Thermodynamics of Gallium Complexation by Human Lactoferrin†

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ABSTRACT: Equilibrium constants for the successive binding of 2 equiv of Ga^{3+} to human lactoferrin have been measured by difference ultraviolet spectroscopy in 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid containing 5 mM bicarbonate at pH 7.4 and 25 °C. Ethylenediamine-*N*,*N*′-diacetic acid was used as the competing chelating agent. Values of the effective binding constants for the stated experimental conditions are $\log K^*_1 = 21.43 \pm 0.18$ and $\log K^*_2 = 20.57 \pm 0.16$. Comparison of these results with literature values for the gallium–transferrin binding constants indicates that lactoferrin binds gallium more strongly by a factor of ~90. The ratios of successive binding constants for the two proteins are essentially identical. A linear free energy relationship (LFER) for the complexation of gallium(III) and iron(III) has been prepared and used to estimate an iron(III)–lactoferrin binding constant for pH 7.4. The LFER prediction is compared with thermodynamic data on iron binding at pH 6.4 and gallium binding at pH 7.4. The results indicate that the ratio of iron binding constants for lactoferrin and transferrin is likely in the range of 50–90.

Gallium-67 is now widely used for imaging of both tumors and soft tissue abscesses. However, the mechanism of gallium concentration in these tissues is still in doubt (Hoffer, 1980; Larson, 1978). Extensive studies have shown that serum transferrin is strongly involved in the serum transport of the gallium(III) ion (Gunasekera et al., 1972; Vallabhajosula et al., 1980; Clausen et al., 1974). Additional studies indicate that the gallium–transferrin complex is actively involved in the uptake of gallium into the tumor cells (Harris & Sephton, 1977; Larson et al., 1979a,b). For these reasons, the gallium–transferrin system has been studied in some detail (Larson et al., 1979a; Harris & Pecoraro, 1983).

Lactoferrin is a second iron binding protein closely related to serum transferrin. It appears that lactoferrin is involved in the accumulation of gallium in abscesses (Hoffer et al., 1977; Weiner et al., 1981a), but the mechanism of this accumulation is still unknown. Lactoferrin is present in high concentrations in polymorphonuclear leukocytes (PMN) (Bennet & Kakocinski, 1978; Mason et al., 1969). One possible mechanism is that the PMN which accumulate in the abscess incorporate gallium, and that this intracellular gallium is tightly bound to lactoferrin (Hoffer, 1980; Gelrud et al., 1974). However, reports on the cellular incorporation of gallium into PMN are conflicting. Weiner et al. (1981a) reported that about 75% of the gallium was taken up by PMN in vitro, and about half of this was bound to lactoferrin. Conversely, Tsan et al. (1978) reported that gallium was bound to the outer membrane of the PMN rather than entering the cell. There is also a report that the gallium localized in abscess induced in rats was present in extracellular fluid with very little activity actually associated with the PMN (DeRoo et al., 1978).

An alternative mechanism of gallium accumulation in abscesses involves the release of lactoferrin from the PMN that have accumulated at the abscess (Weiner et al., 1981a). It has been reported that PMN release about 45% of their lactoferrin in response to a phagocytic challenge (Leffell & Spitznagel, 1975). This free lactoferrin could then compete with serum transferrin for binding of the circulating gallium. Indeed, it appears that gallium localization is due to a more effective uptake from the circulatory system, since mobilization of gallium from other tissues to the abscess does not occur (Gelrud et al., 1974). Regardless of the mechanism, it appears that the binding of gallium to lactoferrin is a major factor in the accumulation of this metal ion in abscesses.

Lactoferrin was originally isolated from bovine milk (Groves, 1960) but has since been found at lower concentrations in a variety of physiological fluids such as seminal fluid, cervical mucus, nasal secretions, and tears (Masson et al., 1966). It is closely related to serum transferrin, with essentially the same molecular weight and similar amino acid sequence (Querinjean et al., 1971), although the two proteins do not cross-react immunologically (Montreuil et al., 1960). Like serum transferrin, lactoferrin binds 2 equiv of ferric ion with the concomitant binding of bicarbonate as a synergistic anion (Masson & Heremans, 1968). However, there are important differences between the two proteins. Lactoferrin has a significantly greater binding affinity for ferric ion (Aisen & Leibman, 1972), which has led to speculation that it may function as a bacteriostatic agent by restricting the availability of this essential nutrient (Masson et al., 1966; Feeney & Komatsu, 1966; Bullen et al., 1974). Lactoferrin also retains its binding affinity at much lower pH than does transferrin (Mazurier & Spik, 1980) and is more stable to denaturation (Krysteva et al., 1976).

We have previously reported on the thermodynamics of gallium binding by human serum transferrin (Harris & Pecoraro, 1983). This paper describes similar studies on the binding of the gallium(III) ion to human lactoferrin. The results are compared with earlier reports on the relative binding affinities of lactoferrin and transferrin for gallium (Weiner et al., 1981b). Because of the close chemical similarities of gallium(III) and iron(III), it is also possible to utilize an iron–gallium linear free energy relationship (LFER) to provide additional insight into the relative iron binding affinities of lactoferrin and transferrin.

