Chloride Acts as a Novel Negative Heterotropic Effector of Hemoglobin Rothschild (β37 Trp→Arg) in Solution†

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Received November 22, 1993®

ABSTRACT: The effects of chloride ion concentration on the rate constants for association of carbon monoxide with human hemoglobin A and a synthetic form of the mutant hemoglobin Rothschild (β37 Trp→Arg) have been investigated by stopped-flow techniques. Previous studies of the structure [Kavanaugh *et al.* (1992) *Biochemistry 31*, 4111] and functional properties [Rivetti *et al.* (1993) *Biochemistry 32*, 2888] of hemoglobin Rothschild crystallized in the T state have demonstrated that the mutant arginine residues create new chloride ion binding sites and that chloride ions act to lower the oxygen affinity of hemoglobin Rothschild in these crystals. The studies reported here demonstrate a parallel effect of chloride ions on the rate of CO association with deoxygenated hemoglobin Rothschild in solution. Although the kinetics of CO binding to this hemoglobin in solution exhibit a Bohr effect, the chloride effect is independent of pH. In addition, we find that other halide ions have similar effects on the rate constants for the association of CO with this hemoglobin variant.

Recent work on hemoglobin crystals by Rivetti *et al.* (1993a) and Mozzarelli *et al.* (1991) has yielded the first information about the functional properties of hemoglobin in the crystalline state. These investigators discovered that crystals of deoxygenated human hemoglobin A (HbA)[1] grown in poly(ethylene glycol) (PEG)[1] could be sufficiently stabilized by increasing the PEG concentration to permit reversible oxygen binding without disruption of the crystal structure. As a result, the equilibrium of oxygen binding to crystals of hemoglobin in the quaternary T state could be measured. For the first time it was possible to measure the functional properties of a crystallographically determined structure of hemoglobin and to ascertain if the properties exhibited in the crystalline state differ significantly from those attributed to the equivalent structural state in solution.

Significant discrepancies were found when the equilibrium dissociation constant for the interaction of oxygen with the T state in the crystal was compared to K_1, the equilibrium dissociation constant for the first oxygen molecule binding to an otherwise deoxygenated hemoglobin tetramer in solution. The hemoglobin crystals were found to have the lower oxygen affinity, which was insensitive to pH, whereas K_1 in solution exhibits a marked pH dependence. Oxygen was found to bind to the crystals with little or no cooperativity, consistent with the crystal lattice preventing a ligand-linked transition in quaternary structure. The apparent differences between T-state properties in the crystals and in solution suggest that the crystal lattice exerts constraints upon the protein in the T state, altering functional behavior. The possible origins of these differences in functional properties have been discussed extensively by Rivetti *et al.* (1993a), but the clear implication is that identity of function, and therefore of structure, in solution and in the crystal cannot be taken for granted.

Following the work on HbA, the study of the structure and functional properties of crystalline hemoglobin was extended to the mutant hemoglobin Hb Rothschild (β37 Trp→Arg). Kavanaugh *et al.* (1992) demonstrated that deoxygenated Hb Rothschild in PEG solutions forms the same type of $P2_12_12$ crystals as deoxygenated HbA. They determined the three-dimensional structure of deoxyHb Rothschild in those crystals and compared it to that of HbA. Rivetti *et al.* (1993b) measured the equilibrium of oxygen binding to similar crystals of two different forms of Hb Rothschild: the naturally occurring mutant protein and a synthetic version in which each β chain also has an additional methionine residue at its amino terminus. The crystallographic analysis revealed an easily discernible chloride binding site at each mutant arginine residue. Oxygen binding measurements demonstrated that, in contrast to the properties of crystals of HbA, the oxygen affinity of crystalline Hb Rothschild is strongly dependent on the concentration of chloride ion. In the absence of chloride, crystals of the synthetic and naturally occurring forms of Hb Rothschild exhibit a 10-fold higher affinity for oxygen than crystals of HbA. However, the addition of chloride ion was found to lower the oxygen affinity of the crystals almost to that of HbA.

