pH 8.0, and 20 mM $NaHCO_3$, loaded onto a DEAE-Sepharose column (Pharmacia), and eluted with 20 mM Tris-HCl, pH 8.0. It was found that the Sigma sample had to be dialyzed extensively against 100 mM sodium perchlorate (at 4 °C) before use to avoid occurrence of spurious heat signals during titration. The C-site-saturated, monoferric hTF (Fe_c–hTF, with bicarbonate bound as the synergistic anion) was prepared according to the method described by Thompson et al. (1986). The recombinant N-terminal half-molecule of hTF, hTF/2N, was obtained by expression in baby hamster kidney cells and purified to homogeneity (Funk et al., 1990; Mason et al. 1991). The concentrations for apo-hTF and apo-hTF/2N were determined spectrophotometrically at 278 nm using extinction coefficients of 93 000 M^{-1} cm^{-1} (Chasteen, 1977) for hTF and 40 000 M^{-1} cm^{-1} for hTF/2N (Lin et al., 1993). The concentration of Fe_c–hTF was determined spectrophotometrically at 465 nm using an extinction coefficient of 2500 M^{-1} cm^{-1} (Thompson et al., 1986). The preparation of Fe–NTA (in a 1:2 molar ratio) has been described previously (Lin et al., 1991). All other chemicals were reagent grade.

Titration Calorimetry. (*a*) *Fe–NTA Binding Experiments.* The titrations of apo-hTF and its N-fragment with Fe–NTA were carried out on a MicroCal OMEGA ultrasensitive titration calorimeter (MicroCal Inc., Northampton, MA). A Windows-based software package (Origin), also supplied by MicroCal Inc., was used for data analysis and plotting. The instrumentation, experimental procedures, and data analysis for the titration calorimeter have been described previously (Wiseman et al., 1989; Lin et al., 1991). The titration experiments were performed in either 100 mM HEPES or 100 mM Tris-HCl, with or without bicarbonate.

For titrations in the absence of bicarbonate, the following procedures were used in preparing solutions and in carrying out experiments to minimize CO_2 contamination. First, acidic deionized water (pH 3.8, HCl only) and preweighed amounts of solid reagents (acidic and basic forms of HEPES, apo-hTF, ferric chloride, and NTA) were placed in a desiccator containing soda lime and ascarite. The desiccator was evacuated using a vacuum pump for 30 min while the water was stirred. The evacuated desiccator, the titration injector syringe, the filling syringe, the Parafilm, and the various pipettes and containers were placed into a glovebag, which was then inflated and purged repeatedly with purified N_2 for 1 h. In this N_2 atmosphere, the desiccator was opened and sufficient acidic water was mixed with appropriate amounts of the HEPES reagents to prepare 0.1 M HEPES buffer, pH 7.5. Aliquots of the buffer were then mixed with appropriate weights of the other solids to prepare the apo-hTF and Fe–NTA solutions which were to be studied in the titration calorimeter. The latter two solutions were loaded into the filling syringe and the injection syringe, respectively, and the tips of the syringes were sealed with Parafilm. After the reaction cell of the calorimeter was exhaustively purged with N_2, the filling syringe was removed from the glovebag, and the apo-hTF solution entered quickly into the cell from the long-needled syringe. Since the calorimeter cell is lollipop-shaped and totally filled (i.e., no air spaces), atmospheric CO_2 must diffuse through about 4 in. of solution in the small-diameter tube before reaching the solution in the active cell volume. Finally, the injection syringe was removed from the glovebag, and its long needle was inserted into the reaction cell and finally seated into the stirring cradle.

Control experiments were carried out in order to quantitate the extent of CO_2 contamination, taking advantage of the fact that ferric ion binding to apo-hTF in the presence of synergistic anions (i.e., either bicarbonate or NTA) results in a pink color due to an absorbance band at 465 nm, while no absorbance occurs when ferric ion binds to apo-hTF in the absence of synergistic anions. The control experiments were prepared and carried out in exactly the same way as the real experiment except the syringe was filled with an acidic $FeCl_3$ solution rather than the Fe–NTA solution. When the normal sequence of injections had been completed at the end of the control experiment, the Fe–apo-hTF solution was quickly removed from the reaction cell and transferred to a cuvette under N_2 purging, and the absorbance was measured at 465 nm. A second control experiment was done which repeated the first exactly, except the apo-hTF solution was allowed to equilibrate first with atmospheric CO_2 before it was loaded into the reaction cell. From the relative absorbance readings obtained in these two control experiments, it was estimated that less than 5% of the protein sites had bound bicarbonate ion when the above procedures were carried out to prevent atmospheric contamination. In addition, these control experiments showed that under CO_2-free conditions all of the heat change resulting from an injection (which includes both heat of binding and heat of neutralization) occurred in a single fast exothermic phase, while an additional slow exothermic phase, temporally correlated with the slow development of color, was seen for the CO_2-equilibrated sample.

(*b*) *Bicarbonate Binding Experiment.* It was found during the course of this investigation that bicarbonate ion can bind, albeit weakly, to apo-hTF in the absence of ferric ion. The normal titration procedure (i.e., apo-hTF in the cell titrated with bicarbonate in the syringe) could not detect this weak binding process, so a different procedure was used. Bicarbonate solutions of various concentrations (0–50 mM in 100 mM HEPES, pH 7.5) in the reaction cell were titrated with an apo-hTF solution (0.3 mM in the same buffer, 10 μL per injection). The heat changes observed at 0 bicarbonate concentration were used as the control and were subtracted from the heat changes found for injection into bicarbonate solutions of various concentrations.