Experimental Procedures

Gallium binding to lactoferrin was monitored by difference UV spectroscopy. The procedure has been previously described in detail (Harris & Pecoraro, 1983). Data were collected with

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a Cary 219 spectrophotometer equipped with a jacketed cell holder maintained at 25 °C by an external circulating water bath. All solutions contained 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.4 and 5 mM sodium bicarbonate. The UV absorbance was monitored at 242 nm. The net absorbance was divided by the analytical lactoferrin concentration to calculate values of $\Delta\epsilon$.

Equilibrium constants were calculated by nonlinear least-squares refinement, in which the two lactoferrin binding constants were varied to minimize the sum of the squares of the residuals between $\Delta\epsilon_{obsd}$ and $\Delta\epsilon_{calcd}$. Equilibrium competition reactions were run vs. ethylenediamine-*N*,*N*'-diacetic acid (EDDA). Formation constants for Ga–EDDA were taken from Harris & Martell (1976). Gallium hydrolysis constants were taken from Baes & Mesmer (1976).

Commercial preparations of 98% human lactoferrin (Sigma) were treated to remove residual iron by dissolution in 0.1 M citrate at pH 2, followed by removal of the citrate by ultrafiltration through an Amicon XM-50 membrane. The pH was slowly raised to neutral by the addition of dilute ammonium hydroxide. Near pH 4.5, there is some precipitation of lactoferrin, which partially redissolves at higher pH. Final solutions were clarified by filtration through a 0.22-μm diaflow filter. The residual iron content of all apolactoferrin solutions was determined with a Perkin-Elmer Model 306 atomic absorption spectrophotometer using flame techniques.

Results

Iron Titrations. The analytical concentration of available iron binding sites in each batch of lactoferrin was determined by spectrophotometric titration at 465 nm with standardized solutions of ferric NTA. The initial portions of these titration curves were linear with slopes of 2800 ± 370 M^{-1} cm^{-1}, which corresponds to the molar absorptivity of the iron–lactoferrin complex at 465 nm. Saturation of the lactoferrin was marked by a sharp break in the titration curve, followed by a flat line reflecting essentially no further increase in absorbance with the addition of excess iron. It was assumed that this break corresponds to the binding of exactly 2.0 equiv of ferric ion. The analytical lactoferrin concentration was taken as half the sum of the iron required to reach the equivalence point in the titration plus the concentration of residual protein-bound iron measured by atomic absorption spectrophotometry. In all cases, the residual iron content of the apolactoferrin was less than 9% of the total iron binding capacity. The molar absorptivity at 278 nm was calculated as the absorbance divided by the analytical apolactoferrin concentration. The average result for four different preparations of apolactoferin was 107 000 ± 2000 M^{-} cm^{-1}. The concentration of apolactoferrin in each sample solution was based on an "apparent" absorptivity that was calculated for each batch of lactoferrin solely on the basis of the concentration of titratable binding sites without regard to residual iron content.

Gallium–Lactoferrin Titrations. Apolactoferrin was titrated with a solution of gallium(III) chloride, and the difference UV spectra recorded after the addition of each aliquot of gallium are shown in Figure 1. There is a strong positive absorbance at 242 nm and a weaker set of peaks around 290 nm. The spectra are characteristic of metal–tyrosyl coordination and are similar to those reported for a variety of metal–transferrin complexes.

Lactoferrin was also titrated with a series of gallium solutions that also contained various concentrations of ethylenediamine-*N*,*N*'-diacetic acid (EDDA). The absorbance at 242 nm was divided by the analytical concentration of lactoferrin to give values of $\Delta\epsilon$, which were normalized for the different

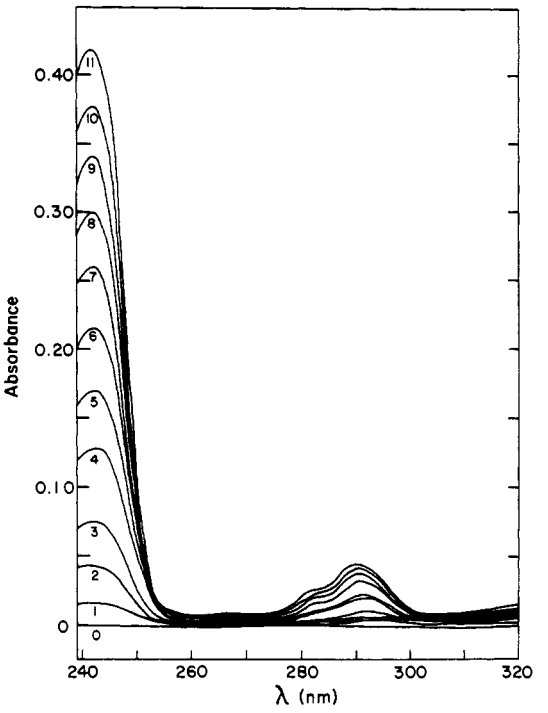

FIGURE 1: Difference UV spectra produced by the titration of 2.0 mL of 1.294×10^{-5} M apolactoferrin with 2.916×10^{-4} M $GaCl_3$: (1) 5, (2) 15, (3) 25, (4) 41, (6) 73, (7) 89, (8) 105, (9) 121, (10) 137, and (11) 161 μL of Ga.

