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Effects of Ionic Strength on Iron Removal from the Monoferric Transferrins

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The effect of ionic strength on the rate of iron removal from the monoferric transferrins by the synthetic catecholate *N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (3,4-LICAMS) has been investigated at pH 7.4 and various temperatures. The apparent activation enthalpies for iron release from C-terminal monoferric transferrin remain the same or slightly decrease from 20 (1) to 16 (5) kcal/mol as the ionic strength is increased from 0.134 to 1.95 M. The break in the Arrhenius plot previously reported for N-terminal monoferric transferrin (which indicates that the protein is undergoing an apparent conformational change) becomes less distinct with the increase of ionic strength. In general, the rates of iron release increase with increasing ionic strength but are most sensitive to ion binding at the lower temperatures. Most remarkably, extrapolations of the rates of iron removal from both monoferric transferrins at all temperatures studied indicate that no iron is released when the ionic strength of the reaction mixture is zero! It is suggested that ion binding to the protein promotes a structural change that is an absolute prerequisite for iron release.

Introduction

The iron transport protein serotransferrin, found in blood serum, is a monomeric glycoprotein of molecular weight ~80 000.[1,2] The protein is bilobal, with each lobe reversibly (and essentially independently) binding a ferric ion concomitantly with a synergistic anion, bicarbonate (or carbonate), in vivo.[1-5] Transferrin is unique in this anion requirement for metal binding. Evidence that the synergistic anion binds directly to the metal and protein has been reported, and it has been suggested that the anion plays a role in the physiological removal of iron from transferrin.[2,6-11]

Nonsynergistic anions also influence the chemistry of transferrin. Price and Gibson found that perchlorate perturbs the EPR spectrum of transferrin.[12] More recent EPR studies have shown that the strengths of anion–protein interactions parallel the lyotropic series (which orders anions according to their relative effectiveness of inducing conformational changes in proteins),[13] two anions bind in each domain of the protein with strong positive cooperativity, and perchlorate induces site-specific spectral changes in the protein that are similar to those observed when cationic lysine residues are chemically modified.[14,15] These nonsynergistic anions are also known to facilitate the removal of iron from transferrin by various chelators and to differentially affect the thermodynamic and kinetic behavior of the metal-binding sites.[16-21]

The mechanistic details of the processes by which anions alter the behavior of transferrin are not known. However, the structure of lactoferrin, a closely related protein, was recently resolved to 3.2 Å and may help in the elucidation of the specific anion–protein interactions.[4,5] Information as to whether anions promote conformational changes at the metal-binding sites or bind to any of the basic sidechains and helix N-termini that exist in the two channels of the metal-binding sites awaits further crystallographic studies.

We have previously reported a detailed kinetic study of iron removal from the diferric and monoferric transferrins by the synthetic catechol sequestering agent *N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (3,4-LICAMS)[22,23] and showed evidence that the N-terminal monoferric transferrin undergoes an apparent conformational change at ~20 °C, which might explain the observation that iron is released faster from the N-terminal site than the C-terminal site at higher temperatures.[24] It was also shown that the rates of iron release from the two sites of diferric transferrin approximately correlate with those obtained for iron removal from the respective monoferric transferrins. Thus, the monoferric transferrins can be used as models for the corresponding sites in diferric transferrin, which is advantageous since the complicated biphasic kinetic behavior of iron release from diferric transferrin can be simplified into two first-order processes of iron release from the N-terminal and C-terminal monoferric transferrins.

In this paper, the effects of anions, or more specifically ionic strength, on the kinetics of iron removal from the monoferric transferrins by 3,4-LICAMS at various temperatures have been examined. No evidence was seen in the Arrhenius plots for iron removal from C-terminal monoferric transferrin to indicate that ions promote temperature-dependent conformational changes, while the magnitude of the break previously observed in the Arrhenius plot for N-terminal monoferric transferrin[24] diminished as ionic strength was increased. Although more pronounced effects were seen at the lower temperatures, the rates of iron release from both monoferric transferrins increased with increasing ionic strength. Most significantly, extrapolations of the rate dependence on ionic strength for both monoferric transferrins at all temperatures indicate that *no* iron is removed by 3,4-LICAMS when the