The discovery of a mutation-specific linkage between oxygen and chloride binding in the crystal offers another opportunity to compare the behavior of hemoglobin in the crystal and in solution. The absence of a Bohr effect in the crystal demonstrates that thermodynamic linkage relationships which occur in solution may be lost when a protein is crystallized. In the studies reported here, we explore whether the linkage between chloride and oxygen binding exhibited in the crystal is to be found in solution. By measuring the rate of association of CO with the deoxygenated tetramer in solution, we have discovered that chloride ions alter the properties of the T state of Hb Rothschild in a manner consistent with the reduction

† This work was supported by research funds from the USPHS National Institutes of Health Program Project Grant P01 HL40453 and the Veterans Administration.

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® Abstract published in *Advance ACS Abstracts,* March 15, 1994.

[1] Abbreviations: HbA, human hemoglobin A; Hb Rothschild, human hemoglobin Rothschild with β37 mutation Trp→Arg; PEG, poly(ethylene glycol); IHP, inositol hexaphosphate; K_1, equilibrium dissociation constant for the first oxygen binding event to an otherwise unliganded hemoglobin; l', overall association rate constant for the binding of carbon monoxide to hemoglobin; l'_1, association rate constant for the interaction of the first carbon monoxide with fully unliganded hemoglobin.

0006-2960/94/0433-4363$04.50/0

in oxygen affinity observed in the crystalline T state. The effect of chloride in solution appears to be independent of the T-state Bohr effect. In addition, bromide and iodide ions are fully as effective as chloride in altering the properties of the T state of Hb Rothschild.

EXPERIMENTAL PROCEDURES

Materials. Native human HbA was obtained from whole blood hemolysates as described by Geraci *et al.* (1969). Isolation and purification of HbA followed those procedures described by Doyle *et al.* (1992). This procedure includes the removal of organic phosphates and all other ions from the hemoglobin solution by passage through a mixed-bed resin column. Hemoglobin concentration was determined spectrophotometrically. The quality of each hemoglobin preparation was assessed by measuring the kinetic parameters for its reaction with CO in 0.1 M HCl–Bistris, pH 7.2, and comparing these to previously established standards.

Human hemoglobin Rothschild (β37 Trp→Arg) was a kind gift from Dr. Gary Ackers. The synthetic form of Hb Rothschild was prepared according to the methods of Hernan *et al.* (1992) by expression of the β globin in *Escherichia coli*. The *E. coli* containing the mutant β globin were obtained from Drs. Roxanne and Joseph Walder of the University of Iowa. This hemoglobin differs from native Hb Rothschild by having an additional methionine attached to the N-termini of both β chains. Unless otherwise stated, all studies were carried out on the synthetic form of Hb Rothschild.

Phosphate buffers used in these studies were prepared free of halide ions by simply mixing the appropriate amounts of monobasic and dibasic phosphates. Phosphate concentration was always kept at 0.1 M because the properties of hemoglobin are sensitive to this parameter. Halide ions were added as the sodium salt to obtain the concentration desired. Halide ion concentrations varied from 0 to 1.0 M. In order to avoid the introduction of extraneous anions with the addition of IHP, the pH of the 200 mM stock IHP solution was adjusted to approximately 5.6 by titration with the protonated form of Amberlite IR-120.

Kinetics of CO Binding. The kinetics of CO recombination following complete photodissociation of ligand were measured as described by Doyle *et al.* (1992). The rates of CO combination with deoxygenated Hb were measured in a stopped-flow apparatus similar to that described by Gibson and Milnes (1964). The procedures used were essentially those first described by Gibson (1959). Reactions were followed in a 2.0-cm cell at 420 and 435 nm at 20 °C. The final heme and CO concentrations were 1.5 and 15.3 μM, respectively. Dithionite was present at a concentration of 2.0 mM to ensure anaerobicity and to maintain the heme groups in the ferrous state. An on-line computer with OLIS (Bogart, GA) instrumentation and software was used for data collection and analysis.