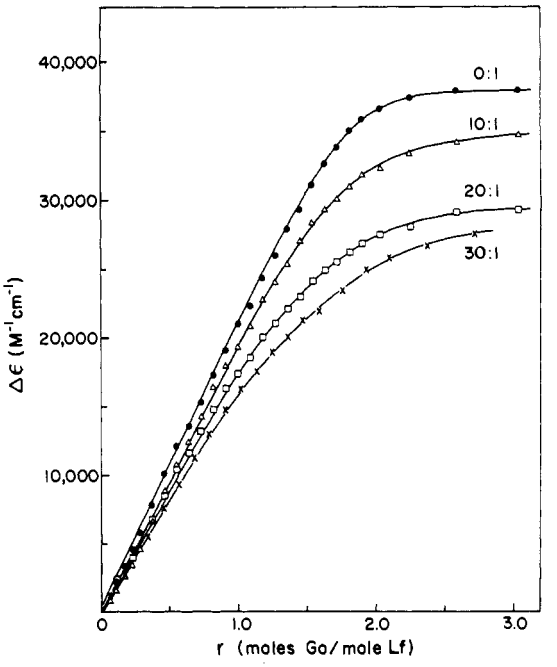

FIGURE 2: Titration curves for the addition of Ga–$(EDDA)_x$ to apolactoferrin, where x = 0, 10, 20, and 30. $\Delta\epsilon$ represents the absorbance at 242 nm divided by the lactoferrin concentration, and r is the molar ratio of gallium to lactoferrin.

titrations, and plots of $\Delta\epsilon$ vs. the ratio of gallium to lactoferrin were prepared as shown in Figure 2. In the absence of any EDDA, the titration curve was linear out to an r value of about 1.5. From the slope of this linear segment, the molar absorptivity of the gallium–lactoferrin complex was found to average 19 900 ± 1400 M^{-1} cm^{-1} per gallium ion. However, the molar absorptivities determined for each individual batch of apolactoferrin, which ranged from 18 000 to 21 000, were used in the equilibrium calculations discussed below instead of the average value.

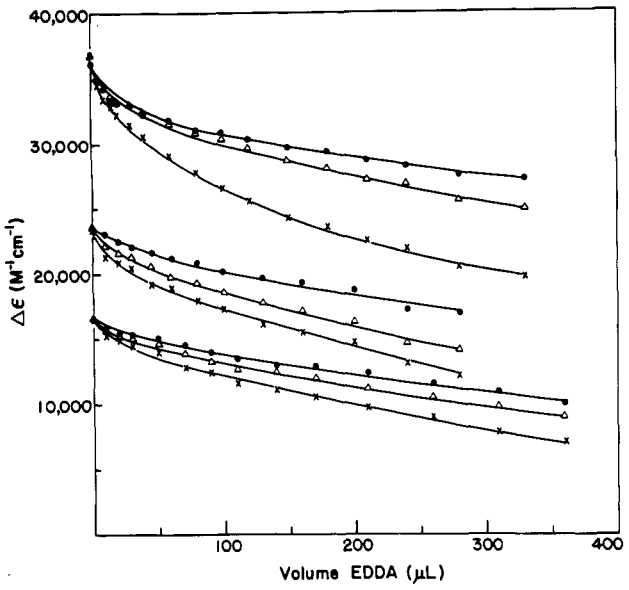

FIGURE 3: Titration curves for the addition of free EDDA to solutions of about 1.5×10^{-5} M lactoferrin that had been allowed to equilibrate with various concentrations of gallium. (Upper curves) [Ga]/[Lf] = 1.66; [EDDA] = 5, 10, and 20 mM. (Middle curves) [Ga]/[Lf] = 1.22; [EDDA] = 10, 20, and 30 mM. (Lower curves) [Ga]/[Lf] = 0.87; [EDDA] = 15, 20, and 30 mM.

On the basis of a molar absorptivity of 19 900 M^{-1} cm^{-1} per gallium, one would expect a final $\Delta\epsilon$ value of about 40 000 for the digallium–lactoferrin complex. Figure 2 shows that the plot of $\Delta\epsilon$ vs. r begins to curve beyond $r = 1.5$ and eventually levels off at a $\Delta\epsilon$ of about 38 000, indicating incomplete saturation of the lactoferrin binding sites. This is likely due to competitive formation of the soluble $Ga(OH)_4^-$ as well as the insoluble $Ga(OH)_3$. Thus, it is difficult to fully saturate lactoferrin with gallium, even in the absence of added chelating agents. Even more serious competition from hydrolysis was observed in studies on gallium–transferrin (Harris & Pecoraro, 1983).