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(1) Chasteen, N. D. *Advances in Inorganic Biochemistry*; Thiel, E. C., Eichorn, G. L., Marzilli, L. G., Eds.; Elsevier: New York, 1983; Vol. 5, pp 201-233.
(2) Aisen, P.; Listowsky, I. *Annu. Rev. Biochem.* **1980**, *49*, 357-393.
(3) Bailey, S.; Jhoti, H.; Garratt, R.; Gorinsky, B.; Lindley, P.; Sarra, S.; Evans, R.; Hasnain, S. Presented at the 8th International Conference on Proteins of Iron Transport and Storage, Quebec, Canada, May 1987; paper 3.
(4) Anderson, B. F.; Baker, H. M.; Dodson, E. J.; Norris, G. E.; Rumball, S. V.; Waters, J. M.; Baker, E. N. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1769-1775.
(5) Baker, E. N.; Rumball, S. V.; Anderson, B. F. *Trends Biochem. Sci.* **1987**, *12*, 350-353.
(6) Schlabach, M. R.; Bates, G. W. *J. Biol. Chem.* **1975**, *250*, 2182-2188.
(7) Campbell, R. F.; Chasteen, N. D. *J. Biol. Chem.* **1977**, *252*, 5996-6001.
(8) Zweier, J. L.; Wooten, J. B.; Cohen, J. S. *Biochemistry* **1981**, *20*, 3505-3510.
(9) Aisen, P.; Leibman, A.; Pinkowitz, R. A.; Pollack, S. *Biochemistry* **1973**, *12*, 3679-3684.
(10) Egyed, A. *Biochem. Biophys. Acta* **1973**, *304*, 805-813.
(11) Aisen, P.; Brown, E. B. *Prog.* Hematol. **1975**, *9*, 25-56.
(12) Price, E. M.; Gibson, J. F. *J. Biol. Chem.* **1972**, *247*, 8031-8035.
(13) Record, M. T.; Anderson, C. F.; Lohman, T. M. *Q. Rev. Biophys.* **1978**, *2*, 103-178.
(14) Folajtar, D. A.; Chasteen, N. D. *J. Am. Chem. Soc.* **1982**, *104*, 5775-5780.
(15) Thompson, C. P.; McCarty, B. M.; Chasteen, N. D. *Biochim. Biophys. Acta* **1986**, *870*, 530-537.
(16) Egyed, A. *Biochim. Biophys. Acta* **1975**, *411*, 349-356.
(17) Pollack, S.; Vanderhoff, C.; Lasky, F. *Biochim. Biophys. Acta* **1977**, *497*, 481-487.
(18) Morgan, E. H. *Biochim. Biophys. Acta* **1979**, *580*, 312-326.
(19) Chasteen, N. D.; Williams, J. *Biochem. J.* **1981**, *193*, 717-727.
(20) Baldwin, D.; de Sousa, D. M. R. *Biochem. Biophys. Res. Commun.* **1981**, *99*, 1101-1107.
(21) Williams, J.; Chasteen, N. D.; Moreton, K. *Biochem. J.* **1982**, *201*, 527-532.
(22) Weitl, F. L.; Harris, W. R.; Raymond, K. N. *J. Med. Chem.* **1979**, *22*, 1281-1283.
(23) Raymond, K. N.; Müller, G.; Matzanke, B. F. *Top. Curr. Chem.* **1984**, *123*, 49-102.
(24) Kretchmar, S. A.; Raymond, K. N. *J. Am. Chem. Soc.* **1986**, *108*, 6212-6218.