Kinetic data were fitted to single- and multi-exponential functions using successive integration and Levenberg–Marquardt fitting routines as supplied by OLIS software. The two fitting procedures gave comparable results. The errors of the estimated rate constants within any one experiment were far smaller than the differences between the results of separate experiments. Therefore, the reported errors are the standard deviations derived from at least three independent experiments.

RESULTS

Previous studies of the structure and functional properties of crystalline Hb Rothschild were carried out on crystals of the T quaternary state of the protein. Therefore, it was important that the parameters measured in solution also reflect the properties of the T state. As pointed out by Sharma *et al.* (1980), one of the distinctive features of Hb Rothschild is its great tendency to dissociate into $\alpha\beta$ dimers. When saturated with ligand, the tetramer is very unstable with respect to this dissociation reaction, and the dimer is the predominant species observed in solution. Although deoxygenation increases tetramer stability, deoxygenated Hb Rothschild is not entirely tetrameric. This tetramer instability complicates the identification of parameters which define T-state properties. As mentioned in the introduction, K_1, the equilibrium dissociation constant for the first oxygen molecule binding to the hemoglobin tetramer, was used to compare the properties of the T state of HbA in solution and in crystals. However, in the case of Hb Rothschild, the ligand-linked tetramer to dimer dissociation reaction so dominates the equilibrium of oxygen binding to the system that it is virtually impossible to determine the dissociation constants for the separate steps in the binding of oxygen to the tetramer. For this reason, we resorted to kinetic measurements. The affinities of hemoglobin for the two ligands oxygen and carbon monoxide exhibit parallel changes in response to alterations in solution conditions. However, the kinetic reflections of affinity changes are very different for the two ligands. Changes in oxygen affinity result primarily in changes in the rate of oxygen dissociation, while a change in CO affinity is reflected primarily in the rate of CO combination. The rate of oxygen dissociation from the fully oxygenated hemoglobin would represent a measure of a combination of R-state and dimer properties. The oxygen pulse technique of Gibson (1973) could potentially be used to measure the rate of oxygen dissociation from the monoliganded tetramer, but it is impossible to eliminate the influence of the $\alpha\beta$ dimer. The kinetics of CO binding offer significant advantages over all other possible measurements. Like HbA, unliganded Hb Rothschild is in the T state, and it is under these conditions that the tetramer is the most stable. Therefore, when the deoxygenated molecule is mixed with CO in the stopped-flow apparatus, a reaction occurs which is rate-limited by the combination of CO with the T state of the protein. Since CO binds much more rapidly with $\alpha\beta$ dimers, the contribution of this species to the observed reaction kinetics is easily discerned and can be separated from that of the tetramer.

The reaction of the hemoglobin tetramer with CO is generally not a kinetically homogeneous process. Because of cooperativity and the related ligand-induced transitions in protein structure, there is an increase in the rate of CO binding, autoacceleration, as the reaction proceeds. It is the initial rate of the reaction which best estimates l'_1, the rate of binding of the first CO molecule to the hemoglobin tetramer. Figure 1 shows the time courses of the reactions of CO with HbA and Hb Rothschild as measured by rapid mixing in the stopped-flow apparatus. By fitting the entire time course for the binding of CO to HbA to a single-exponential function, the autocatalytic nature of this reaction has been made evident (Figure 1A). The time course for the binding of CO to Hb Rothschild has been fitted to a sum of two exponential functions, but only the exponential function for the slow kinetic phase is shown (Figure 1B). The fast phase of the CO binding reaction can be easily seen as a deviation of the experimental points from the exponential curve.