Titrations of apolactoferrin were repeated with gallium solutions that also contained increasing concentrations of EDDA as a competing chelating agent, and typical curves are shown in Figure 2. As expected, increasing the EDDA concentration results in stronger competition with the apolactoferrin and lower values of $\Delta\epsilon$. These data were used to calculate the gallium–lactoferrin binding constants as described below.

A second series of titrations were performed in which gallium was allowed to equilibrate with lactoferrin, and the gallium–lactoferrin complex was titrated with solutions of EDDA. The spectra recorded during the reverse titrations were essentially identical with those from the forward titrations. The only significant difference was due to a small absorbance from the free EDDA, which has a molar absorptivity of 24 M^{-1} cm^{-1}. Even though this is a very small absorptivity, the relatively high concentrations of EDDA needed to compete with the lactoferrin resulted in measurable absorbances due to the accumulation of EDDA. The absorbance data were corrected for this effect prior to the calculations. Titration curves of $\Delta\epsilon$ vs. microliters of EDDA solution are shown in Figure 3.

Calculation of Equilibrium Constants. The sequential binding of 2 equiv of gallium to lactoferrin can be described by the expressions

$$\text{Ga} + \text{apoLact} \overset{K^*_1}{\rightleftharpoons} \text{Ga–Lact} \quad (1)$$

$$\text{Ga} + \text{Ga–Lact} \overset{K^*_2}{\rightleftharpoons} \text{Ga–Lact–Ga} \quad (2)$$

Previous studies on the binding of ferric ion to apolactoferrin have shown that one bicarbonate molecule is bound per metal ion (Masson & Heremans, 1968) and that three hydrogen ions are released per metal ion (Querinjean et al., 1971). Similar results would be expected for the binding of gallium. However, since all the data reported here were collected at pH 7.4 and 5 mM sodium bicarbonate, terms for bicarbonate and hydrogen ion have been excluded from eq 1 and 2, so that K^*_1 and K^*_2 are effective binding constants valid only for these specific experimental conditions. Given these restrictions, one can write the equilibrium constants for gallium binding simply as

$$K^*_1 = \frac{[\text{Ga–Lact}]}{[\text{Ga}][\text{Lact}]} \quad (3)$$

$$K^*_2 = \frac{[\text{Ga}_2\text{–Lact}]}{[\text{Ga}][\text{Ga–Lact}]} \quad (4)$$

It should be understood that free gallium exists as a mixture of hydrolyzed species ranging from $Ga(H_2O)_6^{3+}$ to $Ga(OH)_4^-$. In eq 3 and 4, [Ga] refers to the molar concentration of the $Ga(H_2O)_6^{3+}$ ion.

Equilibrium constants have been calculated from each titration by nonlinear least-squares techniques that minimize the sum of the squares of the residuals between the observed and calculated $\Delta\epsilon$ values for the titration points. Values of $\Delta\epsilon$ can be calculated on the basis of three parameters, the two gallium binding constants and the molar absorptivity of the gallium–lactoferrin complex. The last parameter is obtained independently from the initial slope of the $GaCl_3$ curve in Figure 2. Thus, the data have been refined by using at most the two binding constants as adjustable parameters. In forward titrations where the ratio of EDDA to gallium is 10 or less, there is not sufficient competition for the binding of the first equivalent of gallium to give a reliable value for K^*_1. In these cases, K^*_1 has been set at 10^{23}, which effectively simulates complete binding of the first equivalent of gallium to the lactoferrin, and K^*_2 has been treated as the only adjustable parameter. At high concentrations of EDDA, there is measurable competition for both the first and second equivalents of gallium, and values of both binding constants can be calculated.

Several reverse titration curves are shown in Figure 3. The uppermost curves are for samples containing 1.66 equiv of gallium/mol of lactoferrin. Although the addition of EDDA removes some gallium from the protein, the absorptivity never drops below 20 000. Thus, the competition involves only the binding of the second equivalent of gallium to the lactoferrin, while the first equivalent of gallium is essentially unaffected by the competition. Thus, these data could be used to calculate values of K^*_2 only. The middle set of curves start at an absorptivity of about 24 000 and drop to 14 000–17 000. These data represent competition for both the first and second equivalents of gallium, and thus, values of both K^*_1 and K^*_2 could be calculated. Finally, the third set of samples had only 0.9 equiv of gallium and thus reflect binding of only the first equivalent of gallium to the apolactoferrin. By use of this procedure of varying the ratio of gallium:lactoferrin, values of both binding constants could be calculated with similar concentrations and volumes of the competing chelating agent.

Mean values of the equilibrium constants calculated from the forward and reverse titrations are listed in Table I. There is excellent agreement between the two procedures. The grand means for the total data set are log $K^*_1 = 21.43 \pm 0.18$ and

Table I: Binding Constants for Gallium Lactoferrin

method	n_1[a]	log K^*_1[b]	n_2[a]	log K^*_2[b]
Ga–EDDA + apolactoferrin	5	21.22 ± 0.50[c]	9	20.62 ± 0.20
EDDA + Ga–lactoferrin	12	21.51 ± 0.14	5	20.43 ± 0.16
grand means	17	21.43 ± 0.18	14	20.57 ± 0.16

[a] n = number of replicated titrations for each constant. [b] Equilibrium constants are defined in eq 3 and 4. [c] Uncertainties are 2 times the standard error of the mean.

log K^*_2 = 20.57 ± 0.16. The ratio of K^*_1/K^*_2 is equal to 7.

