Calorimetric Studies of Serum Transferrin and Ovotransferrin. Estimates of Domain Interactions, and Study of the Kinetic Complexities of Ferric Ion Binding†

Lung-Nan Lin,§ Anne B. Mason,⊥ Robert C. Woodworth,⊥ and John F. Brandts*,§

Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003, and Department of Biochemistry, University of Vermont, Burlington, Vermont 05405

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ABSTRACT: Human serum transferrin and hen ovotransferrin have been studied by differential scanning calorimetry (DSC), in an effort to quantitatively estimate the free energy of interaction of the N- and C-domains in each protein and to further understand their interaction with chelated ferric ions. In the case of serum transferrin, separate DSC transitions are observed for the two domains while only a single, coupled transition is seen for ovotransferrin. Although domain interactions are somewhat larger for ovotransferrin (–4100 cal/mol) than for serum transferrin (–3100 cal/mol), the major cause of separated transitions for serum transferrin is that the difference in intrinsic folding stability of the N- and C-domains is about 4-fold larger than for ovotransferrin. Chelated ferric ions bind strongly to each site in both proteins and produce changes in T_m by as much as 30 °C. When apparent binding constants are estimated from DSC results, these appear to be substantially larger than those estimated previously from equilibrium methods at low temperatures, where very long equilibrium times must be used because of slow ligand release. Although second DSC upscans on each protein show good "reversibility", downscans on serum transferrin revealed that liganded forms of the protein are in fact not in true equilibrium during upscanning, which causes T_m values during upscans to be higher than the true reversible T_m values. The likely reason for this kinetic control over unfolding is the slow release of bound ferric ions and those effects, for technical reasons, cannot be totally eliminated by lowering the scan rate.

Transferrins are a class of proteins that bind extracellular iron which they ultimately supply to metabolizing cells through interaction with transferrin receptors on the cell surface (Chasteen & Woodworth, 1990; Crichton, 1990). Studies on human serum transferrin (hTF)[1] and hen egg white ovotransferrin (OTF) reveal many similarities in structure and function. Both are glycoproteins containing a single polypeptide chain of ca. 80 kDa with 50% homology in amino acid sequence (MacGillivray et al., 1983; Jeltsch & Chambon, 1982). X-ray crystallography indicates (Bailey et al., 1988; Anderson et al., 1989, 1990; Baker et al., 1991) transferrins have two homologous domains (frequently called N and C lobes) of nearly equal size, connected by a single short bridging peptide. Each domain consists of two dissimilar subdomains that form a cleft enclosing the binding site for a metal ion and synergistic anion. The binding properties of the two sites in the C- and N-domains are similar, but not identical (Aisen et al., 1978; Williams et al., 1978; Brock, 1985). Titration calorimetric studies of OTF in the presence of bicarbonate as the synergistic anion have shown (Lin et al., 1991) that binding of ferric ion (chelated with nitrilotriacetate (NTA)) to each site occurs in two kinetic phases. Immediately after addition, the chelated ferric ion binds quickly but weakly to the protein site. This is followed by a slow phase, lasting for several minutes depending on temperature, in which the bound chelator NTA is replaced by bicarbonate ion. Binding was shown to be reversible immediately after completion of the fast phase but became essentially irreversible over moderate time periods after insertion of bicarbonate.

The binding of diferric transferrin to its receptor apparently depends on the two-domain structure of these proteins (Brown-Mason & Woodworth, 1984; Brown-Mason et al., 1987). Half-molecules, OTF/2N and OTF/2C, can be prepared proteolytically and neither alone, when saturated with ferric ion, will elicit the physiological response produced by the intact molecule. Mixtures of the two interacting half-molecules saturated with ferric ions will illicit the response. Thus, there appears to be a requirement for both a double-headed molecule and for ferric ion binding to facilitate the strongest interaction of transferrins with transferrin receptors.

Studies of the binding of OTF/2C to OTF/2N using titration calorimetry (Lin et al., 1991) showed clearly that both the free energy and heat of interaction of the two half-molecules were quite dependent on whether neither, one, or both had an attached ferric ion. It seems possible then that iron-dependent changes in domain–domain interactions may produce changes in the structure of transferrins which are required for proper interaction with receptors in order to carry out the final physiological function.

Results from DSC studies can be used to quantitate interactions which occur in multidomain proteins (Brandts et al., 1989). Estimates are based on the assumption that pairwise interactions between domains will persist only so long as *both* domains remain in their native folded state. This being the case, the free energy of pairwise interaction, ΔG_{AB}, between two domains, A and B, will act to stabilize only the transition for the domain with the lower T_m. Estimates of ΔG_{AB} can then be made in two ways. First, if the transition temperature T_A is lower than T_B under normal reference conditions and if some perturbation of A (e.g., strong binding of a ligand to the A domain only) can be introduced which causes T_A to increase above T_B then *transition crossover* will take place

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§ University of Massachusetts.

⊥ University of Vermont.

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[1] Abbreviations: hTF, human serum transferrin; OTF, hen ovotransferrin; NTA, nitrilotriacetate; Fe–NTA, solution consisting of Fe^{3+} and NTA in a 1:2 molar ratio.

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such that ΔG_{AB} will stabilize the B transition after crossover. The difference in free energy of unfolding of the B domain before and after the perturbation will then be ΔG_{AB}. Second, if *isolated domains* in their native state can be studied separately where no interactions occur and compared to the intact protein where interactions occur, then the corresponding difference in the combined free energy of unfolding of both domains will be ΔG_{AB}. In those cases where separate transitions are seen for A and B in the intact protein, then the total value of ΔG_{AB} should be reflected in the downshift of the least stable of the two while the T_m for the more stable should be the same in the isolated domain and in the intact molecule.