Since it appeared from other findings (discussed in the Results section) that bicarbonate was binding primarily or exclusively to the C-site of apo-hTF in the absence of ferric ion, the possibility of binding to the N-site was tested by doing identical experiments but using the Fe_c–hTF derivative or the hTF/2N half-molecule, rather than apo-hTF, since only binding to the N-site could occur in these cases. It was found that the heat changes resulting from the injection of either of these two molecules were very small compared to those observed for apo-hTF and showed no signs of saturative binding as bicarbonate was increased. In view of this, the heat changes observed for the apo-hTF injections were analyzed in terms of bicarbonate binding to the C-site only, assuming a 1:1 stoichiometry. The binding constant, K_b, and the ΔH of binding were obtained by fitting integrated heat data, Q, from apo-hTF injections at various bicarbonate concentrations, C_2, to the equation

$$Q = \Delta H/(1 + 1/K_bC_2)$$

RESULTS

Identification of Two Kinetic Phases in the Binding of Fe–NTA to hTF in the Presence of Bicarbonate. Figure 1 shows

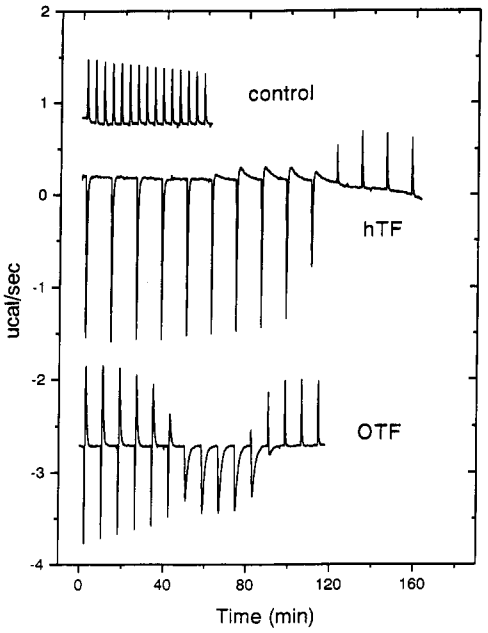

FIGURE 1: Results from titration calorimetry on the binding of Fe–NTA to hTF in the presence of 25 mM bicarbonate, pH 7.5, at 27 °C. The middle trace shows the raw data for 14 injections, 5 μL each, of 1.56 mM Fe–NTA into 1.41 mL of 0.0308 mM apo-hTF. The 15 control injections (upper trace) were carried out in the same way except with no hTF in the cell. The bottom trace shows earlier data from Lin et al. (1991) for 15 injections, 5 μL each, of 1.56 mM Fe–NTA into 0.038 mM apo-OTF under the same conditions.

raw data for the calorimetric titration of 1.41 mL of 0.0308 mM apo-hTF in the sample cell at 27 °C with a 1.56 mM solution of Fe–NTA (2-fold molar excess of NTA) added from the injection syringe. The sample solution and injectant solution are in the same buffer (100 mM HEPES and 25 mM $NaHCO_3$, pH 7.5). For the purpose of comparison, earlier data are shown for the titration of 1.41 mL of 0.038 mM apo-OTF with a 1.56 mM solution of Fe–NTA at 27 °C, using the same buffer (Lin et al., 1991). For the titration of hTF, there are 14 injections of 5 μL each spaced 12 min apart. Figure 1 also shows a control run with no protein in the cell, where 15 5-μL injections were spaced at 4 min intervals.

Three distinct patterns of peak profiles can be recognized for the hTF experiment in Figure 1. Injections 1–5 show only a fast exothermic (i.e., negative deflection) peak, while injections 6–10 exhibit two kinetic phases which include a fast exothermic phase followed by a slower endothermic phase. The last three injections are all endothermic and very similar to the control experiment. It is well-documented that NTA-chelated ferric ion binds preferentially to the C-site of hTF (Aisen et al., 1978; Brock, 1985). The preference of Fe–NTA to bind to the C-site first is so strong that monoferric transferrin (Fe_c–hTF), prepared by mixing Fe–NTA with apo-hTF in a 1:1 molar ratio in 0.1 M HEPES and 25 mM $NaHCO_3$ at neutral pH and then dialyzing out the NTA (Thompson et al., 1986; Bali & Harris, 1990), shows virtually no detectable binding at the N-site. Therefore, the profiles for first five injections in Figure 1, which saturated 50% of the sites of apo-hTF, must result from binding to the C-site, while those for injections 6–10 arise from binding to the N-site. The profiles for injections 12–14 merely represent the endothermic heat of dilution after both sites of hTF have been saturated.

In our previous study of OTF (Lin et al., 1991) we showed that the fast calorimetric phase, which occurs within the instrument response time, corresponds to contact binding of Fe–NTA to the site, the chelator still remaining attached to the ferric ion after binding. The subsequent slow phase was shown to be due to the insertion of bicarbonate ion to replace the NTA at the binding site. These two phases can be clearly seen in the OTF data in Figure 1 where titration of the N-site in injections 1–6 shows a fast exothermic phase followed by a slow endothermic phase, and titration of the C-site in injections 7–11 shows a fast exothermic phase (nearly canceled out by the endothermic heat of dilution) followed by a slow exothermic phase. Although site preferences are reversed for the two transferrins, the same two kinetic phases seen for N-site binding to OTF in the early injections are also seen in the binding of Fe–NTA to the N-site of hTF in the middle injections, i.e., a fast exothermic phase followed by a slow endothermic phase. On the other hand, while binding to the C-site of OTF in the middle injections shows both a fast (canceled by the heat of dilution in Figure 1) and a slow exothermic process, binding to the C-site of hTF in the early injections shows only a fast exothermic phase, and the slow phase is absent. The apparent absence of a distinguishable slow phase for binding to the C-site of hTF could be explained by any of the following three scenarios: (a) The insertion of bicarbonate ion into the C-site of hTF is fast in 25 mM bicarbonate solution, even though insertion at the N-site of hTF and at both sites of OTF is slow. (b) The insertion of bicarbonate ion into the C-site occurs with no net heat change. (c) Bicarbonate ion (at 25 mM) is already bound to apo-hTF prior to the injection of Fe–NTA, so there is no need for insertion when Fe–NTA is added.