Discussion

The molar absorptivity of ferric lactoferrin at 465 nm was determined from the slope of the ferric NTA titration curve to be 2800 M^{-1} cm^{-1} per iron binding site. This result is similar to the value of 2600 M^{-1} cm^{-1} originally reported by Groves (1960) but is higher than the subsequent report of 2040 M^{-1} cm^{-1} (Aisen & Leibman, 1972). Molar absorptivities are often based on dry weight determinations of the protein concentration. The method used here has a distinct advantage in that the absorptivity is based on the analytical concentration of iron in the titrant, which can be determined to a high precision, and would only be affected by a contaminant that binds iron to produce an absorbance at 465. The value does not depend on the precision of the molar absorptivity at 278 nm or on the molecular weight of the protein. Thus, the titration method should provide a very precise determination of the molar absorptivity at 465 nm.

The molar absorptivity of apolactoferrin at 278 nm was 107 000 M^{-1} cm^{-1}, which is significantly greater than the previous reports of about 85 000 M^{-1} cm^{-1} (Aisen & Leibman, 1972; Teuwissen et al., 1972; Krysteva et al., 1979). The reasons for this discrepancy are not clear. It may be related to rather harsh treatment of the apolactoferrin to remove iron (Ainscough et al., 1980). If there were a decrease in the concentration of functional iron binding sites, a higher absorptivity at 278 nm would result from the absorbance of nonfunctional protein. The lower values previously reported for the ferric lactoferrin absorptivity at 465 could also reflect some loss in iron binding capacity.

We have previously reported conditional gallium–transferrin binding constants of log K^*_1 = 19.53 and log K^*_2 = 18.58 for 5 mM bicarbonate at pH 7.4. (Harris & Pecoraro, 1983). On the basis of the results from Table I, lactoferrin binds gallium more avidly than does transferrin by a factor of about 90 for both K^*_1 and K^*_2. The site selectivity for each protein is essentially the same, with a ratio of K^*_1/K^*_2 of 9 for transferrin and 7 for lactoferrin. Thus, the gallium binding properties for the two proteins are remarkably similar.

The relative gallium binding affinities of lactoferrin and transferrin have been previously investigated by direct equilibrium dialysis competition between the two proteins (Weiner et al., 1981b). Although no equilibrium constants were actually reported, we calculate from the data in Figure 1 of their paper that the ratio of the lactoferrin to transferrin binding constants is very close to 1.0. Thus, the data reported here indicate a higher relative gallium binding affinity for the lactoferrin compared to the dialysis results.

Aisen & Leibman (1972) reported that lactoferrin binds iron more strongly than does transferrin by a factor of 300, and this ratio has been widely quoted in the literature (Teuwissen et al., 1972; Van Snick et al., 1974; Baker & Rumball, 1977; Ainscough et al., 1979; Mazurier & Spik, 1980; Weiner et al., 1981b.) However, this ratio was based on the transferrin binding constants reported by Aasa et al. (1963). More recently, a new set of iron–transferrin constants have been reported that differ by about a factor of 10 from those in the earlier publication. The current iron binding constants are log K^*_1 = 20.1 for lactoferrin at pH 6.4 (Aisen & Leibman, 1972) and log K^*_1 = 19.5 for transferrin at pH 6.7 (Aisen et al., 1978). These values differ by only a factor of 4. Even considering that a strict third-order dependence on $[H^+]$ would raise the lactoferrin value to log K^*_1 = 21.0 at pH 6.7, the ratio would increase to only ~30.

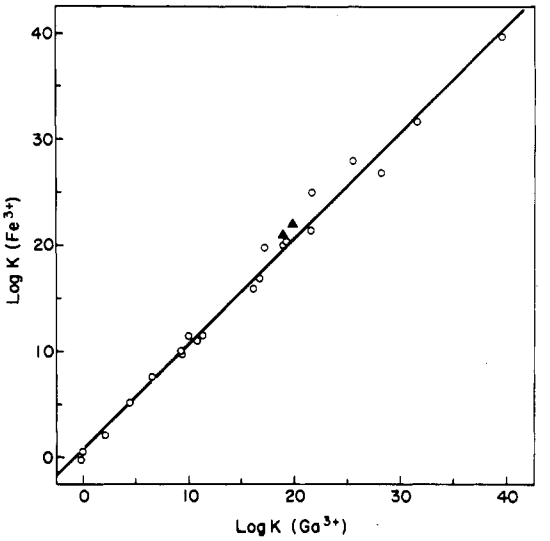

FIGURE 4: Linear free energy relationship for the complexation of iron(III) and gallium(III). Each data point represents the stability constant of a given ligand with gallium(III) as the x coordinate and the iron(III) stability constant with the same ligand as the y coordinate. The points for human serum transferrin are shown as the triangles.