In this paper, DSC results are presented which allow numerical estimates of domain–domain interactions of both hTF and OTF, using both the transition crossover and isolated domains methods. In addition, apparent binding constants for Fe–NTA to both the N- and C-site of the two proteins are estimated using methods described earlier (Brandts et al., 1989; Brandts & Lin, 1990). However, it is found that these estimates do not represent true binding constants since transitions for holo-hTF during downscanning occur at substantially different T_m values than during upscanning, which demonstrates the system is under kinetic, rather than thermodynamic, control.

MATERIALS AND METHODS

Materials. Ovotransferrin (OTF, catalogue no. C-0755, lot 107F-8020; stated purity, 99%) and nitrilotriacetate (NTA, catalogue no. N-0253, lot 37F-0669) were obtained from Sigma Chemical Co. and used without further purification. The N- and C-terminal half-molecules, OTF/2N and OTF/2C, were prepared using the method of Oe et al. (1988), with minor modifications, from ovotransferrin prepared according to the method of Brown-Mason and Woodworth (1984). Human serum transferrin (hTF) used for this study was obtained from (1) Sigma Chemical Co (catalogue no. T-1147, lot 20H-06945; stated purity, 98%) or (2) Calbiochem Co. (catalogue no. 616395, lot 901305; stated purity, 100%) or (3) was prepared essentially as described earlier (Penhallow et al., 1986) except that the sample was equilibrated with 5 mM Tris-HCl, pH 8.0–20 mM $NaHCO_3$, loaded on a DEAE-Sepharose column (Pharmacia), and eluted with 20 mM Tris-HCl, pH 8.0. All three samples gave identical DSC thermal transitions. The concentrations of various protein solutions were determined spectrophotometrically at 280 or 278 nm using the extinction coefficients reported previously (Lin et al., 1993; Lin et al., 1991; Chasteen, 1977). All other chemicals were reagent grade.

DSC Measurements. The differential scanning calorimetry (DSC) experiments were carried out on a MicroCal MC-2 ultrasensitive microcalorimeter (MicroCal Inc., Northampton, MA), interfaced with a personal computer (IBM compatible). A Windows-based software package (Origin), also supplied by MicroCal, was used for data analysis and plotting. The instrumentation and experimental procedures for DSC have been described in detail elsewhere (Brandts et al., 1989; Brandts & Lin, 1990; Bailey et al., 1990). All DSC measurements were carried out in 500 (or 100) mM HEPES, 25 mM $NaHCO_3$, pH 7.5.

Binding Equilibrium Detected by the Optical Method. These experiments were carried out on a Cary 14 spectrophotometer equipped with a water-circulating cuvette holder attached to a Neslab RTE-100 programmable water bath. To improve the protein thermal reversibility and lessen the problems caused by protein aggregation, the pH of the hTF solution (hTF = 0.0058 mM, Fe–NTA = 0.0128 mM in 500 mM HEPES, 25 mM $NaHCO_3$) was lowered to 6.74. The liganded hTF solution was upscanned from 35 to 92 °C and then immediately downscanned from 92 to 35 °C at a scan rate of 60 °C/h with monitoring at 465 nm. The derivative of absorbance changes as a function of temperature was plotted.

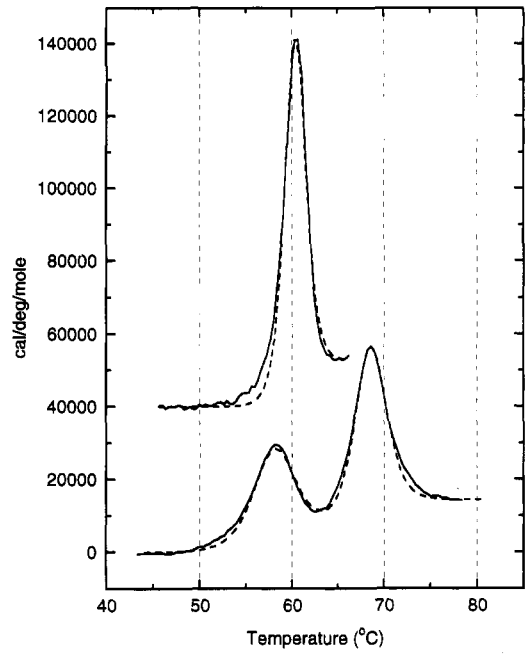

FIGURE 1: DSC scans on apo-OTF (upper trace) and apo-hTF (lower trace) in 500 mM HEPES, 25 mM $NaHCO_3$, pH 7.5. The dashed lines resulted from fitting the data to the two-state model. Protein concentrations were ca. 0.02 mM, and the scan rate was 81.5 °C/h. For clarity, DSC traces in this figure and the subsequent DSC figures have been arbitrarily shifted on the ordinate scale.

RESULTS

DSC Results on apo-hTF and apo-OTF. The buffer used for the DSC experiments was 500 mM HEPES, pH 7.5, containing 25 mM sodium bicarbonate. The scan rate was normally 81 °C/h. The lower scan in Figure 1 was obtained on apo-hTF, after subtraction of a buffer–buffer baseline and after normalization by hTF concentration. Two major transitions are seen with T_m values of 57.62 and 68.35 °C. It will be shown later that the transition of lower T_m occurs in the C-terminal domain and the transition of higher T_m in the N-terminal domain. The dashed line shows the fit obtained assuming both transitions are two-state and that the ΔC_p is the same for each transition. Although the fit to the two-state model is reasonably good, each transition tends to be perceptibly broader than the calculated fit curve so that ΔH values obtained by area integration are slightly larger than those given by the corresponding fit parameter. On the assumption that the integrated area is a more reliable measure of ΔH, the parameter set [T_m, ΔH, ΔC_p] which we will use for the lower transition is [57.62 °C, 152 kcal, 7.2 kcal/deg] and for the higher transition is [68.35 °C, 235 kcal, 7.2 kcal/deg].