In order to obtain a definite answer, the following two experiments were carried out: (a) With buffer containing various concentrations of bicarbonate in the sample cell (no protein; no ferric ion), apo-hTF was injected from the syringe to detect the possible weak binding of bicarbonate to apo-hTF in the absence of ferric ion. Such titrations were also carried out for Fe_c–hTF and hTF/2N to determine the possible binding of bicarbonate to the apo-N site when the C-site is unavailable. (b) Titrations of apo-hTF in the sample cell with Fe–NTA from the syringe were performed at lower bicarbonate concentrations (1 and 5 mM bicarbonate, in both the cell and the syringe) in an attempt both to reduce the extent of preinsertion of bicarbonate (assuming weak binding) prior to ferric ion addition and to slow down the rate of insertion of bicarbonate after ferric ion binding.

The results of experiment a are shown in Figure 2. Figure 2a shows raw data for single injections (10 μL of 0.3 mM apo-hTF) into bicarbonate solutions of various concentrations from 0 to 50 mM. The heats of injection are seen to depend strongly on bicarbonate concentration, indicating that bicarbonate ion does indeed bind to apo-hTF. The identical experiments for Fe_c–hTF and apo-hTF/2N have much smaller heat changes and show no sign of saturation, suggesting that bicarbonate binding to the C-site is primarily responsible for the peak seen for apo-hTF in Figure 2a. Figure 2b shows a plot of the integrated heat changes (after that for the 0 mM bicarbonate control is subtracted) as a function of bicarbonate concentration in the cell. These data were analyzed by assuming a 1:1 complex, according to the equation in Materials and Methods. A binding constant of 273 M^{-1} and a heat of binding of −7.9 kcal/mol were obtained, and the dotted curve shows the calculated behavior using those parameters. This result allowed us to estimate that, in 25 mM bicarbonate solution (i.e., the conditions of Figure 1), apo-hTF should be 87% saturated with bicarbonate prior to the injection of Fe–NTA.

The results of experiment b are plotted in Figure 3, which shows single injections of FE–NTA solution into apo-hTF

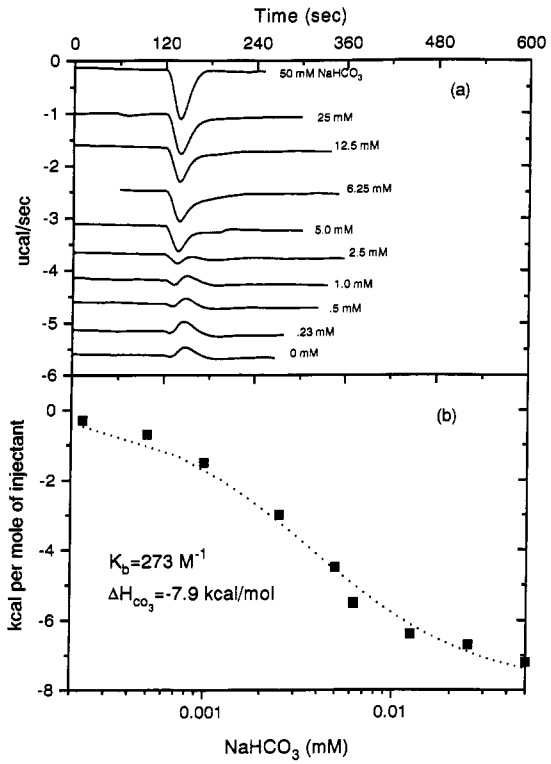

FIGURE 2: Calorimetric titration of bicarbonate solutions of various concentrations with apo-hTF solution; Both bicarbonate and apo-hTF solutions contained 100 mM HEPES, pH 7.5, at 27.0 °C. (a) Raw data for single injections of 0.3 mM apo-hTF into various concentrations of bicarbonate. (b) Plot of the integrated areas for each injection (after subtraction of the control injection into 0 mM bicarbonate) vs bicarbonate concentration, expressed in terms of kcal/mol of apo-hTF injected. The equation given in Materials and Methods was used to fit the data. The best-fit parameters are listed, and the calculated curve is shown as the dotted line.

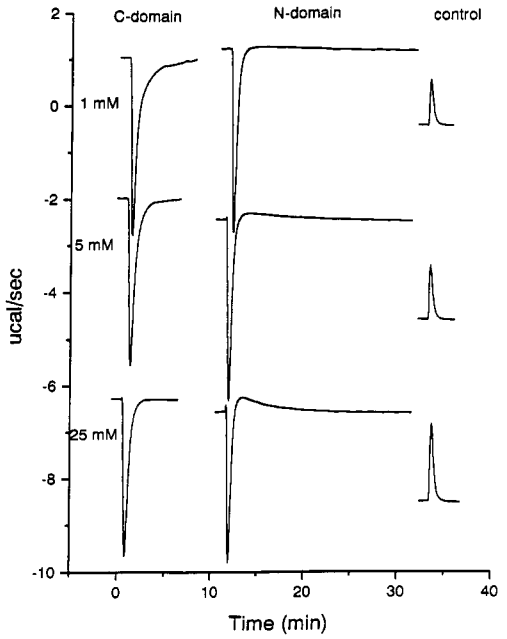

FIGURE 3: Raw data for single 10-μL injections of 3.4 mM Fe–NTA into apo-hTF in 100 mM HEPES, pH 7.5, at 27.0 °C, with bicarbonate concentrations at 1, 5, and 25 mM, respectively (in both solutions). Apo-hTF concentrations are 0.037, 0.040, and 0.05 mM in 1, 5, and 25 mM bicarbonate solutions, respectively. These injections are taken from the sequential titrations where either the C-site or the N-site, but not both together, were titrated.

solution in the reaction cell. Each injection (10 μL of 3.4 mM Fe–NTA) was made at an appropriate point in the saturation profile so that either the N-site or the C-site, but not both together, was being titrated. The primary difference between the three separate experiments is that the bicarbonate concentration in the cell and syringe solutions was varied from 1 to 5 to 25 mM [the bicarbonate concentrations given refer to added bicarbonate and do not include additional bicarbonate (ca. 0.3 mM) from atmospheric CO_2] going from top to bottom. The control injections (no hTF in the cell) at each bicarbonate concentration are also plotted in Figure 3. The data indicate that a slower exothermic phase begins to appear for titration of the C-site as bicarbonate concentration is reduced from 25 to 1 mM, evident from increasing width-at-half-height and increasing negative area. The long kinetic tail is particularly evident at 1 mM bicarbonate, confirming the presence of a process whose rate varies with bicarbonate concentration. Even in 25 mM bicarbonate solution, the width of the injection peak for the C-site is slightly larger than for the fast phase of the N-site, suggesting that the slow phase might be occurring, but that it proceeds faster and to a lesser extent than at the lower concentrations.