We have taken advantage of flash photolysis techniques and the use of IHP in stopped-flow experiments to investigate whether the fast phase results from the presence of $\alpha\beta$ dimers

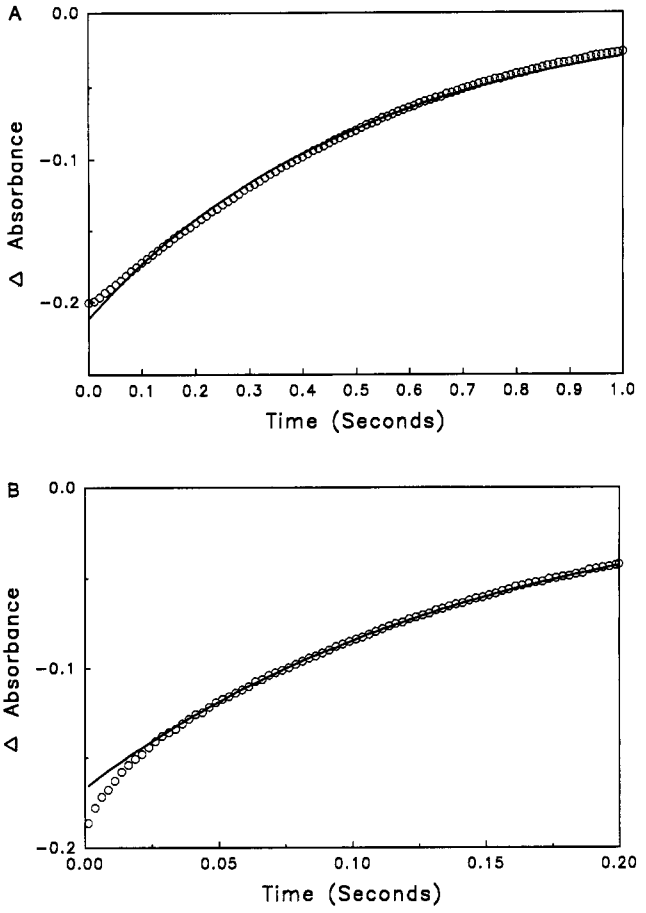

FIGURE 1: Time courses of the binding of CO to HbA (A) and Hb Rothschild (B) at 20 °C and pH 7.2 in the absence of halide ion. The original data appear as open symbols (O). The fitted equations are shown as continuous lines. The data for HbA were fitted to a single-exponential function. Those for Hb Rothschild were fitted to the sum of two exponential functions, but only the equation for the slower kinetic phase is plotted.

in equilibrium with the unliganded Hb Rothschild tetramer. In flash photolysis, one begins with a solution of CO-saturated hemoglobin, photodissociates the CO with a pulse of light, and measures the kinetics of CO rebinding. Because CO-saturated Hb Rothschild is highly dissociated into $\alpha\beta$ dimers (Sharma *et al.*, 1980), the kinetics of CO recombination give a precise measure of the properties of the dimer. The reactions of CO with the $\alpha\beta$ dimer were found to be biphasic, perhaps as a result of kinetic differences between the α and β chains. The rate constants for the two kinetic phases were $(6.1 \pm 0.9) \times 10^6$ and $(3.0 \pm 0.6) \times 10^6$ in the absence of halide ions and $(8.2 \times 0.4) \times 10^6$ and $(4.0 \pm 0.4) \times 10^6$ in the presence of 0.5 M chloride. Within experimental error, the average of the two rate constants under each condition in equal to the rate constant found for the fast phase seen under similar conditions in the stopped-flow experiments. The rate constants found for the fast phase in the stopped-flow experiments were $(5.4 \times 0.2) \times 10^6$ and $(6.4 \pm 0.2) \times 10^6$ in the absence of halide ions and in 0.5 M chloride, respectively.