0020-1669/88/1327-1436$01.50/0

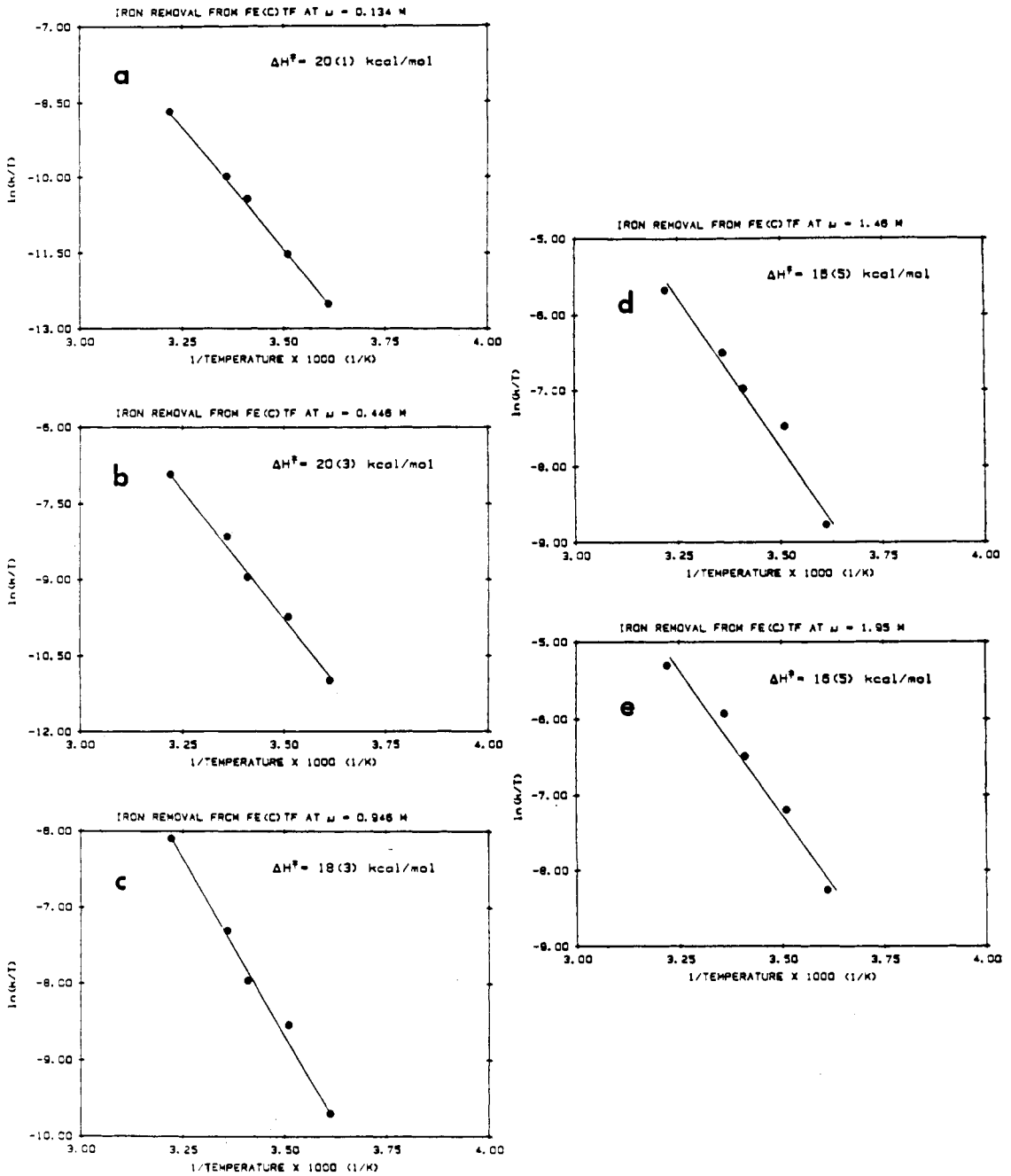

Figure 1. Plots of observed rate constants (min^{-1}) as a function of temperature for iron removal from 0.100 mM C-terminal monoferric transferrin by 12.00 mM 3,4-LICAMS in 0.050 M HEPES buffer, pH 7.4 at the following ionic strengths (adjusted by KCl): (a) 0.134 M; (b) 0.446 M; (c) 0.946 M, (d) 1.46 M; (e) 1.95 M. The activation enthalpies, $\Delta H^{\ddagger}$'s, were determined from the slopes of the lines.

ionic strength of the reaction mixture is zero. The implications of these observations, which have also been extended to the C-terminal and N-terminal sites of diferric transferrin in accordance with the above discussion, will be presented.

Experimental Section

Distilled deionized water was used at all times. Glassware was washed with phosphate-free RBS-pf (Pierce Chemical Co.) and rinsed thoroughly with water. Dialysis tubing (Bethesda Research Laboratories) was boiled in water, placed in a solution containing 0.1% EDTA for short-term storage, and washed several times with water prior to use. All solutions were adjusted to pH 7.4 at the specified temperatures. Urea–polyacrylamide gel electrophoresis was carried out by using standard procedures.[19,25]

Human serum apotransferrin (Calbiochem-Behring) was dissolved in water (20 mg/mL); the protein was dialyzed for a minimum of 9 h at 4 °C against several changes of water to remove contaminating chelates and then against a solution containing 0.050 M *N*-(2-hydroxyethyl)-piperazine-*N'*-ethanesulfonic acid (HEPES, United States Biochemical Corp.) and 0.020 M $NaHCO_3$.[26,27] The molar absorption coefficients were determined by titration of the dialyzed apoprotein with a fresh solution of 2.00 mM ferric nitrilotriacetate $[Fe(NTA)_2]$[27] to be 9.18×10^4 M^{-1} cm^{-1} at 279 nm and 2600 M^{-1} cm^{-1} per iron at 466 nm. Since some time-dependent hydrolysis of $Fe(NTA)_2$ occurs, the $Fe(NTA)_2$ solutions were prepared just prior to use in the following manner: NTA (Eastman Chemical Corp.) was dissolved in water at pH 4.0, ferric chloride at pH 1.0 was added, and the pH was raised to 4.0–4.5 with dilute NaOH.

Monoferric Transferrins. The monoferric transferrins were prepared as previously reported.[24] For C-terminal monoferric transferrin, apotransferrin was dissolved and dialyzed as described above. Freshly prepared $Fe(NTA)_2$ was added to the apotransferrin at pH 7.4 to fill 50% of the sites. The solution immediately turned red and was stirred for 5 min at room temperature. It was dialyzed against a 0.050 M buffer and 0.100 M $NaClO_4$ solution for 3 h at 25 °C and for 1 h at 4 °C to remove

(25) Makey, D. G.; Seal, U. S. *Biochim. Biophys. Acta* **1976**, *453*, 250–256.

(26) Bates, G. W.; Schlabach, M. R. *J. Biol. Chem.* **1973**, *248*, 3228–3232.
(27) Bates, G. W.; Wernicke, J. *J. Biol. Chem.* **1971**, *246*, 3679–3685.