The total integrated heat changes for the C-site, after corrections are made for the respective control runs, are −10.7 kcal/mol in 25 mM bicarbonate, −13.3 kcal/mol in 5 mM bicarbonate, and −16.0 kcal/mol in 1 mM bicarbonate. If 100% insertion of bicarbonate to the C-site occurred only after Fe–NTA binding, then these heats should all have been identical and only the rate of insertion would decrease at the lower bicarbonate concentrations. This is in fact what happens for the N-site, where the total area remains nearly constant at ca. −4.5 kcal/mol but the endothermic tail stretches over much longer time periods in going from high to low bicarbonate concentrations. This is very evident at 1 mM where the slow phase requires ca. 20 min to equilibrate.

If the extent of prebinding of bicarbonate to the C-site depends on bicarbonate concentration for the experiments shown in Figure 3, then the total experimental heat, ΔH_{tot}, should obey the equation

$$\Delta H_{tot} = \Delta H_{Fe} + (1-f)\Delta H_{CO_3} \quad (1)$$

where ΔH_{Fe} is the heat of binding ferric ion after bicarbonate has inserted, and ΔH_{CO_3} is the heat of binding bicarbonate in the absence of ferric ion. The parameter $(1-f)$ is the fraction of C sites not having bound bicarbonate before titration with Fe–NTA and will be equal to

$$1-f = 1/(1 + K_b[HCO_3^-]) \quad (2)$$

The integrated heats from the data in Figure 3 at the three bicarbonate concentrations yield numerical solutions to eqs 1 and 2 and provide estimates of ΔH_{Fe}, ΔH_{CO_3}, and K_b of −9.4 kcal, −8.0 kcal, and 215 M^{-1}, respectively. The latter two parameters agree exceedingly well with the estimates (−7.9 kcal and 273 M^{-1}) of the same two parameters obtained from the data in Figure 2. Note that the two sets of parameter estimates are totally independent of one another since ferric ion binding was involved in the experiment illustrated in Figure 3, while ferric ion was absent in the solutions used to obtain the data in Figure 2, reinforcing the idea that the prebinding model is correct.

Harris (1985) investigated the binding of bicarbonate to apo-hTF in 0.1 M HEPES, pH 7.4, using a spectrophotometric method. In direct binding experiments, the data suggested bicarbonate binding to only a single site on hTF with a binding constant of 460 M^{-1}. However, in competition experiments between bicarbonate and vanadate anions, indirect evidence was found to suggest a second bicarbonate site which is nearly 8 times weaker than the first. Our results using Fe_c–hTF and apo-hTF/2N, discussed earlier, indicate no obvious binding

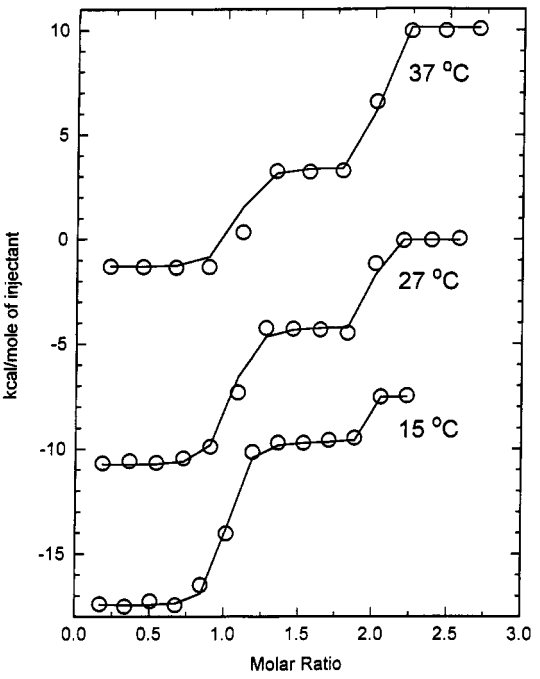

FIGURE 4: Integrated heat changes for the titration of hTF with Fe–NTA at 15, 27, and 37 °C in the presence of 25 mM bicarbonate, after subtraction of respective controls. Solution conditions are as in Figure 1. The open circles are the experimental heats for each injection, while the solid lines are calculated using the binding parameters shown in Table I. The molar ratio is ligand (e.g. Fe–NTA) to macromolecule (apo-hTF). For clarity, data at 37 and 15 °C have been displaced by –7.5 and 11 kcal, respectively.

Table I: Thermodynamic Parameters for Binding of Fe–NTA to apo-hTF in 100 mM HEPES and 25 mM $NaHCO_3$, pH 7.5

		C-site		N-site	
temp (°C)	K_1/K_2	ΔH (kcal/mol)	ΔC_p (cal/deg)	ΔH (kcal/mol)	ΔC_p (cal/deg)
15.5	210	–9.9	–70	–2.0	–200
27.0	150	–10.7	–70	–4.2	–200
37.0	190	–11.4	–70	–6.5	–200

by the N-site, but it is possible that extremely weak binding (i.e., a binding constant of $\sim$50 M^{-1}) could have gone undetected.