Although the higher transition is some 80 kcal larger in area, this is related primarily to the large ΔC_p of 7.2 kcal/deg and the difference of nearly 11 °C in their T_m values; i.e., the two ΔH's are virtually identical when extrapolated to the same temperature. This is in accord with the fact that the N- and C-domains are known to be very similar in size, structure, and amino acid sequence.

A DSC scan on apo-OTF is also shown as the upper trace in Figure 1. In spite of its two-domain nature and close structure–sequence similarity to hTF, only a single peak centered near 60 °C is seen for OTF. The dashed curve shows the best fit to the experimental data, assuming a single two-state transition. The fit is reasonably good but slightly worse than the two-state fit for hTF, discussed above. As with hTF, the transition is broader than expected for a single two-state transition. This could arise if the coupling between the N and C transition, while very strong, is not 100% complete. It will be seen later that the two transitions can be completely uncoupled by binding ferric ions to OTF.

By using the integrated area as the best measure of ΔH, the transition parameters for OTF are [60.24 °C, 314 kcal, 13.2 kcal/deg]. The ΔH of 314 kcal and ΔC_p of 13.2 kcal/deg are within ca. 10% of the sum for *both transitions* in hTF if ΔH's are compared at a common temperature. Since the two transitions cannot be separately resolved in apo-OTF, it will be assumed in later calculations that the heat and heat capacity contributions from the N- and C-domains in apo-OTF are equivalent when compared at the same temperature, as was demonstrated to be true for hTF. Therefore the parameter set [60.24 °C, 157 kcal, 6.6 kcal/deg] will be used to characterize both the N- and C-domain transitions in apo-OTF.

At first glance, it might be supposed that coupling of the transitions for the two domains in OTF, as opposed to separate transitions seen in hTF, is a reflection of stronger domain–domain interactions in OTF. Although OTF will be shown to have slightly stronger interactions between N- and C-domains than does hTF, the major reason the two transitions couple in OTF is that the N- and C-domains have nearly the same stability in the absence of domain interactions.

Reversibility of Transitions for apo-hTF and apo-OTF. The extent of reversibility, measured by relative area recovery, seen on second upscans of the two apo-proteins depended on the temperature at which the first upscan was terminated before cooling the samples in preparation for the second upscan. If heating was terminated at the lowest temperature where the transitions appeared to be completed, then second upscans showed about 80% reversibility of apo-hTF and 55% reversibility for apo-OTF. The T_m values for transitions on the second scan were identical to those on the first scan.[2]

Saturation of Binding Sites of hTF. The five scans in Figure 2 were carried out with different degrees of saturation of the two binding sites of hTF, where chelated ferric ion, Fe–NTA, i.e., Fe^{3+} with a 2-fold molar excess of NTA, was added to achieve binding. The scan of the apo form is shown again as

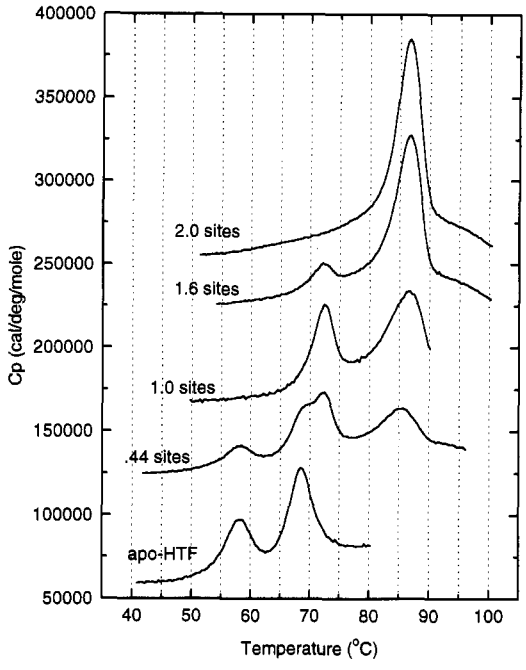

FIGURE 2: DSC scans of hTF with different degrees of saturation with ferric ion. Various amounts of Fe–NTA were added to apo-hTF (ca. 0.02 mM) to achieve the binding. The site saturation was as indicated in the figure. The scan rate was 81.5 °C/h.

the first scan (bottom) with increasing saturation of 0.44, 1.0, 1.6, and 2.0 sites for scans 2–5, respectively. Progressive addition of Fe–NTA to hTF results first in the saturation of the C-site, with the N-site not appreciably titrating until the C-site is completely saturated (Lin et al., 1993). Comparison of scan 1 of apo-hTF with scan 3, where the C-site is completely saturated, shows clearly that it is the low-temperature transition in apo-hTF that occurs in the C-domain. Its T_m shifts higher by more than 28 °C from 57.6 °C in the apo form to 87 °C after saturation of the C-site. This large shift in T_m results in transition crossover so that after ferric ion is bound to the C-site, then it is the N transition which has the lower T_m and is stabilized by ΔG_{AB}. Accordingly, its T_m (72.5 °C) is over 4 °C higher in scan 3 than seen in scan 1 (68.35) for apo-hTF. This effect is also seen in scan 2 at 0.44 site saturation, where roughly one-half of the apo-N domains are attached to an apo-C-domain and not stabilized by ΔG_{AB} and the other half are attached to a Fe-C-domain and stabilized by ΔG_{AB}. This produces two overlapping peaks for the N transition in the temperature region 65–75 °C, where the low-temperature peak coincides with that seen in scan 1 and the high-temperature peak with that seen in scan 3.