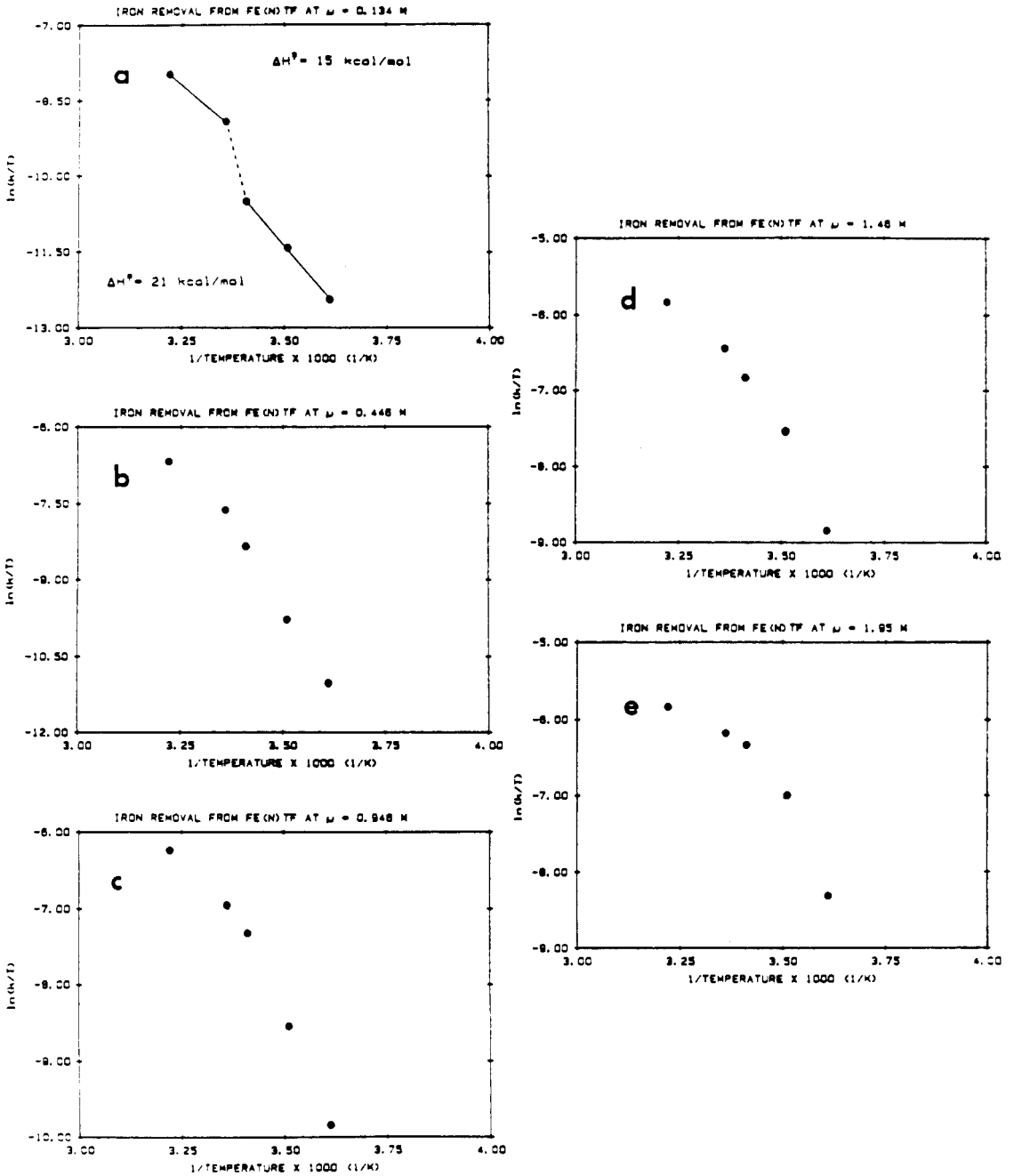

Figure 2. Plots of observed rate constants (min^{-1}) as a function of temperature for iron removal from 0.100 mM N-terminal monoferric transferrin by 12.00 mM 3,4-LICAMS in 0.050 M HEPES buffer, pH 7.4 at the following ionic strengths (adjusted by KCl): (a) 0.134 M; (b) 0.446 M; (c) 0.946 M; (d) 1.46 M; (e) 1.95 M. The activation enthalpies, ΔH°'s, were determined from the slopes of the lines where appropriate.

NTA and unbound iron and $NaHCO_3$ and finally for 9 h against 0.050 M HEPES buffer adjusted to pH 7.4 at the desired temperature. Urea–polyacrylamide gel electrophoresis, used to verify the purity of the C-terminal monoferric transferrin, showed the presence of C-terminal monoferric transferrin with a very small amount of apotransferrin.

For N-terminal monoferric transferrin,[28] apotransferrin was dialyzed at 4 °C against several changes of water and finally against a solution of 0.050 M HEPES buffer and 0.020 M $NaHCO_3$, pH 7.8. Freshly prepared $Fe(NTA)_2$ at pH 4.0 was added to saturated 100% of the sites. The red solution was left overnight at room temperature and then diluted to 0.010 mM transferrin (0.020 mM total iron concentration) with a solution in which the final concentrations of species were 0.100 M HEPES buffer, 1.00 M $NaClO_4$, and 1.00 mM pyrophosphate, pH 7.5. Desferal mesylate (Ciba-Geigy Pharmaceutical Co.) was added as a solution to give a final concentration of 1.00 mM. The solution was left to stand at room temperature for 4 h. Under these conditions, iron was preferentially removed from the C-terminal site as was confirmed by electrophoresis, which showed the presence of N-terminal monoferric transferrin and some apotransferrin. The solution was concentrated by ultrafiltration (Amicon PM-30 membrane) while being stirred under Ar at 4 °C. The N-terminal monoferric transferrin was then dialyzed against several changes of 0.050 M HEPES buffer adjusted to pH 7.4 at the appropriate temperature.

