Effect of Chloride on Oxygen Binding to Crystals of Hemoglobin Rothschild (β37 Trp → Arg) in the T Quaternary Structure†

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ABSTRACT: Oxygen binding to crystals of hemoglobin Rothschild (β37 Trp → Arg) in the T quaternary structure has been investigated by polarized absorption microspectrophotometry. These crystals were grown from poly(ethylene glycol) solutions containing low concentrations of salt. In the absence of chloride, they have a significantly higher oxygen affinity than crystals of human hemoglobin A grown in a similar manner, and exhibit Hill coefficients lower than 1. There is no Bohr effect from pH 6 to 9. We have found that chloride decreases the oxygen affinity of Hb Rothschild crystals, an effect which is absent in crystals of HbA. This dependence of affinity on chloride is almost certainly associated with the chloride binding sites which have been localized crystallographically at the mutant arginine residues (Kavanaugh *et al.*, 1992). Since chloride binding appears to lower the oxygen affinities of both the α and β chains, the linkage between the binding of oxygen and the dissociation of chloride results in significant cooperativity in oxygen binding to the crystals.

Crystals of human deoxyhemoglobin A grown from poly(ethylene glycol) solution at low salt concentration reversibly bind oxygen without cracking or undergoing other irreversible changes. Measurements of the equilibrium of oxygen binding to such crystals (Mozzarelli *et al*, 1991) have yielded the first quantitative information about the functional properties of the crystallographically determined structure of deoxyhemoglobin in the T state (Brzozowski *et al*, 1984; Liddington *et al.*, 1988). Some of these properties are rather different from those of hemoglobin in solution. The Hill coefficient for this binding reaction is close to unity, but the oxygen affinity is significantly lower than that observed for the binding of the first oxygen molecule in solution (Imai, 1982). The crystals exhibit no pH dependence in their oxygen affinity, contrary to the pH dependence observed for K_1 in solution (Imai & Yonetani, 1975; Lee *et al.*, 1988). Recently, crystallographic structures of partially liganded derivatives of hemoglobin in the T state in which the salt bridges remain intact have been reported (Liddington *et al.*, 1988; Luisi *et al.*, 1990; Abraham *et al.*, 1992). These results can be accounted for by a model in which both high- and low-affinity tertiary conformations of the T state, associated with broken and unbroken salt bridges, are populated in solution, but crystallization selects for the subset of structures with intact salt bridges (Rivetti *et al.*, 1993). This nicely explains the absence of a T-state Bohr effect in the crystalline state, in accordance with the Perutz (1970) stereochemical model.

Since the determination by X-ray crystallography of the structural changes induced by amino acid substitutions is commonly used to infer the origins of the effects of mutations on functional properties in solution, it seems essential to determine the influence of such mutations on the functional properties of the protein in the crystal. It is now well accepted that the αβ dimers of human hemoglobin A (HbA)[1] exhibit high oxygen affinity without cooperativity in oxygen binding (Mills *et al.*, 1976; Mills & Ackers, 1979). Bringing these dimers together to form the deoxygenated hemoglobin tetramer results in a drastic reduction in the ligand affinities of the four heme groups. Therefore, it is clear that the ligand affinity is being controlled in large measure by the contact interface between the two αβ dimers, the $\alpha_1\beta_2$ interface. Indeed, the large effects that mutations at this interface have on the binding of ligands to hemoglobin were predicted by the crystallographic studies of Perutz and co-workers (Perutz, 1970; Perutz & TenEyck, 1971). The thermodynamics of these effects have been measured directly by studies such as that of Pettigrew *et al.* (1982).

Numerous naturally occurring mutations in this region of the molecule have been reported. However, Hb Rothschild, β37 Trp → Arg, was particularly attractive for the first study. When this work was begun, a high-resolution crystallographic analysis of deoxyhemoglobin Rothschild in PEG crystals was being undertaken by Kavanaugh *et al.* (1992). Furthermore, Ackers and co-workers were undertaking a systematic examination of the thermodynamics and linkage relationships associated with ligand binding to Hb Rothschild in solution (Turner *et al.*, 1992). An added incentive to choose this particular mutant was its availability in two forms: the naturally occurring human mutant protein and a synthetic form. The latter was produced by expressing the mutant β-globin in *Escherichia coli* and then combining this with normal α chains and heme to produce the hemoglobin variant (Hernan *et al.*, 1992). Since the latter technology promises to make available a virtually endless variety of mutations in the $\alpha_1\beta_2$ interface, including those that have not been found in human subjects, a direct comparison of the naturally

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[1] Abbreviations: HbA, human hemoglobin A; Hb Rothschild, human hemoglobin Rothschild; PEG, poly(ethylene glycol); deoxyHb, deoxygenated hemoglobin; oxyHb, oxygenated hemoglobin; metHb, methemoglobin or ferric hemoglobin; deoxy, deoxygenated; oxy, oxygenated.

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occurring and synthetic variants seemed particularly advantageous.

Hb Rothschild was first described by Gacon *et al.* (1977). In their preliminary study of the functional properties of this mutant, those investigators found that in Bis-Tris buffer, in the absence of organic phosphates, this Hb exhibited a lower overall oxygen affinity than HbA, and also bound oxygen with considerably less cooperativity. Sharma *et al.* (1980) reported a more detailed analysis of the properties of this hemoglobin variant. Most notable was their finding that Hb Rothschild exists as a tetramer when deoxygenated, while the liganded molecule is almost entirely dissociated into dimers. Because of this dissociation, oxygen equilibria measured in solution do not represent the properties of the tetrameric form of the molecule.

