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Heterotropic Effects of Chloride on the Ligation Microstates of Hemoglobin at Constant Water Activity*

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ABSTRACT Dimer-tetramer assembly reactions of the 10 CN-met ligation microstates of hemoglobin (Hb) were analyzed as a function of NaCl concentration while maintaining constant water activity by the addition of compensating sucrose. The assembly free energy for fully ligated cyanomet Hb and for fully oxygenated Hb becomes less favorable by 1.8 kcal when [NaCl] is increased from 0.08 to 0.7 M, whereas that of unligated Hb is practically insensitive to changes in [NaCl]. Values of 1.6 and 0.3 mol chloride release were found for the assembly of fully ligated and deoxy Hb, respectively; i.e., a net release of 1.3 mol chloride is coupled to the ligation of tetramers for both oxygen and cyanomet ligation. The ligation-linked salt component at constant water activity was evaluated to be 1.0 mol for the full oxygenation of the Hb tetramer in agreement with the overall value previously reported. When the detailed salt linkages accompanying all 16 stepwise cyanomet ligation reactions were experimentally resolved, only two "chloride" effects were found. The first chloride effect correlates with the ligation steps, which create tertiary constraint, and the second effect is coupled to the six switchpoints of quaternary T→R transition. The distribution of these chloride effects agrees closely with predictions of the "symmetry rule mechanism." The total chloride release for CN-met ligation is in good agreement with that for oxygenation. Free energy contributions to assembly and cooperativity arising from the osmotic effects of chloride were found to be small for all ligation species.

INTRODUCTION

Chloride ion is a physiologically important allosteric effector for hemoglobin. It is commonly believed that chloride ions bind preferentially to the deoxy T quaternary form of the hemoglobin (Hb) tetramer. Oxygenation-induced switching to the R quaternary form results in the release of chloride. We previously investigated the mechanistic roles of chloride ion (Huang et al., manuscript submitted for publication) by determining the detailed couplings between chloride activity and the energetics of cooperativity for all 10 ligation microstates of cyanomet Hb (Fig. 1). The principal findings were: 1) There are only two chloride effects among the 16 stepwise ligation reactions, and they distribute according to predictions of a previously proposed "symmetry rule mechanism" (Ackers et al., 1992). The first chloride effect is manifested in binding of the first ligand, and the second effect is associated with the six T→R quaternary switchpoints (Fig. 1). The apparent numbers of chlorides released upon ligation of the tetramer relative to its constituent dissociated dimers were found to be 0.8 and 0.7 mol for the "tertiary" and "quaternary" chloride effects, respectively. 2) Dimer-tetramer assembly of the deoxy T interface is practically insensitive to changes in thermodynamic activity of chloride (Chu and Ackers, 1981). On the other hand, the assembly of fully ligated tetramers to form the R interface is destabilized by increasing the activity of chloride, corresponding to 1.8 mol of chloride release.

Because chloride is a weakly binding effector, studies on its allosteric role have usually been conducted in the presence of sufficiently high NaCl concentration to significantly change the activity of water. Recent work on the effects of "osmotic stress" on Hb oxygen binding equilibria showed that some 60 water molecules are coupled to the quaternary T to R transition that accompanies reaction at all four heme sites (Colombo et al., 1992, 1994). It follows that any changes in the chemical potential of water will in principle affect the oxygen binding equilibrium and the energetics of quaternary transition. The findings of Colombo et al. therefore lead to important questions regarding the interpretation of earlier observations of salt effects on Hb: 1) Does the specific distribution of apparent chloride effects determined for the 10 ligation microstates of Hb arise from "artifacts" of the changing osmotic stress due to varying chloride concentration in the experiments, i.e., while masking a more fundamental distribution? 2) Does the osmotic effect of chloride play a significant physiological role in modulating cooperative oxygen binding to Hb? To address these questions, it was necessary to reevaluate the regulatory action of chloride on stepwise ligation of Hb by taking into account the osmotic effects of high [NaCl]. In the present study we evaluated the effects of chloride activity on the energetics of dimer-tetramer assembly and cooperative ligation for the 10 microstates of Hb at constant water activity. Changes in water activity arising from changes in chloride concentration were compensated for by the addition of sucrose, which acts as a neutral osmolyte. This approach eliminates the osmotic effects of NaCl, allowing direct analysis of the allosteric interactions between chloride ions and hemoglo-