Visible Spectroscopy. Kinetic studies of iron removal from the monoferric transferrins by 12.00 mM *N,N',N''*-tris(5-sulfo-2,3-dihydroxybenzoyl)-1,5,10-triazadecane (3,4-LICAMS) were performed in 1-cm quartz cuvettes maintained at constant temperature by using a Hewlett-Packard 8450A double-beam diode array spectrophotometer equipped with thermostated cells. Potassium chloride was added to solutions of 0.100 mM filtered iron transferrin (0.2-μM Gelman Acrodiscs) in order to obtain the desired ionic strengths. The reference cell contained corresponding concentrations of buffer and KCl. The mixing of the protein with ligand was recorded as zero time and scans were taken every 5–240 s depending on the reaction rates. Absorbance values versus wavelength were collected and stored digitally.

Ionic Strength Calculations. Ionic strength calculations were made for the buffer and KCl by using the Debye–Hückel equation[29]

$$\mu = \tfrac{1}{2}\Sigma m_i z_i^2 \qquad (1)$$

(28) Chasteen, N. D., private communication, March, 21, 1985.

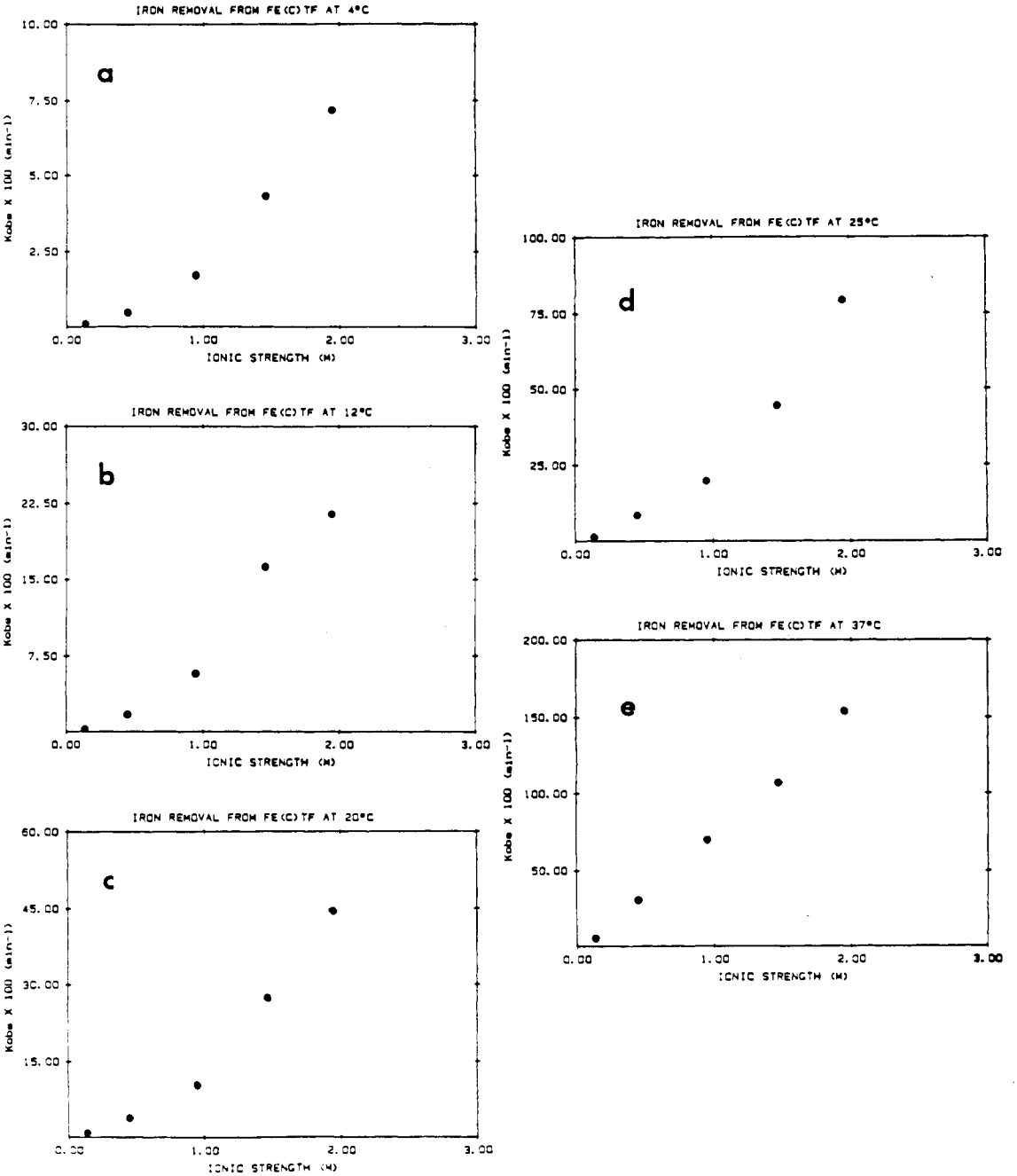

Figure 3. Plots of observed rate constants (min^{-1}) as a function of increasing ionic strength (adjusted with KCl) for iron removal from 0.100 mM C-terminal monoferric transferrin by 12.00 mM 3,4-LICAMS in 0.050 M HEPES buffer, pH 7.4 at the following temperatures: (a) 4 °C; (b) 12 °C; (c) 20 °C; (d) 25 °C; (e) 37 °C.