As saturation progresses from 1.0 to 2.0 sites in the top two scans of Figure 2, the N transition shifts from its T_m value of 72.5 °C seen for the apo-N-domain in scan 3 to a value near 87 °C after complete saturation. Some uncertainty exists in assigning an exact T_m for each domain in the diferric state in scan 5 at the top of Figure 2, since both transitions are included within the same broad peak. In addition to the possibility that the two transitions are not completely coupled in scan 5 and may have slightly different T_m values, there is another factor which could be involved in broadening transition peaks for the liganded species in scans 2–5. In tight-binding systems, when the added ligand is exactly equal to or less than the available number of sites, then at the start of the scan the free ligand concentration will be very low (i.e., of the order of the reciprocal binding constant). As the transition of a liganded species is approached, the free ligand concentration will increase dramatically from ligand release associated with

[2] In this study, we had the opportunity to examine apo-hTF and apo-OTF under the above conditions, where reversibility was good, and under slightly different conditions (using 100 mM buffer, rather than 500 mM buffer, at the same pH), where the reversibility was nearly zero for OTF and decreased from 80 to 30% for hTF. Precipitation at high temperature was moderately heavy for both proteins at the lower buffer concentration. Recently, Galisteo et al. (1991) have pointed out the inadvisability of trying to estimate thermodynamic parameters from DSC data on proteins which show little or no reversibility on second scans. We found, however, that results obtained under the two sets of conditions were very similar for both transferrin proteins, i.e., T_m values were virtually identical (the effect of buffer concentration on T_m was not known) under irreversible conditions, while ΔH, ΔC_p, and transition shapes were very similar within combined errors. When making these comparative scans, the instrument was operated with the cell module rotated by 90°, the configuration suggested by MicroCal for minimizing artifacts caused by precipitate formation. We conclude from this that although minor distortions were introduced by nearly complete irreversibility, the data were still capable of semiquantitative analysis using thermodynamic models. Different proteins are likely to be very different in this regard.

unfolding, and for reversible transitions this will act to broaden peaks and skew them toward the low temperature side.

Using the ΔH at T_m of 235 kcal and ΔC_p of 7.2 kcal/deg obtained for the N transition in apo-hTF, the ΔG_{AB} for interaction of N- and C-domains may be obtained from the T_m shift from 68.35 to 72.5 °C resulting from transition crossover using an equation (Brandts et al., 1989) previously derived, i.e.,

$$\Delta G_{AB}(T_m') = \Delta H_m\left(1 - \frac{T_m'}{T_m}\right) + \Delta C_p\left(T_m' \ln \frac{T_m}{T_m'} + T_m' - T_m\right) \quad (1)$$

where T_m and T_m' are transition temperatures for the apo-N-domain before and after crossover, respectively, ΔH_m is the heat change at T_m, and ΔC_p is the temperature-independent change in heat capacity for the transition. This gives an estimate of −3050 cal/mol for ΔG_{AB} at 72.5 °C, which pertains to interactions between the apo N- and Fe-C-domains in intact hTF.

Apparent binding constants to both C- and N-sites in hTF may also be estimated from the results in Figure 2, using equations (Brandts et al., 1989; Brandts & Lin, 1990) based on thermodynamic modeling of DSC results for ligand–protein systems. Binding of the first ligand to the C-domain of an hTF molecule results in two effects; first, the shift of the C transition from 57.62 to 87 °C and, second, the shift of the N transition from 68.35 to 72.5 °C produced by crossover. The total free energy of binding is equal to the sum of these two factors, which translates into an apparent binding constant of 2.5×10^{20} M^{-1} at 87 °C. Using the measured heat and heat capacity of binding Fe–NTA to the C-site of hTF (ΔH of −10.7 kcal at 27 °C and ΔC_p of −70 cal/deg) previously determined from titration calorimetry (Lin et al., 1993), the apparent binding constant can be extrapolated from 87 °C to give an estimate of 1.1×10^{22} M^{-1} at 25 °C. The estimated binding constant to the N-site is 8×10^{12} M^{-1} at 87 °C and 8×10^{13} M^{-1} at 25 °C.

Saturation of Binding Sites of OTF. The data in Figure 3 show DSC scans as a function of site saturation of OTF using Fe–NTA, progressing from the apo form in scan 1 at the bottom to diferric OTF in scan 5 at the top. Under conditions similar to those of Figure 3, the N-site saturates first at low levels of Fe–NTA (Williams et al., 1978; Brock, 1985; Lin et al., 1991) although the preference of the N-site in OTF is not so strong as the preference for the C-site in hTF. Thus at a saturation level of 0.44 sites in the second scan from the bottom in Figure 3, most of the ferric ions are bound to the N-site (designated as Fe_N–OTF). At first glance, it may seem surprising that *two new peaks* appear at 66 and 76 °C, in addition to the residual peak for apo-OTF at 61 °C, rather than a single new peak which might be expected to occur for the Fe-N-domain. However, for a protein with two interacting domains whose transitions are coupled, a selective perturbation to one domain (e.g., binding Fe^{3+} to the N-site) which acts to increase its stability much more than that of the unaffected domain will uncouple the two transitions and both will then have a T_m higher than the original T_m for the coupled transition (Brandts et al., 1989). The reason for the T_m shift for the "unaffected" domain is that after the perturbation it will receive all of the stabilization from ΔG_{AB} interactions since its domain partner remains folded to higher temperatures. The transition centered at 66 °C is for those apo-C-domains which are attached to Fe-N-domains, while the transition at 76 °C is for the corresponding Fe-N-domains themselves. The small

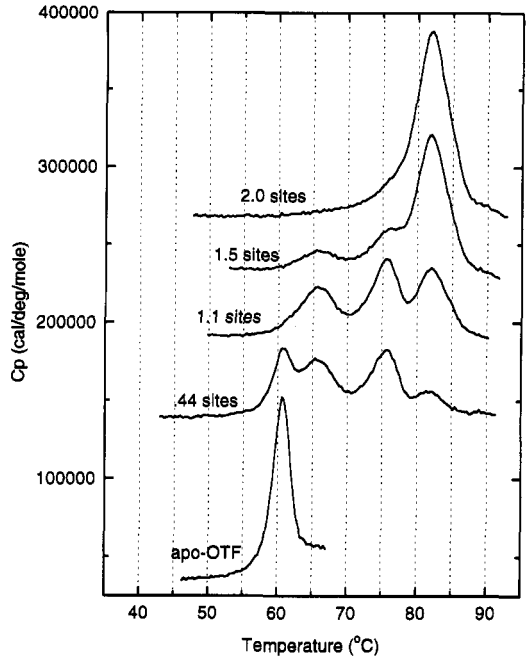

FIGURE 3: DSC scans of OTF with different degrees of iron saturation. Various amount of Fe–NTA were added to apo-hTF (ca. 0.02 mM) to achieve the binding. The scan rate was 81.5 °C/h.