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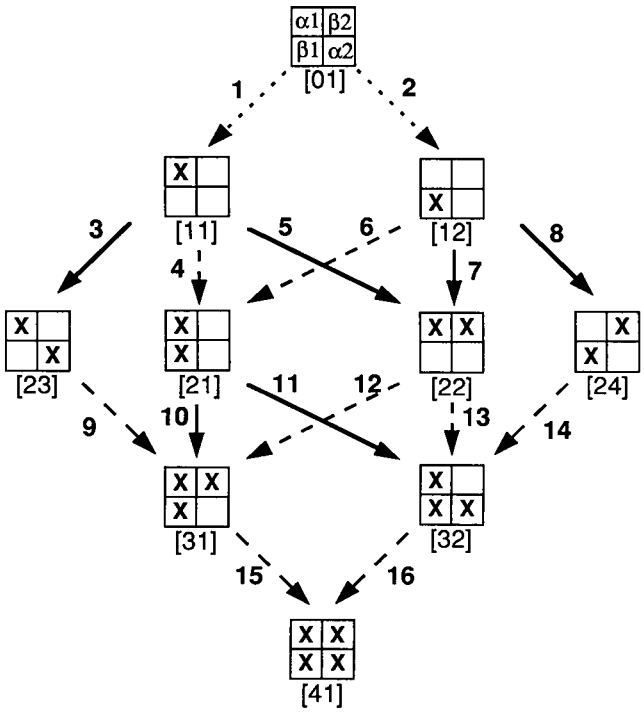

FIGURE 1 Topographic representation of the 10 ligation microstates of hemoglobin and 16 stepwise ligation reactions. Relative orientation of subunits within the tetramers is indicated in the top species [01]. Index [*ij*] denotes the particular species *j* among those with *i* ligands bound ($i = 0$ to 4; $j = 0$ to 4). Ordering of species with respect to *j* is arbitrary. Ligated hemesite (x) in this study contains Fe(III)-CN, and Fe(II) is in each unligated (blank) subunit. Dotted, dashed, and solid arrows denote stepwise ligation accompanied by tertiary constraint transition, null transition, and quaternary switch, respectively (see Discussion for more details).

bin (Colombo et al., 1992, 1994). The osmotic effects by NaCl on the energetics of ligation cooperativity were also analyzed by studying the NaCl dependence of cooperative free energies in the presence and absence of sucrose.

MATERIALS AND METHODS

Purification of hemoglobins A_o and S was achieved by the method of Williams and Tsay (1973). Cyanomet Hb and species [41], [23], and [24] were prepared as described (Daugherty et al., 1994). The purified proteins were stored under liquid nitrogen. Hemoglobins were exchanged to experimental buffer containing appropriate sucrose and NaCl in addition to 0.1 M Tris-base and 1 mM EDTA with a pH of 7.40 measured at 21.5°C. For experiments involving the use of cyanomet Hb, 10 μM KCN was added to the buffers. The total chloride concentrations used were 0.08, 0.18, 0.35, and 0.7 M, with compensating sucrose concentrations of 0.84, 0.74, 0.53, and 0.0 M. The activity of water in these solutions is 1.3 osmol/kg water. Activity coefficients of NaCl in aqueous solution were 0.796 (0.08 M), 0.743 (0.18 M), 0.701 (0.35 M), and 0.666 (0.7 M) (Hamer and Wu, 1972). Osmotic pressures of sucrose and NaCl were reported by Colombo et al. (1994) to be additive within 5%. We assumed the activity coefficients of NaCl in aqueous and sucrose-aqueous solutions to be identical.