where m_i is the molality of the ith species, z_i is the charge of the ion in units of electronic charge, and the sum is taken over all ionic species present.

Because the Debye–Hückel theory assumes isolated point-charged species, this equation is not appropriate for the calculation of ionic strength for the 3,4-LICAMS molecule with its widely dispersed and large number of charges. The other extreme, to treat each charged site of 3,4-LICAMS as a discrete spherical charge, is also not satisfactory. In our calculations of ionic strength for 3,4-LICAMS, we have taken the average value of these two extremes, which would give rise to differences in the calculated μ of a maximum of 6% at μ = 1.00 M.

Results and Discussion

Pseudo-first-order kinetic behavior was obtained for iron removal from the monoferric transferrins by excess 3,4-LICAMS. A higher than necessary concentration of the ligand was used to ensure that the rate constants had reached the saturation level with respect to ligand concentration at each temperature and ionic strength studied. Spectral changes arising from the addition of 3,4-LICAMS to the monoferric transferrins were as previously reported in which the band maximum of the monoferric transferrins at ~466 nm shifted to 495 nm, characteristic of tris(catecholato)iron(III) complexes.[24] The rates of iron removal were calculated by using linear regression analysis of data plotted as ln $[(A - A_\infty)/(A_0 - A_\infty)]$ versus time (not shown) where the absorbance values chosen were at λ 520 nm.

Temperature-Dependence Studies. The temperature dependence of the kinetics of iron removal from the two monoferric transferrins by 3,4-LICAMS at five different ionic strengths was examined. Plots of ln (k/T) versus $1/T$ for the C-terminal and N-terminal monoferric transferrins are shown in Figures 1 and 2, respectively. As discussed previously, at the lowest ionic strength, μ = 0.134 M, a linear relationship is seen for iron removal from the C-terminal monoferric transferrin while two distinct linear regions are observed for iron removal from the N-terminal monoferric transferrin.[24] The apparent activation enthalpies found for the

(29) Moore, W. J. *Physical Chemistry*; Prentice Hall: Englewood Cliffs, NJ, 1972; p 443.