Thermodynamic Parameters for Binding Fe–NTA to hTF in the Presence of Bicarbonate. Each of the injection peaks for hTF in Figure 1 was integrated, as were the control peaks. The hTF heats with control heats subtracted are shown as the middle data set in Figure 4, expressed as kcal/mol of injectant versus the molar ratio of ferric ion to hTF which exists in the cell after each injection. Similar experiments were carried out at 15 and 37 °C, and the results are also shown in Figure 4. For visual clarity the data at 15 °C have been displaced to lower values by –7.5 kcal, and the data at 37 °C were displaced to higher values by 11 kcal. All of these heats correspond to the total heat resulting from the injection and therefore include heats for both the fast and the slow phase for the N-site. The individual heats can only be estimated approximately for the two phases and would correspond to ca. –10 kcal for the fast contact binding phase and 6 kcal for the slow phase at 27 °C, for example, giving a total heat of ca. –4 kcal, as seen in Figure 4.

It is evident by the sharp breaks which occur in all three curves at a molar ratio of 1 that titration of the C-site is nearly completed before the N-site starts to titrate. Analysis of the data in Figure 4 was based on two independent binding sites using a Marquardt algorithm (Lin et al., 1991). The results are plotted as the solid lines in Figure 4, and the best-fit parameters are summarized in Table I. During curve-fitting, two binding enthalpies (ΔH_1 and ΔH_2) and two apparent binding constants (K_1 and K_2)[2] were used as adjustable parameters. The two stoichiometry parameters (n_1 and n_2) were set equal to one another and treated as a single adjustable parameter. Although the absolute values of the apparent binding constants for both sites are very large and not well-defined by curve-fitting, the K_1/K_2 ratio could be crudely estimated and the enthalpies of binding could be determined with good accuracy.

Best values of the stoichiometry parameters were always close to 1.0 and are not included in Table I. The K_1/K_2 ratio necessary to provide a good fit to the data is always larger than 100, confirming that NTA-chelated ferric ion has a strong preference for binding to the C-site at neutral pH. The overall binding enthalpies for the C-site at all three temperatures are more exothermic than for the N-site. If we made corrections in the C-site values to include total bicarbonate binding, the values would be even more exothermic by ca. –7 kcal/mol at 27 °C (i.e., 87% of bicarbonate is bound before Fe–NTA is added, and the heat of bicarbonate binding is –8 kcal/mol). A large portion of this difference arises from the fact that bicarbonate insertion is exothermic for the C-site and endothermic for the N-site. This is the same situation found earlier for binding of OTF (Lin et al., 1991).

From the temperature dependence of the heats shown in Table I, heat capacities of binding (ΔC_p) can be estimated. Both sites have a negative ΔC_p, –70 cal/deg for the C-site and –200 cal/deg for the N-site, suggesting the possibility of a decrease in the exposure of hydrophobic side chains of the protein to water upon binding (Brandts, 1964). To the extent that this is true, the smaller ΔC_p for the C-site (relative not only to the N-site in hTF but to both the N- and the C-site in OTF) could be due to the prebinding of bicarbonate to the C-site, which might partially close the binding cleft to entry by solvent even before ferric ion binds.

In order to determine effects of buffer on the energetics of binding, titration experiments similar to those in 100 mM HEPES were also carried out in 100 mM Tris-HCl buffer solution in the presence of 25 or 1.5 mM $NaHCO_3$. The raw data for these experiments are shown in Figure 5. In 25 mM bicarbonate, the profiles for injection peaks which lead to binding at the C-site (i.e., early injections) in Tris-HCl buffer are very similar to those in HEPES buffer; i.e., only one fast exothermic phase is apparent. The titration of the N-site also shows a fast phase followed by a slow phase, but in Tris buffer both phases are exothermic, while the slow phase is endothermic in HEPES buffer. The titration profiles at 37 °C are very similar to those at 27 °C, as shown in Figure 5, except that heat changes for both sites become more exothermic and the rate of the slow phase increases at the higher temperature. At the lower bicarbonate concentration of 1.5 mM, a slow exothermic phase becomes evident for the C-site, just as was found previously in HEPES buffer.

The total integrated heat changes were obtained from the data in Figure 5, after the respective control runs were subtracted, and analyzed in terms of two independent sites, as described earlier for data in HEPES buffer. Best values

[2] In the presence of bicarbonate, any ferric ion bound to hTF cannot be released back into solution except over very long times. This being the case, true binding constants cannot be estimated from our data. The K_1/K_2 ratios reported in Tables I and II are intended only to indicate the relative preference of a ferric ion to bind to one site or the other, when both sites are available. The site preference is determined by kinetic as well as thermodynamic factors, which are discussed later.

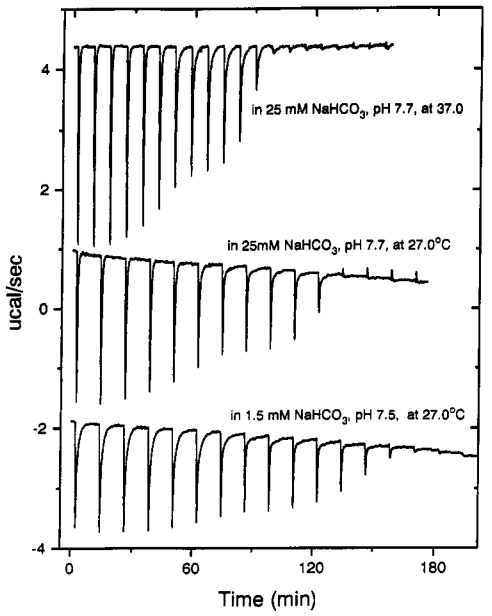

FIGURE 5: Raw data for 5-μL injections of 1.56 mM Fe–NTA into the reaction cell containing 1.41 mM apo-hTF in 100 mM Tris-HCl in the presence of bicarbonate. Apo-hTF concentrations are 0.0343, 0.0317, and 0.034 mM for each experiment (from top to bottom). The bicarbonate concentrations, pH's, and temperatures are as indicated.