IHP binds preferentially to the deoxyhemoglobin tetramer. As a result, IHP shifts the dimer–tetramer equilibrium in favor of the tetramer and increases the proportion of tetramer in the solution. As predicted, in stopped-flow experiments it was found that addition of IHP to final concentrations of 0.1 and 1.0 mM diminished the magnitude of the fast kinetic phase of CO binding (data not shown). Both of these lines of evidence support the hypothesis that the fast phase seen in Figure 1B is the reaction of CO with $\alpha\beta$ dimers which are at

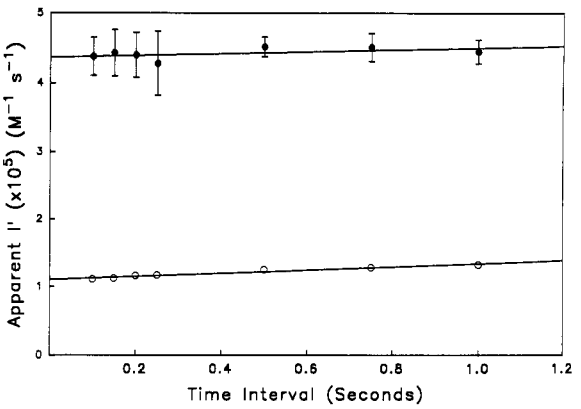

FIGURE 2: Estimations of l'_1. The apparent rate constants, apparent l'_1, for the binding of CO to the unliganded tetramers of HbA (O) and Hb Rothschild (●) were calculated from data obtained over different time intervals from the initiation of the reaction. For HbA, the kinetic data were fitted to a single-exponential function. For Hb Rothschild, the data were fitted to the sum of two exponential functions, and the slower kinetic phase was taken to represent the reaction of the Hb tetramer. The calculated rate constants are plotted as a function of the time interval over which they were obtained. Extrapolation to time zero gives an estimate of l'_1. The standard deviations for Hb Rothschild emphasize the insignificance of the autoacceleration observed. The standard deviations of the data for HbA were smaller than the size of the symbols, demonstrating that the autoacceleration observed for HbA is statistically significant.

equilibrium in solution with the deoxygenated hemoglobin tetramers.

The presence of the rapid, dimer reaction limits the precision with which one can determine l'_1, the initial rate of the reaction of the Hb tetramer with CO. For this reason, conditions were sought in which the dimer population would be absent or negligible. In addition to the effect of IHP, the amount of dimer was found to decrease with decreasing pH and to be a complex function of chloride concentration. However, no conditions were found in which the dimers were absent. Authentic human Hb Rothschild was examined in this regard since the additional N-terminal methionine residue on the β chains of the synthetic mutant has been shown to increase the instability of the tetramer (Doyle *et al.*, 1992). Under all conditions examined, the recombination of CO with the natural mutant exhibited a fast kinetic phase similar to that observed with the synthetic form.

To estimate l'_1, the apparent value of l', the rate of CO reaction with the Hb tetramer, was calculated from data collected over different intervals of time from the initiation of the reaction. For Hb Rothschild, this required fitting the data from each time interval to a sum of two exponential functions. By extrapolating the rate of the reaction to a zero time interval, the initial rate constant, l'_1, was estimated. In Figure 2 the apparent values of l' are plotted as functions of the time interval for HbA and Hb Rothschild. The autoaccelerating nature of the reaction of CO with HbA is again observed. For Hb Rothschild, autoacceleration is not statistically significant due to the increased error of the measurement. Fortunately, the error of the extrapolation to the value of l'_1 is small compared to the large difference in the values of this parameter for the normal and mutant protein.