peak at ca. 82 °C is due to a small amount of Fe-C-domain attached to an apo-N-domain (e.g. Fe_C–OTF) and possibly small amounts of diferric OTF (Fe_2–OTF) which might form even at this low level of saturation.

The peak assignments above are in disagreement with interpretations from previous DSC studies of Donovan and colleagues (Donovan et al., 1976; Donovan & Ross, 1975). They suggested that when Fe–NTA is added to OTF the first ferric ion binds indiscriminately and accordingly assigned the middle two transitions at 66 and 76 °C to the two different monoferric species, Fe_C–OTF and Fe_N–OTF, while we assign these two transitions to the individual domains of a single monoferric species, Fe_N–OTF. Our interpretation is consistent with results obtained using other techniques, which showed that NTA-chelated ferric ion has a strong preference to bind first to the N-site of OTF (Williams et al., 1978; Brock, 1985; Lin et al., 1991).

At 1.1 sites saturated, the transition for apo-OTF is no longer evident, and most of the area resides in the peaks at 66 and 76 °C for Fe_N–OTF and the peak for diferric OTF at 82 °C (which could also include small amounts of unfolding of the C-domain in Fe_C–OTF since preference for the N-site is not absolute). Since the 66 and 76 °C peaks correspond primarily to unfolding of two halves of the same molecules, then each must represent unfolding of the same number of domains. The 76 °C peak is about 30–40% larger in area than the 66 °C peak, and this is in good agreement with our earlier suggestion that the two transitions will be of approximately equal thermodynamic size when peak areas are extrapolated to the same temperature using the ΔC_p of 6.6 kcal/deg determined for the apo-form. This assumes that the heat contribution from release of bound ferric ion upon unfolding of the Fe-C-domain is small relative to the total heat of unfolding, which is known to be the case (Lin & Brandts, 1990).

The domain interaction term and the apparent binding constants for OTF can be calculated using the same procedure as for hTF. Binding of the first ferric ion to the N site results in shift of the C transition from 60.24 to 66 °C, which leads

to a ΔG_{AB} estimate of −3100 cal at 66 °C. Note this is a *minimum magnitude* estimate of ΔG_{AB}. Since the two transitions are coupled in apo-OTF, the C-domain may receive some stabilization from domain interactions while it will receive the total stabilization in the Fe_N–OTF form. Results on domain fragments, discussed below, show that ΔG_{AB} is in fact larger in magnitude than −3100 cal.

The apparent binding constant to the N-site in OTF can be estimated by the combined shifts of both the C and N transition brought about by binding of the first ferric ion, as done earlier for hTF. The binding constant thus determined is 3×10^{13} M^{-1} at 76 °C, which extrapolates to a value of 1.5×10^{14} M^{-1} at 25 °C using the measured values of the heat and heat capacity of binding to the N-site (Lin et al., 1991).

When the second ferric ion binds to the C-site, its transition shifts from 66 to ca. 82 °C. In the process, it crosses over the transition of the N-domain at 76 °C which causes the two transitions to recouple so that both transitions are in the broad peak near 82 °C in the diferric form. The broad nature of this peak may be at least partially due to changes in the concentration of free Fe–NTA resulting from ligand release with progressive unfolding, as pointed out earlier for hTF. From these combined shifts in T_m's, the apparent binding constant to the C-site can be estimated as 2×10^{15} M^{-1} at 82 °C or 1.5×10^{18} M^{-1} at 25 °C. Note that the estimate of apparent binding constant for the C site is substantially larger than for the N site even though it is the N site which saturates first. It was already shown (Lin et al., 1991) that site preference for OTF at room temperature is controlled by kinetic rather than thermodynamic factors.

Kinetic Control of the Binding Process. Estimates of binding constants of ferric ions to transferrins have been reported in the literature on numerous occasions, with considerable disagreement between various estimates over the years. Measurements are difficult because binding constants are so large and ligand release is slow, making equilibrium difficult to achieve (Aisen & Leibman, 1968). There is also a strong tendency for ferric ions to polymerize into polynuclear aggregates near neutral pH. The addition of chelators, such as NTA or citrate, to compete with transferrins for ferric ion binding lessen all of these problems to some extent since they reduce the effective binding constant, break up polynuclear aggregates, and shorten the time for ligand release. The current estimate considered to be most reliable is that of Aisen et al. (1978) for hTF. Equilibrium studies, with very long equilibrium times, were carried out in the presence of high citrate concentration and results back-corrected to zero citrate concentration from the known complexation constants of ferric ion to citrate. Under condition similar to ours, this led to estimates of 4.7×10^{20} M^{-1} for binding at the stronger site and 2.4×10^{19} M^{-1} at the weaker site.

Our DSC estimates for the binding constants to hTF appear to be too large, when compared to these accepted estimates. This is particularly obvious for the C-site, where our estimate of ca. 10^{22} M^{-1} in the presence of 0.05 mM NTA is larger even than the estimate of Aisen et al. (1978) for equilibrium binding in the absence of any chelator.[3] This suggests that T_m values observed for liganded forms of transferrin are too high to represent the equilibrium unfolding process, which would occur at substantially lower temperature if the kinetics of ligand release were faster or if the scan rate were infinitely slow.