In the present study, we have investigated the binding of oxygen to crystals of Hb Rothschild in the T state grown in PEG solutions. In the course of this investigation, we discovered an effect of chloride on oxygen binding to these crystals and have examined this phenomenon.

MATERIALS AND METHODS

Hemoglobin. Hb Rothschild was a kind gift of Dr. Arthur Arnone. Recombinant Hb Rothschild contained the β37 Trp → Arg substitution and an additional methionine residue attached to the N-terminus of the β chains. This β-globin had been expressed in *E. coli* using a T-7 expression system, isolated, and then allowed to fold in the presence of native human α chains and cyanohemin as described by Hernan *et al.* (1992). The resulting product was reduced with dithionite and purified on DE52 (Whatman). It was homogeneous by HPLC and PAGE, and exhibited functional properties similar to those of the natural human Hb Rothschild.

Human HbA was prepared as previously described (Fowler *et al.*, 1992).

Crystals. Crystals of deoxyHb Rothschild and deoxyHbA were grown at room temperature from PEG solutions by a modification of the batch method of Ward *et al.* (1975) and Arnone *et al.* (1980). Crystallization took place from solutions containing 10 mM potassium phosphate buffer, 30 mM sodium dithionite, 1.0 mM Hb in heme equivalents, and PEG 8000 M_r (Sigma) from roughly 10 to 14% w/v, at pH 7.2. All components were carefully deoxygenated before being combined in deoxygenated 8 × 30 mm vials in order to minimize the presence of oxidation products of dithionite. The final solution volume in each vial was 100 μL. Orthorhombic crystals (space group $P2_12_12$) typically appeared overnight. The mother liquor was withdrawn and replaced first by 20% w/v PEG and then by 36% w/v PEG. Both PEG solutions were anaerobic and contained 10 mM phosphate, pH 7.2, and 30 mM dithionite. Crystals were then stored anaerobically in the latter solution at 4 °C until use.

Oxygen Binding Curves of Hemoglobin Crystals. For measurements, crystals of deoxyHb were resuspended several times in a nitrogen-equilibrated, dithionite-free solution containing 36–62% w/v PEG 8000 M_r [purified according to Ray and Puvathingal (1985)], 10 mM potassium phosphate, and 1 mM EDTA, pH 7.2. Catalase (Sigma), 0.1 mg/mL, was added to the solution to decrease hemoglobin oxidation during the long time required to complete oxygen titrations (usually 10 h). Addition of catalase to the crystal suspensions significantly reduces the rate of oxidation of Hb to metHb. The enzyme does not enter the crystals, but appears to effectively reduce the low levels of peroxides that can form even when purified PEG is used. Crystals were then placed on the bottom window of the Dvorak–Stotler flow cell (Dvorak & Stotler, 1971) and covered by an optically isotropic, gas-permeable silicon-copolymer membrane, MEM 213 (General Electric) (Gill, 1981). Helium–oxygen humidified gas mixtures containing oxygen at partial pressures between 0 and 760 torr were prepared with a computerized gas standards generator (ENVIRONICS, series 200) and flowed into the cell using 316 stainless-steel tubing. Oxygen pressures were determined in the outflow from the cell with an oxygen meter equipped with a Clark electrode (OXYAN II, Advanced Products). The flow cell was mounted on the thermostatically controlled stage of a Zeiss MPM03 microspectrophotometer.

Single crystal polarized absorption spectra were recorded between 450 and 700 nm with the electric vector of the incident light parallel either to the *a* or to the *c* crystal axis. These axes coincide with principal optical axes.

Oxygen binding curves were obtained by recording spectra of Hb crystals equilibrated with different oxygen pressures. Fractional saturation of the ferrous heme groups with oxygen was determined by least-squares fitting of the observed spectra to a linear combination of three reference spectra and a base line. The reference spectra are the polarized absorption spectra of deoxyHb, oxyHb, and metHb Rothschild crystals. The procedures for obtaining the reference spectra and the optical theory of single crystal polarized absorption measurements have been previously reported (Eaton & Hofrichter, 1981; Rivetti *et. al.*, 1993).

Curve-Fitting. The simultaneous global fitting of the oxygen binding data to a set of nonlinear equations was accomplished by the Levenberg–Marquardt procedure as found in "SigmaPlot" (Jandel).

RESULTS

Single crystal polarized absorption spectra of deoxy-, oxy-, and metHb Rothschild recorded along the *a* and *c* axes are presented in panels a, b, and c, respectively, of Figure 1. The ratio at each wavelength of the absorbances along the two crystal axes, the polarization ratio, is also indicated. This ratio is a function of the transition moment directions and of the relative orientations of the hemes with respect to the crystal axes, and may change with changes in protein conformation (Eaton & Hofrichter, 1981). The heme absorption bands between 525 and 580 nm and between 630 and 650 nm are for the most part *x,y*-polarized (Eaton & Hofrichter, 1981). The average polarization ratio for these bands was determined from the ratios of the integrated intensities of the spectra obtained along the *a* and *c* crystal axes within these wavelength limits. In Table I, these values for the deoxy, oxy, and met derivatives of Hb Rothschild crystals are compared to those reported for crystals of HbA by Rivetti *et al.* (1993).