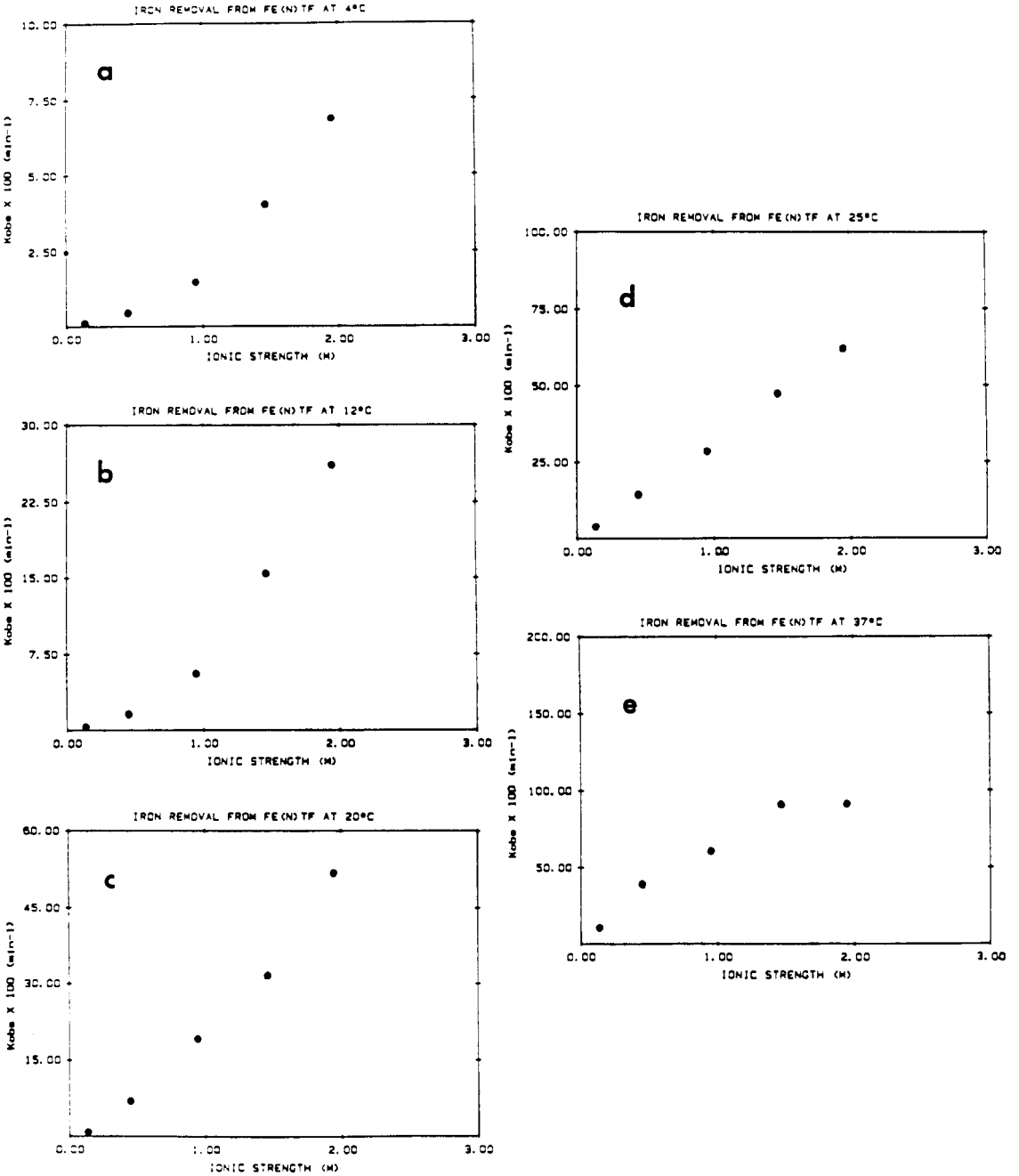

Figure 4. Plots of observed rate constants (min^{-1}) as a function of increasing ionic strength (adjusted with KCl) for iron removal from 0.100 mM N-terminal monoferric transferrin by 12.00 mM 3,4-LICAMS in 0.050 M HEPES buffer, pH 7.4 at the following temperatures: (a) 4 °C; (b) 12 °C; (c) 20 °C; (d) 25 °C; (e) 37 °C.

N-terminal monoferric transferrin are 21 kcal/mol[30] for temperatures below 20 °C and 15 kcal/mol for temperatures above 20 °C. A value of 20 (1) kcal/mol is observed over the entire region of the C-terminal monoferric transferrin. It has been suggested that the break in the Arrhenius plot and the lowered activation enthalpy may result from a temperature-induced conformational change occurring in the N-terminal monoferric transferrin, which allows iron to be released faster from the N-terminal monoferric transferrin and N-terminal site of diferric transferrin than from the C-terminal monoferric transferrin and C-terminal site of diferric transferrin.[24]

The Arrhenius plots for iron removal from C-terminal monoferric transferrin are linear at all ionic strengths studies (Figure 1); hence, there is no evidence for temperature-dependent conformational changes. The apparent activation enthalpies remain the same or slightly decrease from 20 (1) to 16 (5) kcal/mol as ionic strength is increased from 0.134 to 1.95 M.

In contrast, nonlinearity is seen in plots of ln (k/T) versus $1/T$ for iron removal from the N-terminal monoferric transferrin. At the lowest ionic strength, μ = 0.134 M, a definite break in the plot of the data is presented; this is consistent with the previously noted temperature-dependent conformations of the N-terminal monoferric transferrin. At the higher ionic strengths the apparent discontinuities are less evident, indicating that under these conditions the conformational changes are less sensitive to temperature change. However, the higher ionic strength Arrhenius plots do contain two domains that intersect at approximately 20 °C. The

(30) Estimation of the error in the data and derived kinetic parameters was made by repetition of the individual experiments, which gave reproducible rate data with less than a 5% average deviation. In the case of the C-terminal monoferric transferrin, linear behavior of ln (k/T) versus $1/T$ is seen over the entire range of temperatures studied (five different temperatures). The standard deviation of the activation enthalpy (from linear regression) is shown in parentheses in the least significant digits. The same convention for the esd's is used elsewhere. In the case of the N-terminal monoferric transferrin, there are only two (or three) points per region. We feel it is justified to estimate activation enthalpies from these plots given the reproducibility of the data and the behavior of the C-terminal monoferric transferrin.

activation enthalpies for the high-temperature domains are lower than those for the low-temperature domains.

Ionic Strength Dependence Studies. The observed rates of iron removal from the C-terminal and N-terminal monoferric transferrins are plotted against ionic strength at each of five different temperatures ranging from 4 to 37 °C in Figures 3 and 4, respectively. These rates generally increase with increasing ionic strength over the entire temperature range. We interpret this as due to ions binding to sites in the protein that promote a change to a conformation from which metal release is possible.