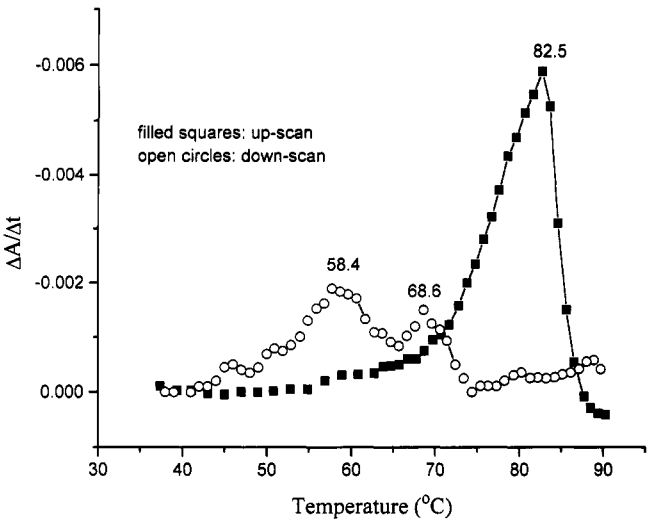

FIGURE 4: Spectrophotometric thermal scans (monitored at 465 nm) of the binding equilibrium of Fe–hTF ([hTF] = 0.058 mM; [Fe–NTA] = 0.0128 mM) in 500 mM HEPES, 25 mM $NaHCO_3$, pH 6.74. The data were plotted in derivative format (i.e. $\Delta A/\Delta t$). The Fe–hTF solution was upscanned (solid squares) from 35 to 92 °C and then immediately downscanned (open circles) to 35 °C, at a scan rate of 60 °C/h.

Kinetic effects in the DSC results on hTF were revealed by comparing results from two different tests for "reversibility". If the first upscan on holo-hTF is followed by rapid cooling to room temperature or below, then a second upscan shows ca. 60% reversibility with all of the refolded protein participating in the transition of the holo-form at a T_m identical to that seen on the first upscan. On the other hand, if the first upscan is followed immediately by a downscan, then no transition is seen at the T_m of the holo form and refolding is not observed until the T_m's of the two apo-domains are reached at much lower temperature. Although this could be crudely demonstrated by DSC downscans under the above conditions, results showed very poor overall reversibility due to the high protein concentrations necessary for downscanning compounded by the long amount of time which the protein spent in the unfolded state during the upscan and subsequent downscan.

Better results were obtained using optical methods to monitor upscans/downscans at a lower concentration of hTF and at slightly lower pH, 6.74, which substantially reduced problems from irreversible aggregation and precipitation at high temperature. Some results are shown in Figure 4 in the

[3] Although the chelation constant of NTA to ferric ion is available for acid pH values (ca. 10^{16} M^{-1} for the 1:1 complex below pH 4), the formation of hydroxo complexes prevents determination near neutral pH. Because of this, we were unable to "correct" our DSC estimate of binding constants obtianed in the presence of NTA to the values which would be expected in the absence of NTA. We did carry out a DSC study where citrate ion, rather than NTA, was used as the chelator over the range of citrate concentration from 1 to 33 mM. The T_m values for the ferric ion complexes were very similar to low concentration of citrate as reported above at low concentration of NTA. Using the method of Aisen et al. (1978) and the same chelation constants which they used for citrate, we corrected our DSC estimates of binding constants to zero citrate concentration. The binding constants so determined varied from 10^{34} M^{-1} with extrapolation from the lowest citrate concentration, to 10^{26} M^{-1}, from the highest. These studies were carried out on the diferric form so correspond to an average of the two sites in hTF. Since the values are citrate-dependent and substantially larger than those obtained by Aisen et al. using very long equilibration times, this reinforces the conclusion based on studies using NTA; i.e., T_m values are controlled by kinetic factors within the time frame of DSC experiments and therefore cannot be used to estimate true binding constants.

derivative format, where the squares represent the first upscan and circles correspond to the subsequent downscan. On the upscan, the large broad peak near 82.5 °C corresponds to the transition of the holo-form of hTF. In the downscan, there is no transition at a T_m corresponding to the peak seen in the upscan, but two transitions occur at lower temperatures, 58.4 and 68.6 °C, very close to the T_m values seen for the C- and N-domains, respectively, when upscans are carried out in the absence of ferric ion. Note that optical measurements were made at 465 nm so the signal is actually monitoring the rebinding of ferric ion to hTF, which apparently occurs concomitantly with or subsequent to refolding to each apo-domain.

In spite of the good apparent reversibility displayed in second upscans, downscan results show that the protein is not in reversible equilibrium with the Fe–NTA at the T_m of holo-transferrin. Apparently, released ferric ion cannot rebind at the T_m of holo-transferrin, and in order for rebinding to occur the temperature must be lowered to where the domains refold spontaneously in the absence of ferric ion. Since the system is not in reversible equilibrium even when Fe-NTA is present, the apparent binding constants do not represent true binding constants even though they could have some qualitative validity relative to one another.

In theory, DSC upscans could be done at scan rates slow enough to reveal the true thermodynamic T_m for liganded forms of transferrin. In practice, upscans carried out at slower scan rates gave only a marginal indication of kinetic factors, since T_m shifted down by less than 2 °C when the scan rate was lowered from 80 to 20 °C/h. This is an indication that the temperature dependence of the off rate (i.e., E_a for ligand release) is very large so that variations in the upscan rate are not so revealing of kinetic control as are downscans. The difficulty in going to even lower scan rates than 20 °C/h is that protein concentrations must be increased substantially and this, along with the inordinately long time spent at high temperature, exacerbates the precipitation problem.