From the crystallographic structure, it is possible to calculate the relative contribution of an individual heme, *j*, to the absorption by *x,y*-polarized transitions along a particular crystal axis, μ. For such transitions, the absorption intensity is proportional to $\sin^2 z_j\mu$ where $z_j\mu$ is the angle between the *z* vector normal to the plane of heme *j* and the crystal axis μ. The values of $\sin^2 z_j\mu$ for the α and β chains of Hb Rothschild are presented in Table II along with the sum of these values, $\sum_j \sin^2 z_j\mu$, for each crystal axis. The ratio of this sum for the *a* axis to that for the *c* axis is the predicted value for the polarization ratio and is equal to 1.62, similar to the value of 1.68 measured directly (see Table I). Lacking structural information about the oxygenated and oxidized derivatives of the crystals of Hb Rothschild, we are unable to calculate predicted values for their polarization ratios. However, from

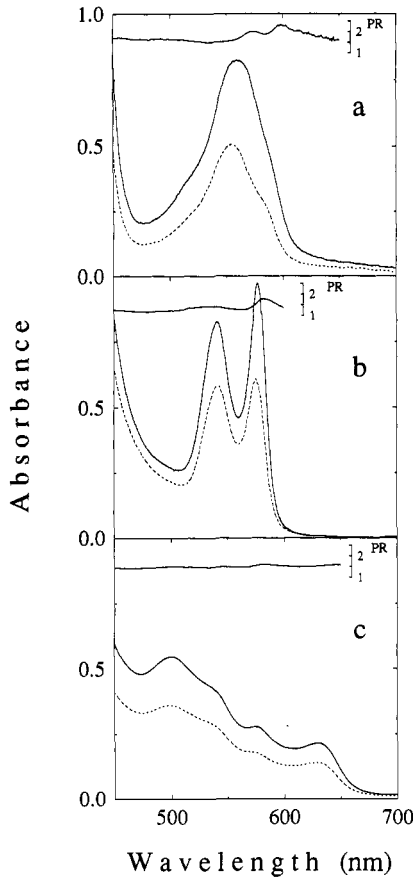

FIGURE 1: Reference spectra of crystals of Hb Rothschild. (a) Polarized absorption spectra of pure deoxyHb Rothschild crystals were recorded along the *a* (—) and *c* (– – –) axes of crystals suspended in 30 mM sodium dithionite, 36% w/v PEG 8000 M_r, 10 mM potassium phosphate, and 1 mM EDTA, pH 7.2, at 15 °C. (b) Polarized absorption spectra of pure oxyHb Rothschild crystals were obtained by recording spectra along the *a* (—) and *c* (– –) axes at oxygen pressures between 160 and 760 torr, at 8 °C where the oxygen affinity is higher, and by calculating the spectra at infinite oxygen pressure, as previously described (Rivetti *et al.*, 1993). (c) Polarized absorption spectra of pure metHb Rothschild were recorded along the *a* (—) and *c* (– –) axes of crystals, first suspended in a solution containing 3 mM potassium ferricyanide and then resuspended several times in solutions containing 36% w/v PEG 8000 M_r, 10 mM potassium phosphate, and 1 mM EDTA, pH 7.2. In separate experiments, the pH dependence of the spectra of metHb Rothschild crystals was determined (data not shown). For each set of spectra, the polarization ratio, PR, has been calculated as a function of wavelength, and is presented.

Table I: Average Measured Polarization Ratios between 525 and 580 nm and between 630 and 650 nm for T-State Crystals of Hb Rothschild and HbA

derivative	polarization ratio	
	Hb Rothschild	HbA[a]
deoxygenated	1.68	1.77
oxygenated	1.40	1.41
aquometHb	1.54	1.61

[a] From Rivetti *et al.* (1993)

Table I it is evident that the changes in polarization ratio which accompany oxygenation or conversion to the aquomet form are similar for crystals of Hb Rothschild and HbA.

The spectral changes associated with oxygen binding to crystals of Hb Rothschild at 21 °C in 36% w/v PEG 8000 M_r, 10 mM phosphate, pH 7.2, are shown in Figure 2. Hill plots of the data for natural and recombinant Hb Rothschild and for HbA are shown in Figure 3. The fractional saturations used in these Hill plots were calculated by using the reference

Table II: $\sin^2$ Values of the Angles between the Vectors Normal to the Heme Planes, z_j, and the Axes of Crystals of Hb Rothschild[a]

subunit	principal crystal axis		
	a	*b*	*c*
α_1	0.745	0.521	0.734
α_2	0.758	0.823	0.419
β_1	0.880	0.866	0.254
β_2	0.796	0.648	0.557
$\sum \sin^2 z_{j\mu}$	3.179	2.858	1.964

[a] Based upon the crystallographic data of Kavanaugh *et al.* (1992) calculated using the procedure described by Rivetti *et al.* (1993).