Analytical gel permeation chromatography

Dimer-tetramer assembly free energies of oxy Hb and cyanomet species [23], [24], and [41] at various chloride concentrations were determined by large zone chromatography (Ackers, 1970; Valdes and Ackers, 1979). Experiments for species [23] and [24] were performed in a Coy anaerobic chamber. A Sephadex G-100 column with a bed volume greater than 30 ml was used. The column temperature was maintained at 21.5°C. Species [23] and [24] samples were deoxygenated under a flow of humidified N_2 for 1 h and then placed into the anaerobic chamber for another 15–20 min to ensure full deoxygenation. The samples were maintained below 10°C while agitating slowly on a shaker plate during deoxygenation. The required concentration of hemoglobin was obtained by diluting the sample with anaerobic buffer to achieve a total volume of 15–20 ml. Elution profiles were monitored using a Shimadzu UV-150-02 spectrophotometer. The plateau protein concentration ranged from 0.01 to 300 μM.

Values of the weight-averaged partition coefficient, $\bar{\sigma}_w$, of a hemoglobin sample were evaluated from the determined elution volumes (V_e) using the relationship

$$\bar{\sigma}_w = \frac{V_e - V_o}{V_i}, \tag{1}$$

where V_i and V_o are the included and excluded volumes, respectively, determined using glycylglycine and blue dextran in "small zone" experiments. The weight-averaged partition coefficient depends upon the equilibrium fractions of dimers (f_D) and tetramers (f_T), and their respective partition coefficients, σ_D and σ_T, according to

$$\bar{\sigma}_w = \sigma_D f_D + \sigma_T f_T. \tag{2}$$

The fraction of dimer, $f_D = 1 - f_T$, is related to the assembly equilibrium constant K_2 and total protein concentration P_t as follows:

$$f_D = \frac{-1 + \sqrt{1 + 4K_2P_t}}{2K_2P_t}. \tag{3}$$

Data of $\bar{\sigma}_w$ versus P_t were fitted to Eqs. 2 and 3 to resolve the equilibrium constant K_2 from which the assembly free energy was evaluated ($\Delta G_2 = -RT \ln K_2$).

Tetramer-dimer dissociation kinetics

Dissociation rate constants for deoxy Hb at different solution conditions were determined by haptoglobin (Hp) trapping experiments (Ip et al., 1976). Because Hp forms essentially irreversible complexes with Hb dimers but does not bind to tetramers, mixing Hp with Hb leads to a net dissociation of tetramers, as the dimers are complexed rapidly by Hp. The rate of forming the Hp-Hb complex is fast relative to the tetramer-dimer dissociation rates, giving rise to a pseudo-first-order process that reflects dissociation kinetics of the tetrameric species (Ip and Ackers, 1977; Turner et al., 1982). The experiment was initiated by mixing Hb (10 μM) with a twofold molar excess of Hp in a split-cell cuvette that was subsequently monitored spectrophotometrically over time at 430 nm. Anaerobicity was maintained by the addition of 0.1% dithionite in the buffer, and the cuvette was capped and sealed.

Cryogenic isoelectric focusing analysis of hybrid populations

Six species ([11], [12], [21], [22], [31], and [32]) were studied in hybrid mixtures with their corresponding parent species (see Fig. 2 for construction of hybrid species). Once the parent species are mixed, dimer rearrangement reactions take place to yield a hybrid. Sample mixing was performed under anaerobic conditions. An enzyme system containing *Aspergillus niger* catalase (0.3 mg/ml), glucose oxidase (1.8 mg/ml), and glucose (0.6%) was added to the sample to ensure full deoxygenation. The samples were aliquoted and sealed in vials, which were placed in large rubber-sealed vials containing 0.1% sodium dithionite solution to prevent oxygen leakage during sample incubation at 21.5°C. At a desired incubation time, the sample was quenched in a 1:1 mixture of ethylene glycol and standard buffer at −30°C. The sample was then loaded into the cryo-