Similar upscan and downscan experiments were carried out on apo-hTF, except the process was monitored using the change in absorbance of aromatic residues at 292.5 nm. It was found that within errors the T_m values of transitions for both the C- and N-domain were the same for upscans and downscans. This indicates that the kinetic control seen in the presence of Fe–NTA has to do with ligand binding or release rather than with an intrinsic property of the protein.

Since no other protein–ligand system has to our knowledge been observed during downscanning, we reexamined the RNase A/2′CMP system studied earlier only by upscanning (Brandts & Lin, 1990) to satisfy ourselves that at least some systems behave in the expected thermodynamic manner. Results from DSC upscans and downscans (Lin & Brandts, unpublished data) showed that this system is in true reversible equilibrium. It should be pointed out that the binding constant for the RNase system studied was only $\sim 10^6$ M^{-1} and ligand release is known to be fast on a DSC time scale (Cathou & Hammes, 1965; Erman & Hammes, 1966). Other very tight systems with slow ligand release could behave similarly to transferrins.

Dependence of ΔG_{AB} on pH. pH changes, particularly on the acid side of neutrality, exert strong effects on the binding and release of iron from transferrins (Aisen et al., 1978; Brock, 1985). We were interested in determining if domain interactions might also be influenced by pH changes. Results from DSC scans on both OTF and hTF are shown in Figure 5 at pH values of 6.3, 7.5, and 8.5 and at intermediate levels of

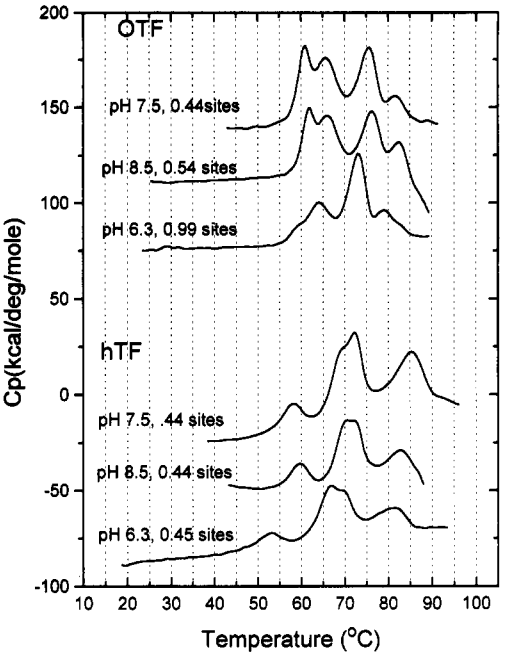

FIGURE 5: DSC scans of hTF and OTF (in 500 mM HEPES, 25 mM $NaHCO_3$) at pH 6.3, 7.5, and 8.5. The degree of iron saturation and the pH of the solutions are indicated. The experimental conditions were the same as those for Figures 2 and 3.

saturation with Fe–NTA. Reversibilities over this pH range, from second upscans, were similar to or better than indicated earlier for pH 7.5.

There is a definite trend for T_m values for the two apo-forms to shift higher with increasing pH. This is very evident for hTF in going from pH 6.3 to 7.5, where the difference is 5 °C. It can also be seen that the two transitions for hTF at intermediate temperature, corresponding to transitions of the apo-N domain before and after perturbation crossover, overlap more at pH 8.5 than at pH 7.5, which suggests a weaker ΔG_{AB} at the more alkaline pH. To make numerical estimates of ΔG_{AB}, we will use the same parameter sets presented earlier for apo-hTF and apo-OTF at pH 7.5 and assume they are also appropriate at pH 6.3 and 8.5. This is in keeping with numerous DSC studies on other proteins (Privalov, 1979) which show very small effects of pH on ΔH and ΔC_p, even when there are large effects on T_m values. Proceeding in this manner, estimates of ΔG_{AB} for hTF are −2500, −3050, and −2100 cal/mol at pH 6.3, 7.5, and 8.5, respectively, while minimum magnitude estimates for OTF are −2700, −3100, and −2600 cal/mol. These data show that domain interactions are maximal near physiological pH for both transferrins and can decrease as much as 30% with a change of one unit in pH.

Studies on Domain Fragments of OTF. Studies on intact OTF provide only a minimum magnitude estimate for ΔG_{AB} because of coupling of the two transitions in the apo-protein. To get an absolute estimate it is necessary to study the domain fragments of OTF, which have been prepared proteolytically as described in the experimental section. Shown in Figure 6 are scans carried out on the apo- and ferric forms of both the N fragment and the C fragment at pH 7.5. These are shown relative to the scan of intact OTF. The ΔH and T_m of the apo-C fragment are 150 kcal and 59 °C and for the apo-N fragment are 120 kcal and 52 °C. Although ΔC_p cannot be accurately estimated from these data on fragments, the ΔH values are in reasonable agreement with expected values from extrapolations for the intact apo-protein. Using the ΔH, T_m, and ΔC_p arrived at earlier for the intact molecule and

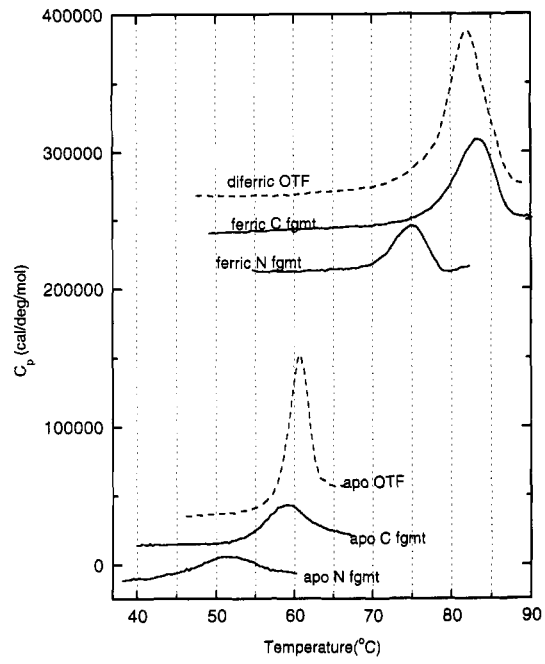

FIGURE 6: DSC scans on the N and C fragments of OTF in 500 mM HEPES, 25 mM $NaHCO_3$, pH 7.5. The experimental conditions were the same as those for the intact OTF shown in Figure 3. For purpose of comparison, the DSC scans for the intact apo-OTF and differic OTF are also plotted as dotted lines.

extrapolating down to the T_m values of the two fragments gives an estimate of ΔG_{AB} of −4100 cal/mol. This is significantly larger than the minimum estimate of −3100 cal/mol based on partial crossover of transitions.