Table II: Thermodynamic Parameters for Binding of Fe–NTA to apo-hTF in 100 mM Tris-HCl and 25 mM $NaHCO_3$, pH 7.5

		C-site		N-site	
temp (°C)	K_1/K_2	ΔH (kcal/mol)	ΔC_p (cal/deg)	ΔH (kcal/mol)	ΔC_p (cal/deg)
27.0	150	−12.63	220	−9.33	520
37.0	undefined	−14.85	220	−14.56	520

of the heat parameters for the two sets of data in 25 mM bicarbonate are shown in Table II (the data in 1.5 mM bicarbonate are not included, since the very slow kinetics for the N-site made integration uncertain). Estimates of K_1/K_2 could not be made accurately at 37 °C because total heat changes for the two sites were close to identical. These data show that the apparent heat of binding is 2–3 kcal more exothermic for the C-site, and 5–8 kcal more exothermic for the N-site, in Tris than in HEPES buffer. If we assume that the intrinsic binding enthalpies for the two sites are the same in the two buffers, the differences in apparent heat of binding in the two buffers can be attributed to the release of protons during the binding process and the larger heat of ionization of Tris relative to HEPES (11.3 vs 3.92 kcal at 25 °C). The heat values indicate that ca. 0.5 proton is released when ferric ion binds to the C-site and ca. 1 proton is released when ferric ion and bicarbonate bind to the N-site. These data strongly suggest that more proton release at the N-site occurs during bicarbonate insertion since the heat change of the slow phase is more affected by the buffer change than that of the fast phase. Although the ΔC_p value is still larger for the N-site than the C-site, both are larger than the corresponding values in Table I obtained using HEPES buffer. The most straightforward interpretation of this is that proton release might vary a little with temperature. If this is true, then the total ΔC_p cannot be attributed soley to hydrophobic factors.

Binding of Fe–NTA to hTF in the Absence of Bicarbonate. It was shown for OTF (Lin et al., 1991) that when binding of Fe–NTA takes place in the absence of bicarbonate, an equilibrium is quickly established and the relative preference of ligands for the two sites is determined by the binding

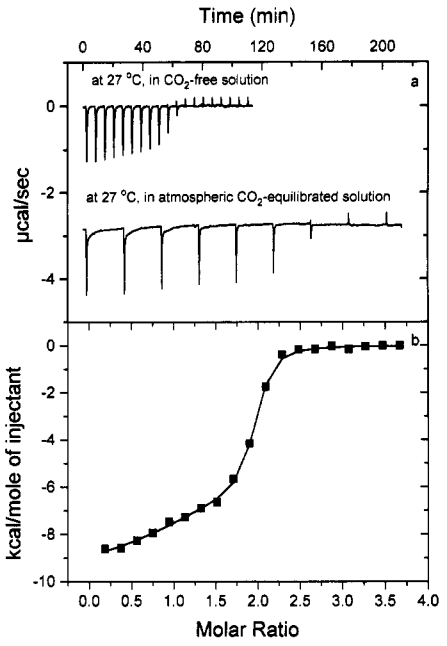

FIGURE 6: Calorimetric titrations of apo-hTF with Fe–NTA in 100 mM HEPES, pH 7.5, in the absence of bicarbonate and in the presence of bicarbonate resulting from equilibration with atmospheric CO_2 (both at 27.0 °C) (a) Upper curve: raw data resulting from injections of 5 μL of 1.45 mM Fe–NTA into 1.41 mL of apo-hTF (0.028 mM) in the CO_2-free solution. Lower curve: raw data resulting from injection of 5 μL of 1.56 mM Fe–NTA into 1.41 mL of apo-hTF (0.021 mM). (b) Integrated heats for each injection, after subtraction of the control injection, for the data obtained in CO_2-free solution, shown in panel a. The solid line is the calculated curve using the best-fit parameters shown in Table III.

constants at each site. Also, the slow kinetic phases seen in the presence of bicarbonate, attributed to bicarbonate replacement of NTA at the binding site, disappear almost entirely.

Similar experiments on hTF were also carried out in 100 mM HEPES, pH 7.5, at both 27 and 37 °C using solutions where dissolved CO_2 had been removed to the maximum extent possible (see Materials and Methods). Figure 6a (upper curve) shows raw data for the titration of apo-hTF (0.028 mM) with Fe–NTA (1.45 mM, 5 μL per injection) at 27 °C. Integrated data (after subtraction of small control heats) are in Figure 6b. Also shown in Figure 6a is a curve for titration of apo-hTF (0.021 mM) with Fe–NTA (1.56 mM, 5 μL per injection) in a buffer solution equilibrated with atmospheric CO_2 (ca. 0.3 mM bicarbonate in the solution). It is seen in Figure 6a that, in the CO_2-free solution, no slow heat changes are observed, while in CO_2-equilibrated solution, the first 3 injections show a small, slow exothermic phase very similar to that seen for binding to the C-site in 1 mM $NaHCO_3$ in Figure 3.[3] These results indicate that binding of Fe–NTA in the absence of bicarbonate also occurs sequentially, with the C-site being titrated first. However, the preference is probably not as strong as that in the presence of 25 mM bicarbonate, as indicated by a more gradual change in injection heats near the point of 50% saturation in Figure 6b, compared to the very abrupt change seen in Figure 4 with bicarbonate present.

[3] In the CO_2-equilibrated data in Figure 6, the titration of the N-site in injections 4–6 does not show the slow endothermic phase associated with bicarbonate insertion. This probably has to do with its very slow kinetics. Even in the earlier data (Figure 3) at 1 mM added bicarbonate (1.3 mM total bicarbonate), the amplitude of the slow phase is very small. Since the bicarbonate concentration in CO_2-equilibrated solution is about 4-fold lower yet, the amplitude of the slow phase will be further reduced in accordance with the longer time to reach equilibrium.