The effects of chloride ion concentration on the values of l'_1 for HbA and Hb Rothschild are shown in Figure 3. Only at a concentration of 1.0 M does chloride appear to alter the properties of HbA, but the increase in association rate may actually reflect the effect of chloride on the activity coefficient of CO. In contrast, chloride ion has a profound effect on the kinetics of binding of CO to the T state of Hb Rothschild. A

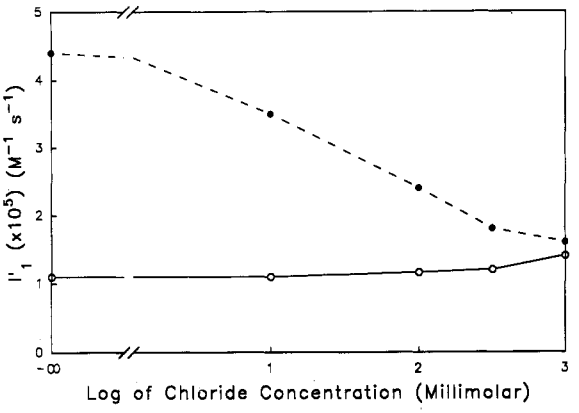

FIGURE 3: Effect of chloride concentration on the value of l'_1. The values of l'_1 for HbA (○) and Hb Rothschild (●) at 20 °C and pH 7.2 are plotted as functions of the log of the chloride concentration.

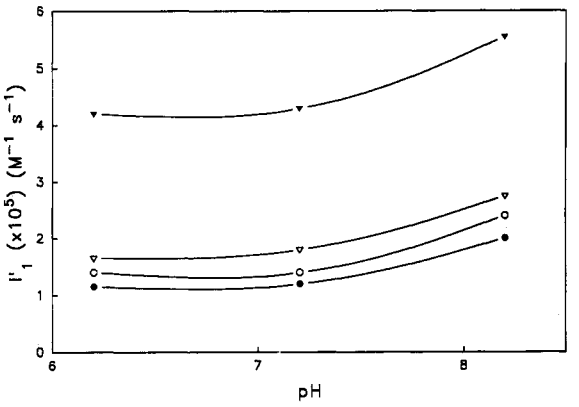

FIGURE 4: pH dependence of the effect of chloride on l'_1. Estimates of l'_1 in the absence of chloride (closed symbols) and in 0.5 M chloride (open symbols) for HbA (●, ○) and Hb Rothschild (▼, ▽) are plotted as functions of pH.

chloride ion concentration of 10 mM significantly decreases the rate of CO binding to Hb Rothschild, and concentrations of 0.5 M and greater decrease this rate almost to the value for HbA. As shown in Figure 4, the value of l'_1 for Hb Rothschild is dependent on pH, but the chloride effect is superimposed on the pH dependence and is independent of it. This kinetic Bohr effect is in contrast to the lack of a pH effect on the oxygen affinity of crystals of Hb Rothschild as reported by Rivetti *et al.* (1993).

Kavanaugh *et al.* (1992) demonstrated a unique chloride binding site in crystals in human Hb Rothschild by replacing chloride with bromide. This prompted examination of the effects of a variety of halide ions on l'_1. The effects of fluoride, bromide, and iodide ions are presented in Figure 5 along with those of chloride ion. At a concentration of 10 mM, the effects of bromide and iodide are similar to one another and greater than that of chloride. At 0.5 M and above, the effects of chloride, bromide, and iodide are the same within experimental error. Fluoride exhibits no tendency to reduce the value of l'_1 for Hb Rothschild, and at high concentration actually appears to increase this rate constant.

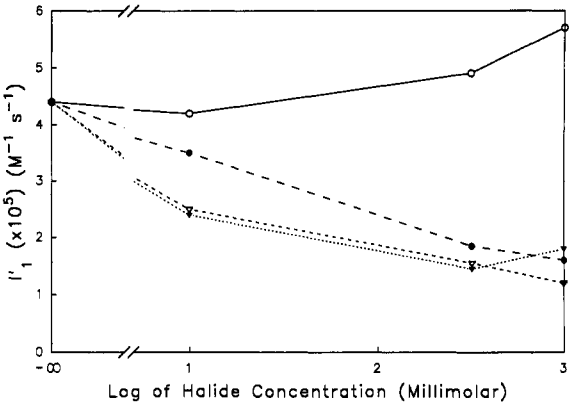

FIGURE 5: Effect of various halides on l'_1. Estimates of l'_1 for Hb Rothschild in the presence of fluoride (○), chloride (●), bromide (▽), and iodide (▼) are plotted as functions of halide concentration. No differential effect of these ions on the value of l'_1 for HbA was observed (data not shown).