Binding of Fe–NTA shifts the transition of the N fragment to higher temperature by ca. 23 °C while for the C fragment the shift is ca. 25 °C. In view of the kinetic factors discussed above, no conclusions can be drawn regarding true binding constants.

DISCUSSION

Earlier studies (Brandts et al., 1989) showed that the free energy of domain–domain interactions in proteins can be estimated from DSC results using the assumption that interactions will act to raise the T_m of the least-stable domain of the interacting pair if the two domains show separate unfolding transitions. The method of transition crossover can be readily applied to hTF, since binding the first ferric ion to the C-domain shifted its T_m from a temperature below that of the N-domain to a temperature substantially above it. Although the N-domain did not participate directly in the binding process, its T_m was nevertheless shifted upward by some 4 °C since subsequent to binding it is stabilized by domain interactions. The estimate of ΔG_{AB} made from the DSC data is −3050 cal, which suggests moderately strong interactions between the N and C lobes, consistent with the rather small area of contact seen in the crystallographic structure of transferrins (Baker et al., 1991).

In contrast to hTF, the two domains of apo-OTF participate in a single, nearly coupled transition. Because of this, transition crossover can only be partially accomplished when the first ferric ion is bound, leading to a minimum magnitude estimate of −3100 cal for ΔG_{AB}. From study of the N and C fragments, a total value of −4100 cal was found for ΔG_{AB}, indicating marginally stronger domain interactions than for hTF.

The onset of coupling of transitions in a two-domain protein requires that ΔG_{AB} becomes equal in magnitude to the difference in free energy of unfolding of the two isolated domains. Only when ΔG_{AB} exceeds the difference in intrinsic free energies by 2000–2500 cal (Brandts et al., 1989) does coupling become complete so that a single two-state transition model is able to accurately fit the entire transition envelope. Our studies on the isolated domain fragments of OTF indicate that the C fragment is more stable than the N fragment by approximately 2500 cal, while ΔG_{AB} is −4100 cal, leading to a difference between the two of 1600 cal. According to this, the coupling should be less than complete, which is in agreement with the observation that the OTF transition is a little too broad to be fit perfectly by a single two-state transition.

Although studies on the domain fragments of hTF were not carried out, the transitions of the two domains are separated in intact hTF and the difference in free energy can be estimated as 6000 cal. If this is added to the ΔG_{AB} which was found or hTF, then the difference in intrinsic free energy of the isolated domains will be ca. 9000 cal or nearly four times as large as for OTF. Thus, although domain–domain interactions are slightly weaker for hTF than for OTF, the major difference leading to observation of two separated transitions for hTF is the 4-fold larger difference in intrinsic stability of the two domains.

The apparent binding constants of ferric ions to hTF evaluated from DSC upscans are 1.1×10^{22} M^{-1} for the C-site and 8×10^{13} M^{-1} for the N-site in 25 mM bicarbonate, extrapolated to 25 °C, when the concentration of chelator NTA is twice that of added ferric ion. The corresponding estimates for OTF are 1.5×10^{18} and 1.5×10^{14} M^{-1}. Considering the relatively high concentration of chelator present, these estimates appear to be much larger than accepted values (see footnote 2) obtained after lengthy equilibrium at low temperature using direct binding techniques. This indicates that the observed T_m values on upscans are controlled by kinetic, rather than thermodynamic, factors at the scan rates which were used. We feel that the true T_m's for the reversible unfolding–refolding for liganded forms of transferrins are substantially lower than the observed T_m's seen during upscans, and that this leads to overestimates of binding constants. At the true T_m, the unfolding reaction is so slow relative to the scan rate that the reaction does not progress perceptibly toward completion. It seems likely that the rate of unfolding is limited by slow ligand release, since this is known to be extremely slow for transferrins at lower temperatures and since the kinetic complexities of unfolding were not seen for the apo-proteins. As the temperature is raised above the true T_m, ligand release and unfolding will become faster until a temperature is reached where unfolding can occur quickly, but irreversibly. When carrying out experiments at slower scan rates down to 20 °C/h for liganded forms of hTF, we did find the observed T_m decreased by ca. 2 °C while T_m for the apo-form was nearly unchanged at a lower scan rate.

During downscanning, on the other hand, T_m values obtained from optical studies which monitor ferric ion binding occur at virtually the same temperature as does protein refolding in the absence of Fe–NTA. Thus, in the time allowed by the downscan rates which were used, ferric ion is unable to bind until the ligand-free protein has refolded spontaneously. It was also shown that not all proteins behave in this manner, since the presence of 2′CMP in RNase A solutions did permit refolding of the protein at higher temperatures during downscans relative to refolding in the absence of 2′CMP. The unusual nature of the binding pocket and the requirement for association of the synergistic anion prior to or simultaneous

with ligation to ferric ion may make it difficult for binding to occur in short time periods. Transferrins are unusual among metalloproteins in that none of the four amino acid ligation sites lies within four residues of another in the primary sequence. The synergistic anion requires an intact α-helix 7 and correct positioning of Arg 124 to bind and partially neutralize positively charged groups in the binding cleft. These factors may place time constraints on the formation of the nascent binding sites in the absence of complete protein refolding.

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