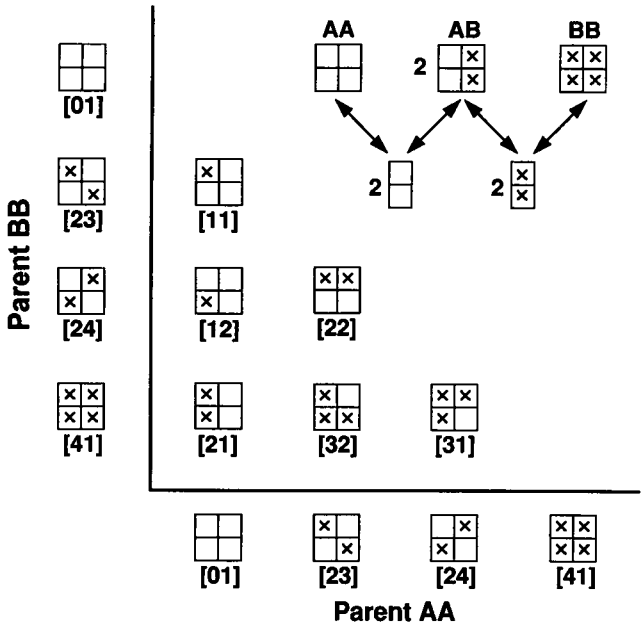

FIGURE 2 Hybridization scheme for construction of the six hybrid species from their parent molecules.

isoelectric focusing gel tubes and focused overnight at −25°C until three distinct bands were resolved.

Quantitation of the three species populations for a hybrid mixture was obtained by integrating the area under each peak. Using the determined equilibrium fractions (f_{AA}, f_{BB}, and f_{AB}) the deviation free energy (δ) of the hybrid species can be obtained by the following equation:

$$\delta = -RT \ln \frac{f_{AB}}{2\sqrt{f_{AA} f_{BB}}}, \quad (4)$$

where AA, BB, and AB denote the two parent and the hybrid species, respectively. Using assembly free energies of the parent species determined by the other techniques, assembly free energies of the hybrids can be calculated (LiCata et al., 1990):

$$\delta = {}^{AB}\Delta G_2 - \frac{1}{2}({}^{AA}\Delta G_2 + {}^{BB}\Delta G_2). \quad (5)$$

Data fitting and error estimation

All data fitting and error analyses were carried out using a nonlinear least-squares program, NONLIN (Johnson and Frasier, 1985). In cases where no standard error was determined for experimental data points, all data were weighted equally in the fitting. When standard errors were determined for each experimental data point, each point was weighted by its standard error in the fitting.

RESULTS

Dissociation rate constants for deoxyhemoglobin

Tetramer-dimer dissociation kinetics of deoxy Hb were found to be well described by a single exponential function at all conditions of this study; the resulting rate constants (k_r) are given in Table 1. When water activity is not controlled by compensation sucrose, k_r decreases slightly with increasing NaCl concentration (Doyle et al., manuscript submitted for publication) and with increasing sucrose concentration at constant concentration of NaCl (unpublished observations). In the present study, decreases in NaCl concentration were compensated for by increased sucrose concentration, so that the activity of water remained constant at 1.3 osmol/kg water for all solution conditions. The determined dissociation rate constants were found to be practically insensitive to changes in NaCl activity (Table 1).