The ratio of iron binding constants at pH 7.4 cannot be directly calculated because an iron–lactoferrin constant at pH 7.4 has never been reported. However, we can estimate this parameter using a linear free energy relationship (LFER) for gallium and iron complexation. The LFER consists of a plot of log K(M) vs. log K(M′), where M and M′ represent the metal ion of interest and a chemically related reference metal ion, respectively, and the log K's are the binding constants with a series of low molecular weight ligands. Larson et al. (1978) constructed such a LFER based on only seven ligands for Ga^{3+} and Fe^{3+} and attempted to predict the gallium–transferrin constant. The procedure was unsuccessful primarily because they selected an older, incorrect literature value for the iron–transferrin constant. In the present study, the number of ligands used to construct the LFER has been increased from 7 to 21, and this LFER has been used to predict the iron–lactoferrin constant at pH 7.4 from the experimentally determined gallium–lactoferrin constant.

The iron–gallium LFER is shown in Figure 4. Twenty-five pairs of iron and gallium binding constants were found in the literature (Martell & Smith, 1974; Harris & Martell, 1976; Motekaitis & Martell, 1980). Twenty-one of the 25 were used in the LFER. Four were rejected on the basis of inconsistencies in either the iron or the gallium value. The plot in Figure 4 leads to the linear equation:

$$\log K_{Fe} = (1.00 \pm 0.02) \log K_{Ga} + (0.68 \pm 1.17) \quad (5)$$

This equation was first applied to the transferrin binding constants, since both iron and gallium values have been reported. The gallium constants were measured in 5 mM bicarbonate, while the iron constants were measured by Aisen et al. (1978) at ambient bicarbonate (0.20 mM). Thus, it is necessary to adjust the values to a common set of conditions. Although metal binding to apotransferrin is often written as

the reaction of metal ion, bicarbonate, and apotransferrin, the binding actually occurs in two steps (Kojima & Bates, 1981). These steps are

$$HCO_3^- + \text{apoTr} \rightleftharpoons HCO_3\text{–Tr} \quad (6)$$

$$M^{n+} + HCO_3\text{–Tr} \overset{K'}{\rightleftharpoons} M\text{–}HCO_3\text{–Tr} \quad (7)$$

We have recently determined the equilibrium constants for the stepwise binding of bicarbonate to apotransferrin to be $10^{2.68}$ and $10^{1.8}$, in 0.1 M HEPES (Harris, 1985) and $10^{2.97}$ and $10^{2.39}$ in 0.01 M HEPES (W. R. Harris, unpublished results). Effective metal binding constants (K^*) have been converted to K' values, which correspond to the equilibrium shown in eq 7 for the reaction of the metal ion with the bicarbonate–transferrin binary complex. This constant is independent of the bicarbonate concentration and thus can be used to compare results obtained under different experimental conditions. The details of this correction procedure will be reported separately.

These corrected values are log K'_1 = 21.70 and log K'_2 = 20.41 for iron–transferrin and log K'_1 = 19.71 and log K'_2 = 18.76 for gallium–transferrin. These two sets of values are also plotted in Figure 4. The fit to the LFER is quite good. The iron–transferrin constants calculated from eq 5 are log $K'_1 = 20.39 \pm 1.2$ and log $K'_2 = 19.44 \pm 1.2$. Thus, it appears that metal ion complexation by transferrin, and presumably by lactoferrin, can be treated by this method to predict order of magnitude binding constants.

The regression parameters for eq 5 have been recalculated by including the transferrin binding constants for iron and gallium in the least-squares fit. This gives values of 1.01 ± 0.02 for the slope and 0.72 ± 1.2 for the intercept. The first binding constant for ferric–transferrin at 5 mM bicarbonate is calculated to be $10^{21.66}$. The gallium–lactoferrin value from Table I has been used with the revised regression parameters for eq 5 to predict an iron–lactoferrin binding constant of $10^{22.36}$ at 5 mM bicarbonate, pH 7.4. The ratio of these iron binding constants for lactoferrin and transferrin is only 5. Given the uncertainty in log K values derived from the LFER, this value could vary from 0.3 to 80. Since the LFER underestimates the ferric transferrin binding constants, it may be more likely that the ferric lactoferrin constant derived from the LFER is also low. Thus, the true ratio of lactoferrin:transferrin constants may be more likely to fall in the upper range from 5 to 80. This result is consistent with the actual binding data of ferric lactoferrin, which indicates a ratio of lactoferrin to transferrin binding constants of 30 at pH 6.7 if one uses the most recent data on ferric transferrin from Aisen et al. (1978). The gallium system is similar to iron, with a ratio of gallium–lactoferrin to gallium–transferrin binding constants of 90. Thus, there is excellent consistency in the equilibrium data for iron and gallium with transferrin and lactoferrin. The older estimate of 300 for the ratio of the iron binding constants of lactoferrin and transferrin was based on an incorrect ferric transferrin binding constant and should be disregarded.