Evidence for anion binding is supported by the literature. For example, in 1961, Wishnia and co-workers found from electrophoretic mobility and isoionic pH studies that ~17 chloride ions bind to ovotransferrin.[31] Folajtar and Chasteen reported that two anions bind to each domain of serotransferrin with strong positive cooperativity.[14] They found that different anions similarly altered EPR spectra of ferric transferrin, indicating that the anions are not directly coordinated to the metals but possibly bind to cationic amino acids such as lysines or the positive dipoles of helix N-termini that exist in the metal-binding sites.[4,5,14,15] The observed changes in the EPR spectra may be due to the binding of anions to the protein to cause conformational changes. Indeed, an allosteric mechanism in which one anion binds causing conformational changes in the protein that enhance the binding of the second anion would explain the observed positive cooperativity of anion binding.[14]

Earlier studies have shown nonsynergistic anion binding can significantly affect the rate of iron release from ferric transferrins by chelators.[16-21] Baldwin and de Sousa showed that increasing NaCl and LiCl concentrations caused rates of iron removal from C-terminal monoferric transferrin by EDTA at 37 °C to be increased in a parabolic fashion, while increasing concentrations of $NaClO_4$ increased the rates in a hyperbolic fashion.[20] However, increasing amounts of NaCl and $NaClO_4$ reportedly *decreased* the rates of iron release from N-terminal monoferric transferrin, while LiCl caused the rates to be increased in a parabolic manner.[20] Williams et al. found that NaCl increased the rates of iron release at 37 °C from both C-terminal and N-terminal monoferric transferrins in parabolic and hyperbolic manners, respectively, when desferrioxamine B was used as the metal chelator in the absence of mediators.[21] The difference in behavior of the Li^+ and Na^+ salts may be due to the fact that Li^+ is higher in the cation lyotropic series[13] than Na^+. The Baldwin study used very high concentrations of EDTA, with a correspondingly high ionic strength. We have seen that there are ion-specific changes in the rate of iron removal from transferrin under such conditions, and we attribute to this the differences in the results of the Baldwin and Williams studies.

One simple mechanism to illustrate ion binding is

$$\mathrm{FeTf} + n(\mathrm{ions}) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{FeTf}\cdot n(\mathrm{ions})$$

$$\mathrm{FeTf}\cdot n(\mathrm{ions}) + \mathrm{L} \xrightarrow{k_2} \mathrm{FeL} + \mathrm{Tf} + n(\mathrm{ions})$$

where Tf = the bicarbonate complex of transferrin and L = a hexadentate ligand. Under the steady-state approximation for FeTf·n(ions), the observed pseudo-first-order rate constant becomes

$$k_{\mathrm{obsd}} = \frac{k_1 k_2[\mathrm{L}]}{k_{-1} + k_2[\mathrm{L}]}[\mathrm{ions}]^n$$

In Figures 3 and 4, we see that the rates of iron release from the monoferric transferrins are parabolically dependent on ionic strength in the low-temperature regime. At 25 °C, the rate of increase slows down for iron removal from N-terminal monoferric transferrin and a linear dependence is seen; at 37 °C, the rate reaches saturation level. For C-terminal monoferric transferrin, the linear behavior occurs at 37 °C; perhaps at higher temperatures, a hyperbolic behavior would be obtained. The behavior at 37 °C observed for iron removal from the monoferric transferrins by 3,4-LICAMS in the presence of various concentrations of KCl is very similar to that observed by Williams et al., in which desferrioxamine B was used as the metal chelator and NaCl as the salt.[21] The parabolic dependence indicates that multiple binding of ions is easier at the lower temperatures. Since entropy loss associated with ion binding is less at the lower temperatures, it should be more difficult for ions to bind at the higher temperatures, accounting for the decelerated dependence of the observed rate on ionic strength at the higher temperatures. The saturation of the rate at 37 °C for N-terminal monoferric transferrin is not yet understood. However, this cannot be due to saturation of ion-binding sites in the protein since more ions actually bind at the lower temperatures, and such leveling effects are not observed in these regions.

The most remarkable observation is that extrapolations of the data of the rate dependencies on ionic strength for both monoferric transferrins at all temperatures indicate that no iron is removed by 3,4-LICAMS when the total ionic strength of the reaction mixture is zero! This implies that the presence of anions (and/or cations) are *necessary* for iron release. Thus, the electrostatic interactions and/or conformational changes induced by ions in solution are necessary for promoting iron release. It should be mentioned that reexamination of the data from studies published by Baldwin and de Sousa and Williams et al.[20,21] in terms of observed rate versus ionic strength rather than salt concentration gives results similar to those reported here.

Conclusion

In the present study, it is shown that ionic strength plays an essential role in iron removal from transferrin. Although it has been known that the presence of salts greatly affect the kinetics of iron removal, it is novel that no iron is released when the ionic strength of the medium is zero. The structure of lactoferrin,[4,5] shows that the N-terminal site is more accessible to solution than the C-terminal site. But the kinetics of iron release from the two monoferric transferrins and, hence, the metal binding sites are both affected by ionic strength in similar manners. Simple electrostatic interactions, which serve only to neutralize charge near the metal sites, cannot account for this since the N-terminal site would be expected to be affected to a greater extent than the C-terminal site. Thus, the overall picture is that ions induce conformational changes that play a prerequisite role in the metal release from transferrin to strong chelating agents.

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Registry No. Fe, 7439-89-6.

(31) Wishnia, A.; Weber, I.; Warner, R. C. *J. Am. Chem. Soc.* **1961**, *83*, 2071-2080.