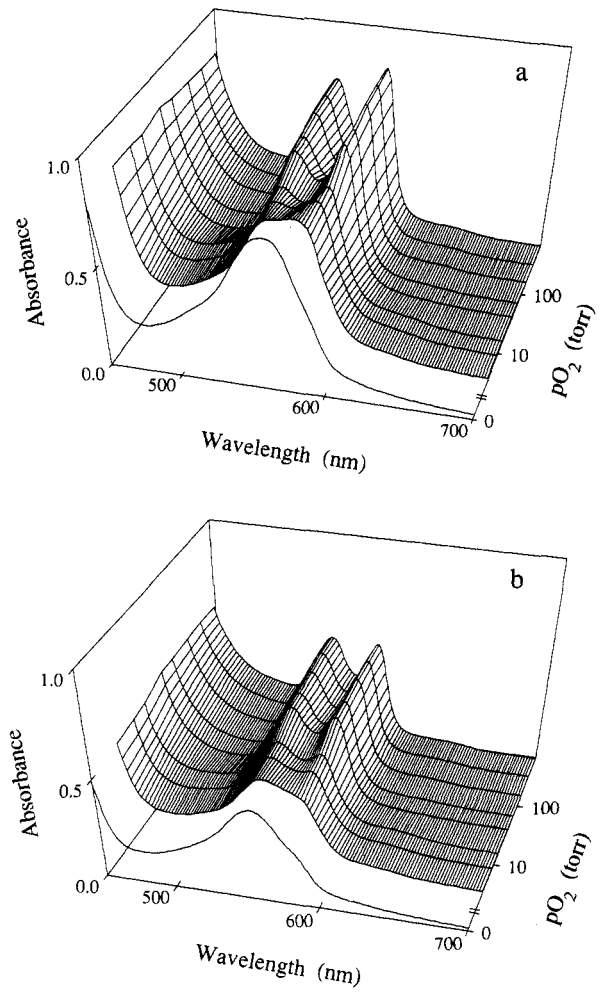

FIGURE 2: Spectral changes upon oxygen binding to Hb Rothschild crystals. Crystals of Hb Rothschild, suspended in a solution containing 36% w/v PEG 8000 M_r, 10 mM potassium phosphate, and 1 mM EDTA, pH 7.2, were equilibrated stepwise with increasing oxygen partial pressures between 0 and 760 torr at 21 °C. At each oxygen pressure, polarized absorption spectra were recorded using linearly polarized light with the electric vector parallel either to the *a* (panel a) or to the *c* (panel b) crystal axis. One crystal was used between 0 and 36 torr and a second between 36 and 760 torr. The absorption intensities of the two crystals were scaled with respect to the reference spectra. Oxygen pressure is plotted on a log scale so that distances is proportional to chemical potential. The spectra obtained at zero oxygen pressure are displayed but are obviously not on the same scale. Almost perfect isosbestic points are observed when spectral variations due to metHb formation during the experiment and baseline offset are taken into account.

spectra in Figure 1a–c. The apparent oxygen tension required for half-saturation ($p50$) of the Hb Rothschild crystals is 22 and 17 torr as measured along the *a* and *c* crystal axes, respectively. Natural and recombinant Hbs Rothschild have

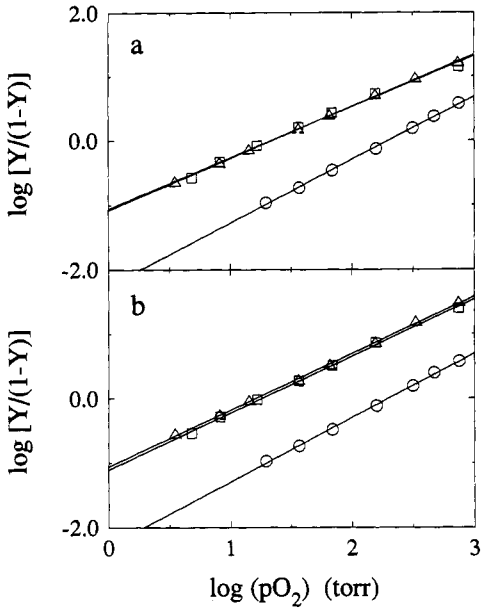

FIGURE 3: Hill plots of oxygen binding data for naturally occurring Hb Rothschild, recombinant Hb Rothschild, and HbA. Crystals of natural Hb Rothschild (△), recombinant Hb Rothschild (□), and HbA (○) were suspended in 36% w/v PEG 8000 M_r, 10 mM potassium phosphate, and 1 mM EDTA, pH 7.2, at 21 °C. The Hill coefficients, n, obtained along the a axis (panel a) are 0.80, 0.79, and 0.98 and along the c axis (panel b) are 0.88, 0.88, and 0.99. The $p50$ values for the a axis are 22, 22, and 200 torr, and for the c axis are 16, 18, and 198 torr. The fraction of metHb in the three experiments varied between 0.03 and 0.26. In contrast to HbA, a slight decrease in oxygen fractional saturation with increasing metHb content was observed at low levels of fractional saturation. This effect might be due to preferential oxidation of high oxygen affinity hemes. Moreover, at low fractional saturation with oxygen, crystals of Hb Rothschild approached equilibrium more slowly than those of HbA.

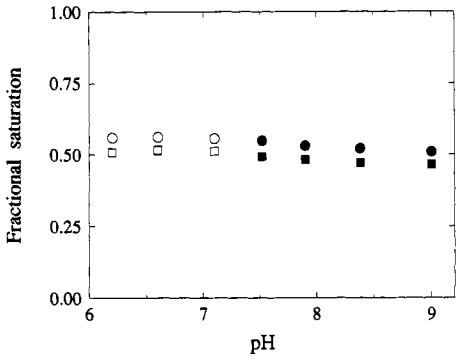

FIGURE 4: Dependence of oxygen fractional saturation of Hb Rothschild crystals on pH. Crystals of Hb Rothschild were suspended in a solution containing 36% w/v PEG 8000 M_r, 1 mM EDTA, and either 10 mM potassium phosphate (open symbols) at pH between 6.2 and 7.2 or 10 mM potassium borate (closed symbols) at pH between 7.5 and 9.0, at 23 °C, and equilibrated at constant oxygen pressure. Polarized absorption spectra were recorded on a crystal between pH 6.2 and 7.5 and on a second crystal between pH 7.5 and 9.0. Oxygen-fractional saturations are reported for the a axis (□, ■) and the c axis (○, ●). The fraction of metHb present during the experiment varied between 0.05 and 0.3.