DISCUSSION

One of the central goals of biochemistry is to understand how amino acid sequence determines protein structure and how structure in turn determines the functional properties of the protein. To develop this understanding requires knowledge of structure, for which X-ray crystallography has been the primary source. The assignment of structure by crystallography assumes that the structure and function in the crystal duplicate those in solution. The studies of Mozzarelli *et al.* (1991) and Rivetti *et al.* (1993a) demonstrate that for human HbA this is not precisely true. In the crystal, the oxygen affinity of the T state is lower than in solution, and the heterotropic pH effect is absent. Although these discrepancies can be explained by a number of models which hypothesize minimal distortions of structure and function by crystallization, the experimental results merely establish the inequality of functional properties, not the magnitude of the structural differences from which this inequality arises.

The studies reported here were designed to explore further the relationship between functional behavior in solution and in the crystal. For the first time, functional properties first identified in a crystalline protein have been demonstrated in solution. The effect of chloride ions on the kinetics of CO binding to the deoxygenated Hb Rothschild molecule is completely consistent with the effect that this anion has on the oxygen affinity of crystals of this hemoglobin. This suggests that in spite of a lack of identity, a relationship does exist between the functional properties of hemoglobin in solution and the crystal.

This result is consistent with the hypothesis that within the crystal lattice ligand-linked conformational transitions of the protein are constrained by lattice forces. At one level, the validity of this proposal is obvious. The lattice of the crystalline T state is incompatible with a change in the hemoglobin quaternary structure to the R state. This incompatibility probably explains the general instability of crystals of deoxygenated hemoglobin when oxygenated. The hypothesis also predicts that when conditions are found which permit reversible oxygen binding without damage to the crystal, the protein remains in the quaternary state in which it was originally crystallized.

There is now considerable evidence that ligand-linked conformational changes in hemoglobin are not limited to the large quaternary transition between the R and T states of the protein. In solution, tertiary changes within the quaternary T state almost certainly explain the T-state Bohr effect. Just as lattice forces in the crystal can constrain quaternary structure, they also seem to constrain ligand-linked changes in tertiary structure. Ligand-linked changes in the tertiary structure of the T state have been identified within the crystal (Liddington *et al.*, 1988), but these are not the same as the changes which occur in solution since, in the crystalline T state, no Bohr effect is observed.

The pH dependence of K_1 in solution has prompted the prediction that ligand binding to the T state is associated with sequential breaking of critical salt bridges which are postulated to be the source of protons for the Bohr effect. Brzozowski *et al.* (1984) and Liddington *et al.* (1988) have reported that in partially liganded crystals of the T state of HbA the salt bridges are intact, consistent with the lack of a Bohr effect and with the crystal imposing a constraint on this structural change. However, the binding of chloride ions to crystals of Hb Rothschild and the linkage between chloride binding and oxygen affinity demonstrate that the crystal lattice does not preclude the structural changes associated with all heterotropic effects. If the differences between functional properties in solution and in the crystal are indeed due to lattice constraints, then it would seem that, although heterotropic effects observed in solution might be attenuated or absent in the crystal, heterotropic effects observed in the crystal should always be present in solution, perhaps in amplified form. The effects of chloride on the properties of Hb Rothschild reported here support this idea. Further studies of the properties of hemoglobins in the crystalline state and in solution are warranted.

ACKNOWLEDGMENT

We thank Dr. Laura Kwiatkowski and Alice DeYoung for valuable suggestions.

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