The method used above for making bicarbonate corrections is new. In the past, it was usually assumed that the effective binding constant would increase linearly with the bicarbonate concentration. This is essentially correct at low saturation of apotransferrin with bicarbonate, but the magnitude of the bicarbonate–transferrin binding constants are such that this linear approximation holds only at less than millimolar concentrations of bicarbonate. Thus, the corrected iron and gallium binding constants reported in Table III of Harris & Pecoraro (1983) are incorrect. In fact, the ratios between the iron and gallium binding constants for transferrin in 5 mM HCO_3^- are 140 and 60, respectively, rather than in the range of 200–400 as reported previously.

Registry No. Ga, 7440-55-3; Fe, 7439-89-6.

REFERENCES

Aasa, R., Malmstrom, B. G., Saltman, P., & Vanngard, T. (1963) *Biochim. Biophys. Acta 75*, 203–222.

Ainscough, E. W., Brodie, A. M., & Plowman, J. E. (1979) *Inorg. Chim. Acta 33*, 149–153.

Aisen, P., & Leibman, A. (1972) *Biochim. Biophys. Acta 257*, 314–323.

Aisen, P., Leibman, A., & Zweier, J. (1978) *J. Biol. Chem. 253*, 1930–1937.

Baes, C. F., & Mesmer, R. E. (1976) *The Hydrolysis of Cations*, Wiley, New York.

Baker, E. N., & Rumball, S. V. (1977) *J. Mol. Biol. 111*, 207–210.

Bennet, R., & Kokocinski, T. (1978) *Br. J. Haematol. 39*, 509–521.

Bullen, J. J., Rogers, H. J., & Griffiths, E. (1974) *Microbial Iron Metabolism* (Neilands, J. B., Ed.) p 517, Academic Press, New York.

Butler, J. E. (1972) *Biochim. Biophys. Acta 295*, 341–351.

Clausen, J., Edeling, C. J., & Fogh, J. (1974) *Cancer Res. 34*, 1931–1937.

DeRoo, M., Hrogmartans, M., Van Der Schuerin, B., & De Geest, G. (1978) *Int. J. Nucl. Med. Biol. 5*, 100–104.

Feeney, R. E., & Komatsu, S. K. (1966) *Struct. Bonding (Berlin) 1*, 149–206.

Gelrud, L. G., Arseneau, J. C., Milder, M. S., Kramer, R. J., Swann, S. J., Canellos, G. P., & Johnston, G. S. (1974) *J. Lab. Clin. Med. 83*, 489–495.

Groves, M. L. (1960) *J. Am. Chem. Soc. 82*, 3345–3350.

Gunasekera, S. W., King, L. J., & Lavender, P. J. (1972) *Clin. Chim. Acta 39*, 401–406.

Harris, A. W., & Sephton, R. G. (1977) *Cancer Res. 37*, 3634–3638.

Harris, W. R. (1985) *Biochemistry 24*, 7412–7418.

Harris, W. R., & Martell, A. E. (1976) *Inorg. Chem. 15*, 713–720.

Harris, W. R., & Pecoraro, V. L. (1983) *Biochemistry 22*, 292–299.

Hekman, A. (1971) *Biochim. Biophys. Acta 251*, 380–387.

Hoffer, P. (1980) *J. Nucl. Med. 21*, 282–285.

Hoffer, P. B., Huberty, J., & Khayam-Bashi, H. (1977) *J. Nucl. Med. 18*, 713–717.

Krysteva, M. A., Mazurier, J., & Spik, G. (1976) *Biochim. Biophys. Acta 453*, 484–493.

Larson, S. M. (1978) *Semin. Nucl. Med. 8*, 193–203.

Larson, S. M., Allen, D. R., Rasey, J. S., & Grunbaum, Z. (1978) *J. Nucl. Med. 19*, 1245–1249.

Larson, S. M., Rasey, J. S., Allen, D. R., & Nelson, N. J. (1979a) *J. Nucl. Med. 20*, 837–842.

Larson, S. M., Rasey, J. S., Allen, D. R., & Grunbaum, Z. (1979b) *J. Nucl. Med. 20*, 843–846.

Leffell, M. S., & Spitznagel, J. K. (1975) *Infect. Immunol. 12*, 813–820.

Martell, A. E., & Smith, R. M. (1974) *Critical Stability Constants*, Plenum, New York.

Masson, P. L., & Heremans, J. F. (1968) *Eur. J. Biochem. 6*, 579–584.

Masson, P. L., Heremans, J. F., & Dive, C. (1966) *Clin. Chim. Acta 14*, 735–739.

Masson, P. L., Heremans, J. F., & Schonne, E. (1969) *J. Exp. Med. 130*, 643–658.