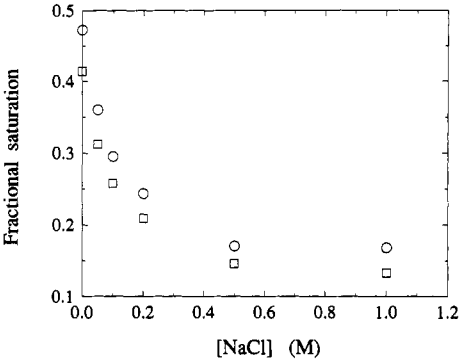

FIGURE 5: Dependence of oxygen fractional saturation of Hb Rothschild crystals on chloride concentration. Crystals of Hb Rothschild were suspended in a solution containing 36% w/v PEG 8000 M_r, 10 mM potassium phosphate, 1 mM EDTA, pH 7.2, and increasing concentrations of sodium chloride. Chloride concentration is calculated on the basis of the total volume of the solution. The actual volume occupied by water is 1.4 times less. Polarized absorption spectra were recorded upon equilibration of the crystals to an oxygen pressure of 14.2 torr, at 21 °C. Apparent oxygen fractional saturations are reported for the a axis (□) and the c axis (○). The fraction of metHb varied between 0.13 and 0.33. Four crystals were used in the experiment.

the same oxygen affinities which are about 10-fold higher than that of HbA. The oxygen affinity of crystals of HbA grown as described in the present paper is identical to that of crystals grown as described by Rivetti *et al.* (1993). The Hill coefficient for the binding of oxygen to crystals of HbA is close to unity while the values obtained for Hb Rothschild are less than 1, 0.80 and 0.88 along the a and c crystal axes, respectively. Such Hill coefficients indicate considerable heterogeneity in the affinities of the oxygen binding sites and, as will be seen, are consistent with the presence of two populations of sites whose affinities differ by a factor of 7.

As was found for crystals of HbA (Mozzarelli *et al.*, 1991), the crystals of Hb Rothschild exhibit no Bohr effect. Figure 4 illustrates the effect of pH on the oxygen fractional saturation of such crystals at a fixed oxygen tension. Data were obtained using phosphate buffers between pH 6.2 and 7.2 and borate between pH 7.5 and 9.0.

An unexpected result was obtained when oxygen binding was examined in the presence of buffers containing chloride ions. In contrast to what is observed for HbA crystals (Rivetti *et al.* 1993), chloride anions markedly decrease the oxygen affinity of Hb Rothschild crystals (Figure 5). The effect of NaCl concentration is a reduction in oxygen saturation of roughly 3-fold upon raising the concentration of NaCl from 0 to 1 M.

The full effect of chloride can only be detected by examining the way this ion alters the shape of the oxygen saturation curve. However, it was observed that in the presence of high concentrations of NaCl, Hb Rothschild crystals deteriorate when fractional saturation with oxygen is high. Just as the presence of PEG at concentrations of 36% w/v and higher was found to stabilize HbA crystals against melting (Grabowski *et al.*, 1978) and cracking upon oxygenation (Mozzarelli *et al.*, 1991), a still higher PEG concentration, 62% w/v, was found to prevent the NaCl-induced deterioration of Hb Rothschild crystals.

In the absence of NaCl, data were collected for Hb Rothschild in both 36% and 62% w/v PEG (Figure 6), and no significant effect of PEG concentration on oxygen affinity was observed. Hill plots obtained in the presence of several concentrations of sodium chloride are presented for the a (Figure 6a) and c (Figure 6b) crystal axes. Addition of NaCl reduced the fractional saturation with oxygen at all oxygen tensions. However, the effects are not uniform, the reduction in the apparent oxygen affinity being much greater at low levels of saturation than at high. This unequal response to NaCl produces the phenomenon of cooperativity in the binding of oxygen as indicated by the changes in the slopes of the Hill plots in Figure 6. In fact, at NaCl concentrations above 0.1 M, the Hill coefficient at half saturation becomes greater than 1.

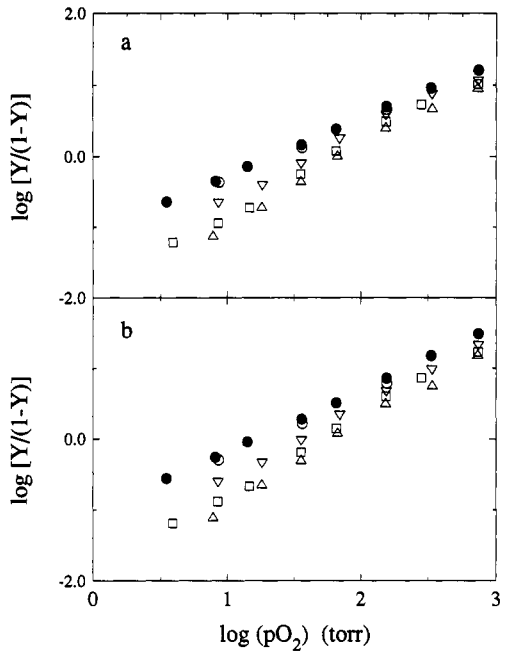

FIGURE 6: Hill plots of oxygen binding data for Hb Rothschild crystals in the presence of chloride ions. Oxygen binding curves were determined for Hb Rothschild crystals suspended in a solution containing either 36% (closed symbols) or 62% (open symbols) w/v PEG 8000 M_r, 10 mM potassium phosphate, and 1 mM EDTA, pH 7.2, in the absence (○, ●) and in the presence of 100 (▽), 200 (□), and 500 mM (△) sodium chloride, at 21 °C. Data collected along the *a* and the *c* axis are presented in panels a and b, respectively.

DISCUSSION

The crystals of Hb Rothschild examined in this study are grown from poly(ethylene glycol) solutions at low salt concentration under anaerobic conditions. On the basis of the close similarity between calculated and observed polarization ratios, these crystals appear to be identical to those from which Kavanaugh *et al.* (1992) have determined the three-dimensional structure of this hemoglobin.