Association rate constant and assembly free energy

The second-order rate constant (k_f) for dimer-tetramer assembly of deoxy Hb has been extensively investigated (Ip et

TABLE 1 Sodium chloride dependence of association rates, dissociation rates, and free energies for dimer-tetramer assembly of deoxygenated hemoglobin

[NaCl] (molar)	Sucrose (molar)	Ethylene glycol (molar)	Viscosity η*	$k_f^{o\#} \times 10^{-5}$ ($M^{-1}\,s^{-1}$)	$k_f^{\S} \times 10^{-5}$ ($M^{-1}\,s^{-1}$)	$k_f^{\P} \times 10^{6}$ (s^{-1})	$^{o}\Delta G_2$ (kcal/mol)
0.08			1.01	12.5		21 ± 2	−14.5 ± 0.2
0.18			1.02	8.5		18 ± 5	−14.4 ± 0.2
0.35			1.03	5.0		7.8 ± 0.8	−14.6 ± 0.2
0.70			1.07	3.3		7.5 ± 0.8	−14.3 ± 0.2
0.08	0.84		2.57	12.5	4.9	7.2 ± 0.8	−14.6 ± 0.2
0.18	0.74		2.24	8.5	3.9	6.8 ± 0.3	−14.5 ± 0.2
0.35	0.53		1.75	5.0	3.0	6.7 ± 1.1	−14.4 ± 0.2
0.08		1.07	1.18	12.5	10.7	11.2 ± 1.0	−14.8 ± 0.2
0.18		0.91	1.15	8.5	7.5	11.9 ± 1.0	−14.6 ± 0.2
0.35		0.62	1.12	5.0	4.6	10.3 ± 1.0	−14.4 ± 0.2

Other solution conditions: 0.1 M Tris-base, pH 7.40, 21.5°C, 1 mM EDTA.

* Wolf et al. (1972).

\# Association rate constant determined in the absence of sucrose and ethylene glycol from Doyle et al. (1996). Standard errors are approximately 30%.

§ Corrected association rate constant in the presence of sucrose or ethylene glycol (eq 7).

¶ Dissociation rate constants for 0.08, 0.35 and 0.7 M NaCl in the absence of sucrose and ethylene glycol were from Doyle and Ackers (1996).

al., 1976; Ip and Ackers, 1977; Turner et al., 1982; Doyle et al., manuscript submitted for publication). The rate and the apparent activation enthalpy (ΔH = 3.71 ± 0.26 kcal) of assembly (Ip and Ackers, 1977) are consistent with characteristics of diffusion-controlled macromolecular assembly. For diffusion-controlled bimolecular collision, the second-order rate constant is inversely proportional to solution viscosity (η) at temperature T:

$$k_f = C\frac{T}{\eta}. \quad (6)$$

Here C is a constant. Taking advantage of this relationship, the rate constants (k_f) may be estimated in the presence of sucrose by correcting for the change in solution viscosity:

$$k_f = k_f^o \frac{\eta^o}{\eta}, \quad (7)$$

where k_f^o is the determined rate constant at a given [NaCl] value in the absence of sucrose. Values of k_f^o as a function of [NaCl] without the addition of sucrose have been determined (Doyle et al., manuscript submitted for publication). The parameters η and η^o are viscosities of the buffer at a given concentration of NaCl with and without sucrose, respectively. The determined values for k_f at various concentrations of NaCl with compensating sucrose are given in Table 1. Assembly free energies can be calculated from the association and dissociation rate constants [$\Delta G_2 = -RT \ln(k_f/k_r)$]; the results are given in Table 1. It was found that the assembly equilibrium of deoxy Hb is insensitive to changes in chloride activity (with constant water activity) within the concentration ranges studied (0.08–0.7 M chloride).