Table III: Thermodynamic Parameters for Binding of Fe–NTA to apo-hTF in 100 mM HEPES, pH 7.5

	C-site		N-site	
temp (°C)	K (M^{-1})	ΔH (kcal/mol)	K (M^{-1})	ΔH (kcal/mol)
27.0	3.3×10^7	–8.7	3.9×10^6	–5.6
37.0	2.3×10^7	–9.9	3.0×10^6	–6.2

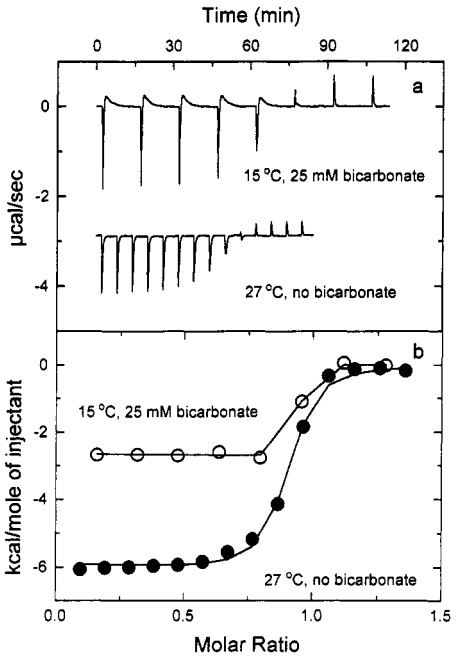

FIGURE 7: Calorimetric titrations of apo-hTF/2N with Fe–NTA in 100 mM HEPES, pH 7.5, in the presence and absence of bicarbonate. (a) Raw data for 5-μL injections of 1.56 mM Fe–NTA into 1.41 mL of apo-hTF/2N. The protein concentrations are 0.035 mM in the presence of 25 mM bicarbonate (upper trace) and 0.079 mM in its absence (lower trace). (b) Integrated heat changes for each injection after subtraction of the control injection. The solid lines are calculated from the best-fit parameters.

The integrated data were analyzed in terms of two independent sets of sites in the same manner as those in the presence of bicarbonate. In this case, the apparent binding constants should be true thermodynamic constants since ferric ion binding in the absence of bicarbonate was shown to be quickly reversible for transferrins (Lin et al., 1991). The results are summarized in Table III, and the best-fit curves are plotted as solid lines in Figure 6b. The binding constants for both sites, in the range of ca. 10^7 M^{-1}, are only of moderate strength in contrast to the very tight binding in bicarbonate solutions. The ratio of binding constants is about 10, compared to more than 100 in the presence of bicarbonate.

The heat changes in the absence of bicarbonate, ranging from –8.7 kcal/mol at 27.0 °C to –9.9 kcal/mol at 37.0 °C for the C-site, are close to those obtained in 25 mM bicarbonate (cf. Table I). The reactions which occur are different, however, since here Fe–NTA is binding to an empty site with the chelator NTA remaining attached, while in 25 mM bicarbonate Fe–NTA is binding to sites which for the most part already have bound bicarbonate, and the chelator is left free in solution. The heat changes for the N-site binding are –5.6 kcal at 27.0 °C and –6.2 kcal/mol at 37.0 °C. These heats are at least several kilocalories smaller in magnitude than indicated for the contact binding process observed in the presence of 25 mM bicarbonate even though the net reactions should be very similar in the two cases. However, in 25 mM bicarbonate the N-site being titrated is interacting with a C-terminal domain having a bound ferric ion and bicarbonate ion, while in the absence of bicarbonate the N-site being titrated is interacting with a C-domain partially titrated with Fe–NTA but with no bicarbonate anion. We showed in earlier studies on solutions containing interacting N- and C-fragments of OTF that the heat of binding to a site in one half-molecule can vary by as much as 10 kcal depending on whether or not its interacting partner has an empty or filled site.

Binding of Fe–NTA to the hTF/2N Half-Molecule. Less-extensive calorimetric titrations were also carried out for the recombinant N-terminal fragment (hTF/2N) using the same HEPES buffer. The upper data set in Figure 7a shows the titration in 25 mM bicarbonate at 27 °C, and the lower data set is for the titration in the absence of bicarbonate at 15 °C. The titration profiles in the presence of bicarbonate are qualitatively similar to those for the binding to the N-site in intact hTF shown in Figure 1 (cf. injections 6–10), showing a fast exothermic phase followed by a slow endothermic phase. For the titration in the absence of bicarbonate, only a fast exothermic phase is apparent.

The total integrated heats for each injection (after the respective heats are subtracted) are shown in Figure 7b. These data were fit with a model based on one set of sites (Wiseman et al., 1989), and the best-fit curves are plotted as the solid lines. The total heat change for binding to the N-fragment in the presence of 25 mM bicarbonate was found to be less exothermic than for the N-site binding in the intact hTF (–2.5 vs. –4.2 kcal/mol), perhaps dues to ligand-dependent changes in domain–domain interactions in intact hTF. Careful examination of peak profiles in Figures 1 and 7 suggests that the heat changes of the fastest phase (i.e., contact binding) for intact hTF and hTF/2N are nearly identical, ca. –10 kcal, and the difference in total heats arises primarily because heats for bicarbonate insertion are nearly 2 kcal more endothermic for hTF/2N than for intact hTF. It also appears, making allowance for the different temperatures of the two experiments shown in Figure 7a, that the total binding process in the absence of bicarbonate has a heat change similar to that of the fast process in the presence of bicarbonate.

In the absence of bicarbonate, a binding constant of 4×10^6 M^{-1} and a heat of binding of –5.9 kcal/mol were obtained for hTF/2N at 15.5 °C. These two parameters are comparable to those for binding to the N-site in intact hTF (3.3×10^6 M^{-1} and –5.6 kcal/mol at 27 °C) in the absence of bicarbonate, shown in Table III. These data suggest that the presence of the C lobe in intact hTF has only a small effect on the binding parameters to the N-site in the absence of bicarbonate.