Mazurier, J., & Spik, G. (1980) *Biochim. Biophys. Acta 629*, 399-408.
Montreuil, J., Tonnelat, J., & Mullet, S. (1960) *Biochim. Biophys. Acta 45*, 413-421.
Motekaitis, R. J., & Martell, A. E. (1980) *Inorg. Chem. 19*, 1646-1651.
Querinjean, P., Masson, P. L., & Heremans, J. F. (1971) *Eur. J. Biochem. 20*, 420-425.
Teuwissen, B., Masson, P. L., Osinski, P., & Heremans, J. F. (1972) *Eur. J. Biochem. 31*, 239-245.
Tsan, M. F., Chen, W. Y., Schoffel, U., & Wagner, H. N. (1978) *J. Nucl. Med. 19*, 36-43.
Vallabhajosula, S. R., Harwig, J. F., Siemsen, J. K., & Wolf, W., (1980) *J. Nucl. Med. 21*, 650-656.
Van Snick, J. L., Masson, P. L., & Heremans, J. F. (1974) *J. Exp. Med. 140*, 1068-1084.
Weiner, R., Hoffer, P. B., & Thakur, M. L. (1981a) *J. Nucl. Med. 22*, 32-37.
Weiner, R. E., Schreiber, G. J., Hoffer, P. B., & Shannon, T. (1981b) *Int. J. Nucl. Med. Biol. 8*, 371-378.

Molecular Basis for the Anti-Sickling Activity of Aromatic Amino Acids and Related Compounds: A Proton Nuclear Magnetic Resonance Investigation†

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ABSTRACT: High-resolution proton nuclear magnetic resonance spectroscopy and relaxation techniques have been used to investigate the interactions of sickle cell hemoglobin (Hb S) and human normal adult hemoglobin (Hb A) with *p*-bromobenzyl alcohol, L-phenylalanine, L-tryptophan, and L-valine. With the exception of valine, all these compounds inhibit the polymerization of deoxy-Hb S [Noguchi, C. T., & Schechter, A. N. (1978) *Biochemistry 17*, 5455]. Using transferred nuclear Overhauser effects among the proton resonances of the compound of interest and the corresponding longitudinal relaxation rates (T_1^{-1}), we have shown that the binding of each of the compounds investigated to deoxy-Hb S is comparable to that to deoxy-Hb A. Intermolecular transferred nuclear Overhauser effects have been observed between proton resonances of the anti-sickling compounds and specific protons situated in the heme pockets of Hb. On the basis of these results, we suggest that one binding site, common to all compounds with anti-sickling activity, is at or near the heme pockets in the α and β chains of both deoxy-Hb S and deoxy-Hb A. The proton T_1^{-1} values of the histidyl residues situated over the surface of the hemoglobin molecule indicate that a second binding site is located at or near the β6 position, containing the mutation in Hb S (β6Glu → Val). The binding of the compounds investigated to the latter site induces conformational changes in the amino-terminal domains of the β chains. Our present NMR results support the following two mechanisms for the anti-sickling activity: (i) the allosteric mechanism, in which alterations in the local conformation(s) at intermolecular contact site(s) between Hb S molecules in the polymer occur as a result of binding of an anti-sickling compound to adjacent regions on the Hb S molecule; (ii) the competitive mechanism, in which the binding of an anti-sickling compound blocks one or more of the intermolecular contact sites between Hb S molecules in the polymer.

Sickle cell hemoglobin (Hb S)[1] is a mutant hemoglobin (β6Glu → Val) occurring in individuals with sickle cell anemia or sickle cell trait. In the deoxygenated form, under physiological conditions, Hb S molecules can polymerize into long fibers, which can then distort and rigidify the red blood cells. The polymerization of Hb S is the main molecular process responsible for the clinical manifestations in patients with sickle cell anemia. A large variety of compounds have been found to inhibit the polymerization of deoxy-Hb S either by reacting covalently to modify the Hb S molecule or by noncovalent interactions [for recent reviews, see Noguchi & Schechter (1985) and Schechter et al. (1985)]. Among the noncovalent agents, inhibition of polymerization has been demonstrated for aromatic amino acids (L-phenylalanine, L-tryptophan, and L-tyrosine) (Noguchi & Schechter, 1978), alkylureas (Elbaum et al., 1974), phenyl derivatives (Behe & Englander, 1979; Ross & Subramanian, 1977, 1978), benzyl esters of aromatic and hydrophobic amino acids (Gorecki et al., 1980a), and benzyloxy and phenoxy acids (Abraham et al., 1984). In addition, phenylalanine-containing di- and tripeptides and various oligopeptides have been shown to be able to inhibit Hb S polymerization (Noguchi & Schechter, 1978; Gorecki et al., 1980b; Franklin et al., 1983, Votano & Rich, 1985).

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[1] Abbreviations: Hb S, sickle cell hemoglobin; Hb A, human normal adult hemoglobin; Phe, L-phenylalanine; Trp, L-tryptophan; Tyr, L-tyrosine; His, L-histidine; CFA, 2-(*p*-chlorophenoxy)-2-methylpropionic acid; p-BrBzlOH, *p*-bromobenzyl alcohol; NMR, nuclear magnetic resonance; ppm, parts per million; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; T_1^{-1}, longitudinal relaxation rate; NOE, nuclear Overhauser effect; TRNOE, transferred nuclear Overhauser effect; EDTA, ethylenediaminetetraacetic acid.

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