The structure of the deoxyHbA determined from crystals grown from PEG solutions (Kavanaugh *et al.*, 1992) is essentially identical to that found by Fermi *et al.* (1984) in crystals grown at high salt concentration. This structure has been commonly assumed to be that of "T-state" hemoglobin. However, one must use this label advisedly since the relationship between the quaternary structure observed crystallographically within these crystals and that of the deoxygenated molecule in solution is not necessarily a simple one. Studies of the binding of oxygen to PEG crystals of HbA have clearly established that HbA in the crystalline state exhibits functional properties very different from those observed in solution (Mozzarelli *et al.*, 1991; Rivetti *et al.*, 1993). As already mentioned, in solution the interaction of two deoxygenated α–β dimers to form a deoxygenated tetramer results in a large alteration in the properties of the oxygen binding sites. It is reasonable to expect that changes in the $\alpha_1\beta_2$ interface can alter the functional properties of the T state in solution. The situation in the crystal is less clear. Given that the intermolecular interactions within the crystal lattice can alter functional properties, one wonders to what extent the effects of these interactions might be dominant. Since the molecular packing of Hb Rothschild crystals is very similar to that of HbA crystals (Kavanaugh *et al.*, 1992), the large effect of the β37 Trp → Arg substitution on functional properties of Hb crystals, as evidenced by the present study, establishes unequivocally the sensitivity of the crystalline tetramer to the nature of the contacts at the $\alpha_1\beta_2$ interface. The higher oxygen affinity of Hb Rothschild with respect to HbA in T-state crystals parallels the indication derived by kinetic measurements (Sharma *et al.*, 1980) that the Trp → Arg mutation increases the oxygen affinity of the T state in solution. Equilibrium measurements of oxygen binding to the T state of Hb Rothschild in solution are not available, since the binding equilibrium is dominated by the dimer–tetramer equilibrium, and it has not been possible to separate the individual steps of oxygen binding to the tetramer (Turner *et al.*, 1992).

The structural constraints of the crystal lattice not only prevent the T to R transition but also seem to forbid the conformational response to ligand binding which is normally exhibited by the T state in solution, since crystals of both HbA and Hb Rothschild exhibit no Bohr effect. However, with Hb Rothschild, we have now demonstrated that there is sufficient conformational response to oxygen binding to result in a thermodynamic linkage between this binding event at the heme groups and the binding of chloride ions.

This difference in chloride sensitivity between HbA and Hb Rothschild can be explained by the presence of chloride binding sites in Hb Rothschild which are absent in HbA. Just such a pair of sites was discovered by Kavanaugh *et al.* (1992) by X-ray crystallographic analysis of the same type of PEG crystals used in the present study. Their crystals were grown and examined in the presence of 100 mM chloride. In order to visualize the chloride binding sites, they soaked crystals in buffers in which chloride ion was replaced by bromide. This resulted in two very distinct electron density peaks per tetrameric Hb Rothschild molecule in the electron density difference map. These peaks were adjacent to the mutant arginine residues, explaining their uniqueness to Hb Rothschild. No other chloride binding sites were found. The replacement of a tryptophan residue with an arginine alters bulk and hydrophobicity, but, very importantly, it also inserts a positively charged guanidinium ion into this location. It is perhaps not surprising that the neutralization of this charge with chloride reduces somewhat the functional perturbation resulting from this substitution, decreasing the oxygen affinity of Hb Rothschild crystals and making them more similar to crystals of HbA.

The linkage between chloride binding and oxygen affinity requires a reciprocal effect (Wyman, 1964). This reciprocity offers an explanation of the cooperativity observed in oxygen binding to these crystals in the presence of chloride ions. The key to this explanation is the observation that chloride binding affects the oxygen affinities of all of the heme groups. Qualitatively, the situation can be described as follows. The binding of oxygen lowers the affinity of Hb Rothschild for chloride ion. Therefore, as the tetramer is progressively saturated with oxygen, chloride ions progressively dissociate. This results in there being more chloride ions bound at the beginning of the oxygen saturation process than near its end. Therefore, oxygen affinity will be lower at low oxygen saturation than when full saturation is approached. The effect will obviously be absent when no chloride is present and would disappear once more if the chloride ion concentration were raised sufficiently high that the chloride binding sites remained fully occupied when the hemoglobin was saturated with oxygen. However, it is likely that the latter would require unattainable concentrations of chloride.

In order to model this system precisely, we should simultaneously deal with the linkage between four oxygen and two chloride binding sites. However, to do so requires the determination of numerous distinct linkage terms. A simpler

model which illustrates the phenomenon reasonably well can be constructed by assuming that a single chloride binding site influences the two subunits (α and β) between which it is located. Such a formulation becomes trivial if one assumes the oxygen binding sites to be homogeneous. However, binding site homogeneity cannot accommodate Hill coefficients below 1.0. Since we observe values as low as 0.8, there must be intrinsic heterogeneity among the oxygen binding sites. As will be discussed, this heterogeneity is also a necessary condition for the differences in the apparent binding isotherms measured along the two crystal axes. In analogy to HbA crystals, we assign this heterogeneity to functional differences between the α and β chains (Rivetti *et al.*, 1993).