DISCUSSION

The binding processes for the C-site and the N-site in hTF are very different. Bicarbonate ion is able to bind, albeit weakly, to the C-site of apo-hTF in the absence of ferric iron. By two independent methods, a binding constant of ca. 250 M^{-1} and a heat of binding of –8 kcal/mol were obtained for binding of bicarbonate to the C-site of apo-hTF. There is no indication in our data that the N-site of hTF is able to bind significant bicarbonate in the absence of ferric ion. Thus, in 25 mM bicarbonate solution, most of the C-sites of apo-hTF are filled with bicarbonate while the N-sites are largely free of bicarbonate. Upon the titration of apo-hTF with Fe–NTA, then, only a fast, exothermic phase is apparent for the C-site binding. Two kinetic phases, a fast exothermic phase followed by a slow endothermic phase (i.e., in HEPES buffer) which lasts more than 7 min at 25 °C, are seen for the N-site binding. The fast phase for the N-site apparently incorporates the NTA-chelated ferric ion into the binding site, while the slow phase involves insertion of bicarbonate and removal of NTA, in agreement with processes found earlier for both sites in OTF (Lin et al., 1991).

The actual processes involved in ferric ion binding to the C-site are complicated by this prebinding and depend on the concentration of bicarbonate in solution. In 25 mM bicarbonate, ca. 87% of the C-sites already have bicarbonate bound in apo-hTF, and these sites need only bind a nonchelated ferric ion to acheive the full complement of bound ligands. This is a fast process. The other 13% of sites have no prebound bicarbonate and therefore will first bind Fe–NTA (fast) and then replace the NTA with bicarbonate from solution (slower). Because of the preponderance of the first process, the overall reaction appears to occur quickly. It is only at lower bicarbonate concentrations of 1 and 5 mM, where the C-site is presaturated to a much lesser extent and where the rate of bicarbonate insertion is slowed down by lower concentrations of free bicarbonate, that the slower exothermic phase becomes clearly evident (Figure 3).

In the titration time frame, the apparent binding constant[1] of ferric ion binding to the C-site is a couple hundred times larger than that for binding to the N-site in the presence of 25 mM bicarbonate, indicating a strong preference of ferric ion for the C-site when both sites are available. This strong preference arises from three separate factors: (1) Binding to the C-site was shown to be about 10 times stronger in the absence of bicarbonate. Thus, in the presence of bicarbonate, the fast equilibrium which is established between those sites not containing bicarbonate will provide a short-term thermodynamic preference toward the C-site. (2) At moderate concentrations of bicarbonate, most of the C-sites (but few or none of the N-sites) prebind bicarbonate and are then able to bind ferric ion quickly, whereupon they are no longer able to dissociate ferric ion except over very long periods of time. (3) Even under conditions where prebinding of bicarbonate is small (see the results in Figure 3 at 1 and 5mM bicarbonate), bicarbonate insertion into the C-site occurs approximately 5-times faster than into the N-site. Although it has little to do with preference over the time span of our experiments here, there are studies which appear to show that the true thermodynamic binding constant to the C-site may also be somewhat stronger than to the N site at pH 7.4 (Aisen et al., 1978).

The heat changes for the binding of Fe–NTA to hTF in the presence and absence of atmospheric CO_2 have previously been investigated by Binford and Foster (1974) using batch microcalorimetry. They found that the binding enthalpies for the C- and N-site of hTF at 25 °C in the presence of bicarbonate are almost identical, –10.90 and –10.43 kcal/mol, respectively. In the absence of bicarbonate, the binding enthalpies for the C- and the N-site were also found to be the same, –4.64 and –4.52 kcal. They concluded that the two binding sites of hTF are equivalent and independent. We disagree with this conclusion since our data consistently show that the two sites are thermodynamically and kinetically different.

The calorimetric data obtained on hTF/2N are not as extensive as on the whole molecule. Although large differences in the energetics of binding, relative to the N-site in intact hTF, are not seen, there are small differences in the presence of bicarbonate which might be attributable to the absence of interactions with the C domain. In the absence of bicarbonate no significant differences were observed.

The binding reactions of ferric ion to hTF show major differences from, but also some similarities to, those of OTF, and these can be quickly summarized. For example, in the presence of 25 mM bicarbonate, the insertion of bicarbonate into the N-site of OTF is much faster than into the C-site, while the opposite is true for hTF (Figure 1). Bicarbonate is able to prebind to the C-site in hTF but not to the C-site of OTF.[4] Although binding to each site in hTF is more exothermic than for the corresponding site in OTF (after correct for prebinding of bicarbonate to the the C-site of hTF) at 27 °C, binding to the C-site in each molecule is more exothermic than binding to the N-site in the same molecule. The primary reason for this is that bicarbonate insertion occurs exothermically for the C-site of each transferrin and endothermically for the N-site (i.e., in HEPES buffer). Finally, in the absence of bicarbonate, Fe–NTA binding to the C-site of hTF is more exothermic and stronger than to the N-site of hTF, while the exact opposite relationship was found for OTF. This factor undoubtedly contributes significantly to the reversed site preference of the two molecules in the presence of bicarbonate, since it gives a short-term initial advantage to the C-site for hTF and to the N-site for OTF, whereupon bicarbonate insertion essentially freezes the distribution for very long periods of time.

It was also shown in earlier studies on OTF, using half-molecule fragments, that significant interactions occur between the N- and the C-domain and that the interactions change depending on the attachment of ferric ions to either or both sites. Although a method for preparing the recombinant N-fragment of hTF has been available for some time (Funk et al., 1990; Woodworth et al., 1991), difficulty has been encountered in expressing the C-terminal fragment. A new strategy is being undertaken, aimed at obtaining the recombinant C-lobe so that future studies can be pursued. It seems possible that further differences between these two transferrin molecules will be revealed in the pattern of interaction and communication between the two binding sites.

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[4] Oe et al. (1989) were not able to detect binding of bicarbonate to the C-terminal half-molecule of OTF in the absence of ferric ion, agreeing with our earlier (Lin et al., 1991) interpretation of the calorimetric data. However, Oe et al. reported the opposite situation for the N-terminal half-molecule and for the N-site in intact OTF at pH 8.0, while Lin et al. found no calorimetric evidence of prebinding of bicarbonate to the N-site of OTF at pH 7.4. Whether this discrepancy results from differences in solution conditions or from other factors is not known.

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