Let us assume that one Cl^- binding site is thermodynamically linked to one α-chain heme and one β-chain heme. In the absence of chloride, the oxygen dissociation constants are K_a for the α chains and K_b for the β chains. For the binding of oxygen to this dimeric unit, the sequence of events can be written as

$$\alpha\beta + 2O_2 \overset{K_1}{\rightleftharpoons} (\alpha\beta)O_2 + O_2 \overset{K_2}{\rightleftharpoons} (\alpha\beta)(O_2)_2$$

or as

$$\begin{array}{ccc} \alpha\beta + 2O_2 & \xrightarrow{K_a} & \alpha O_2\beta + O_2 \\ \Big\uparrow K_b & & \Big\uparrow K_b \\ \alpha\beta O_2 + O_2 & \xrightarrow{K_a} & \alpha O_2\beta O_2 \end{array}$$

where $K_1 = K_aK_b/(K_a + K_b)$ and $K_2 = K_a + K_b$. Assume that the binding of a Cl^- ion raises both dissociation constants for oxygen by a factor c such that in the presence of saturating amounts of Cl^- ${}^{Cl}K_a = cK_a$ and ${}^{Cl}K_b = cK_b$. This is in fact a two-state model in which the two states are defined by the presence or absence of a chloride ion. It can be shown that for any value of $[Cl^-]$ the apparent value of K_1, $\bar{K}_1$, is given by

$$\bar{K}_1 = K_1(1 + [Cl^-]/K_{Cl})/(1 + [Cl^-]/cK_{Cl}) \qquad (1)$$

and the apparent value of K_2, $\bar{K}_2$, is given by

$$\bar{K}_2 = K_2(1 + [Cl^-]/cK_{Cl})/(1 + [Cl^-]/c^2K_{Cl}) \qquad (2)$$

where K_{Cl} is the dissociation constant for chloride ion in the absence of oxygen. Assuming that all of the $\alpha\beta$ units function independently of one another, we can write

$$Y = \frac{pO_2/\bar{K}_1 + 2(pO_2)^2/\bar{K}_1\bar{K}_2}{2 + 2pO_2/\bar{K}_1 + 2(pO_2)^2/\bar{K}_1\bar{K}_2}$$

where Y is the fractional saturation of the ferrous heme groups with oxygen. The present formulation attributes binding site heterogeneity to differences between the α- and β-subunits of the crystalline hemoglobin. If this is correct, then the monoliganded $\alpha\beta$ unit will be a population in which a disproportionately large fraction of the high-affinity subunits will be liganded. Since the orientations of the α- and β-heme with respect to any given crystal axis are not necessarily the same, their contributions to the absorbance or to absorbance changes associated with ligand binding may also differ. If this is the case, then the absorbance change will be a nonlinear function of ligand saturation since the absorbance change associated with the formation of the monoliganded $\alpha\beta$ unit will not be the simple average of that associated with binding ligand to an α chain and to a β chain. This function will also vary with the crystallographic axis along which the measurements are made. This can account for the differences observed in the estimated values of Y obtained from data collected with light polarized parallel to the a axis and to the c axis of the crystal of Hb Rothschild. The value of Y estimated from absorbance measurements, $Y(\text{est})$, will be given by

$$Y(\text{est})_\mu = \frac{\nu_\mu pO_2/\bar{K}_1 + 2(pO_2)^2/\bar{K}_1\bar{K}_2}{2 + 2pO_2/\bar{K}_1 + 2(pO_2)^2/\bar{K}_1\bar{K}_2} \qquad (3)$$

where ν is a unitless factor and μ refers to the crystallographic axis along which the spectral measurements are made. By simultaneously fitting all of our binding data to eq 1 and 2 and to two forms of eq 3, with $\mu = a$ and $\mu = c$, we have solved for K_1, K_2, K_{Cl}, c, ν_a, and ν_c, and obtained:

$$K_1 = 5.8 \pm 1.7 \text{ torr}, K_2 = 52 \pm 10 \text{ torr}$$

$$K_{Cl} = 33 \pm 8 \text{ mM}, c = 15 \pm 3$$

$$\nu_a = 0.79 \pm 0.17, \nu_c = 1.00 \pm 0.17$$

The fit of the data is shown in Figure 7. The values of K_1 and K_2 indicate K_a and K_b values of 6.5 and 46 torr, but from this analysis, it is impossible to determine which value should be assigned to which subunit. Rivetti *et al.* (1993) report that in crystals of HbA the α chains have on average a 5-fold greater oxygen affinity than the β chains. This determination was based upon a detailed analysis of oxygen binding to HbA crystals, taking into account the relative projections of the heme planes of deoxyHb, oxyHb, and metHb along the a and c crystal axes. Such an analysis cannot be performed on data obtained from Hb Rothschild crystals, since no information about the structure of the liganded T state of Hb Rothschild is available. However, if one examines the data in Table I, one sees that the sum of the $\sin^2$ values for the α chains is a greater fraction of the total $\sum \sin^2 z_j\mu$ along the c axis than along the a axis. Since the apparent affinity for oxygen is greater along the c axis, we assign the greater affinity to the α chains, giving $K_a/K_b = 0.14$.

It can be shown that

$$\nu_\mu = \frac{2[(\Delta\epsilon_a/\Delta\epsilon_b)_\mu + K_a/K_b]}{(1 + K_a/K_b)[(\Delta\epsilon_a/\Delta\epsilon_b)_\mu + 1]}$$

or

$$(\Delta\epsilon_a/\Delta\epsilon_b)_\mu = \frac{2(K_a/K_b) - \nu_\mu(1 + K_a/K_b)}{\nu_\mu(1 + K_a/K_b) - 2} \qquad (4)$$

where $\Delta\epsilon_a$ and $\Delta\epsilon_b$ are the changes in the extinction coefficients of the α and β chains, respectively, that result from oxygen binding. With the values of K_a and K_b being significantly different, $\nu_c = 1$ indicates that the α and β chains contribute equally to the spectral changes along the c axis associated with oxygen binding. A value of $\nu_a = 0.79$ indicates a difference along the a axis between $\Delta\epsilon_a$ and $\Delta\epsilon_b$.

Rivetti *et al.* (1993) concluded that in T-state crystals of HbA, α and β chains contribute equally to absorbance along the a axis but make different contributions along the c axis; i.e., $\nu_a = 1$ and $\nu_c > 1$. The absolute values of ν_a and ν_c are strongly coupled in our fitting functions, and the estimates of these parameters are associated with significant standard errors. It is therefore unclear whether the crystals of these two hemoglobins actually differ in this regard.

Our model with its many simplifying assumptions is remarkably successful in reproducing the principal characteristics of the data. Beginning with a dimeric unit, which in the absence of chloride is without cooperativity in oxygen

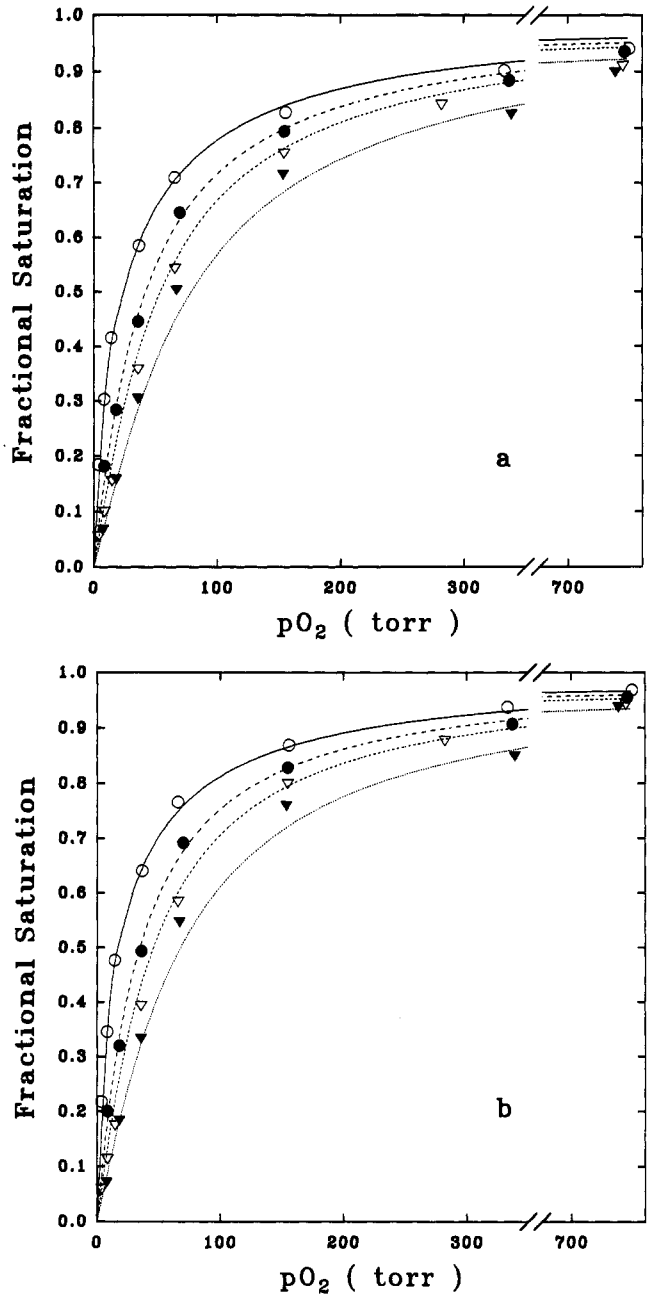

FIGURE 7: Linkage relationship between oxygen fractional saturation and chloride concentration. The oxygen binding curves reported in Figure 6a,b are simultaneously fitted to eq 1–3 by least-squares analysis. The experimental data (symbols) and the fitted curves are reported for measurements carried out in the absence (○—○) and in the presence of 100 (●– –●), 200 (▽– – –▽), and 500 mM (▼···▼) sodium chloride. Data obtained along the *a* and *c* axis of the crystal appear in panel a and in panel b, respectively.

binding, the model achieves cooperative oxygen binding in response to the binding of chloride. It does this by creating a thermodynamic linkage between the two heme sites of the dimer via their common thermodynamic linkages to the chloride binding site. Heterogeneity in the spectral properties and oxygen binding affinities of the α and β subunits accommodates the apparent differences in the oxygen binding properties of the protein as measured along the two crystal axes. At both 100 and 200 mM chloride, the model reproduces the data within experimental error. However, it fails to reproduce the experimental results at 0.5 M chloride. One of our simplifying assumptions is that the activity of chloride is a linear function of concentration. In 62% PEG and 0.5 M chloride, it is doubtful that this assumption is reasonable. On the other hand, this misfit may indicate that the model is too simple. Each Hb Rothschild contains four oxygen binding heme groups and two mutation-specific, chloride binding sites. There is the potential for thermodynamic linkage among all six sites, each linkage being characterized by a distinct coupling constant. Such an approach presents us with more adjustable parameters than can be meaningfully assigned by the data available.

The occurrence of two affinity states within the same crystal lattice and quaternary structure is intriguing and should enhance the possibility of observing the structural basis for this modulation of ligand affinity. The structure of deoxyHb Rothschild reported by Kavanaugh *et al.* (1992) does not appear to contain features indicative of an unusually high T-state oxygen affinity. However, this structure was determined from crystals in 100 mM chloride. From the dissociation constant, 33 mM, which we have estimated for the binding of chloride to deoxyHb Rothschild in the crystal, one can calculate that their crystals were 75% saturated with chloride. Therefore, the predominant structure of deoxyHb Rothschild in these crystals was the chloride-bound form for which the estimated oxygen affinity is similar to that of HbA. A crystallographic analysis of chloride-free crystals might reveal the molecular basis for the oxygen affinity difference reported here.

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