To address the question of whether there is a specific effect of sucrose on the assembly equilibrium in addition to the nonspecific osmotic effect, we carried out kinetics experiments by using ethylene glycol (EG) as a compensating osmolyte. The viscosities of the EG solutions are much lower than those of the corresponding sucrose solutions, and therefore much smaller corrections (Eq. 7) were required for the association rate constants measured in the EG solutions. The dissociation rates in various concentrations of EG were experimentally determined with the haptoglobin kinetics technique (see Materials and Methods). The resulting rate constants and the evaluated assembly free energies for deoxy Hb are given in Table 1. It is noted that the determined ΔG_2 for sucrose and EG solutions at a given concentration of NaCl are highly consistent, indicating that assembly equilibrium of deoxy Hb is independent of the chemical nature of the osmolytes used. These results are in accord with the findings of Colombo et al. (Colombo et al., 1992, 1994; Colombo and Bonilla-Rodriguez, 1996) and provide a cross-check on the validity of the method used in the present study for correcting association rate constants in these chemically distinct osmolyte solutions.

Assembly free energies of oxy Hb and cyanomet species [23], [24], and [41]

For these species the dimer-tetramer assembly free energies were determined by large-zone analytical gel chromatography experiments. At each chloride concentration, three or more zones were employed for each species, with plateau concentrations that varied by at least 10-fold. Elution volumes were determined as the centroids of leading and trailing boundaries. The centroid values were transformed into weight-averaged partition coefficients using the excluded and included volumes of the column (Materials and Methods). Fig. 3 shows the weight-averaged partition coefficient versus total protein concentration for cyanomet Hb [41], [23], [24], and oxy Hb at 0.08 M chloride with 0.84 M sucrose. The determined weight-averaged partition coefficients (σ_D and σ_T) for oxy Hb, along with those for the cyanomet species, were fitted simultaneously to Eq. 2 with common dimer and tetramer partition coefficients for all species. Confidence intervals obtained for assembly equilibrium constants thus include the uncertainty from "floating" σ_D and σ_T during analysis. The chloride dependence of assembly free energies for Fe/FeCN species [23], [24], and [41] are given in Fig. 4, and those for oxy Hb are given in Fig. 5.

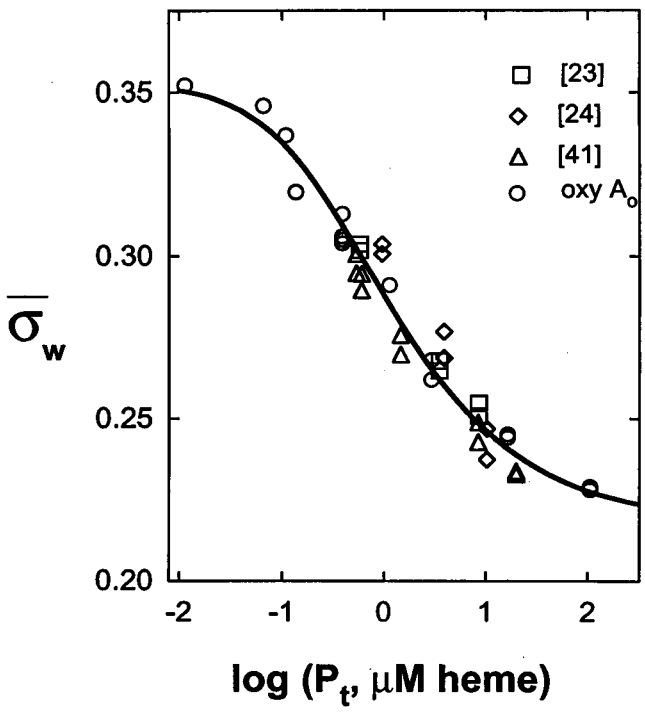

FIGURE 3 Analytical gel permeation chromatography results showing weight-averaged partition coefficients versus Hb concentrations in micromolar heme unit for Fe/FeCN species [23] (□), [24] (◇), [41] (△), and oxy Hb (○). Experimental conditions were 0.1 M Tris-base, 0.08 M chloride, 0.84 M sucrose, 1 mM EDTA, 10 μM KCN, pH 7.4, and 21.5°C. Results from both leading and trailing edges of each elution profile are given. Curves represent best-fit parameters for the dimer-tetramer equilibrium of oxy Hb.

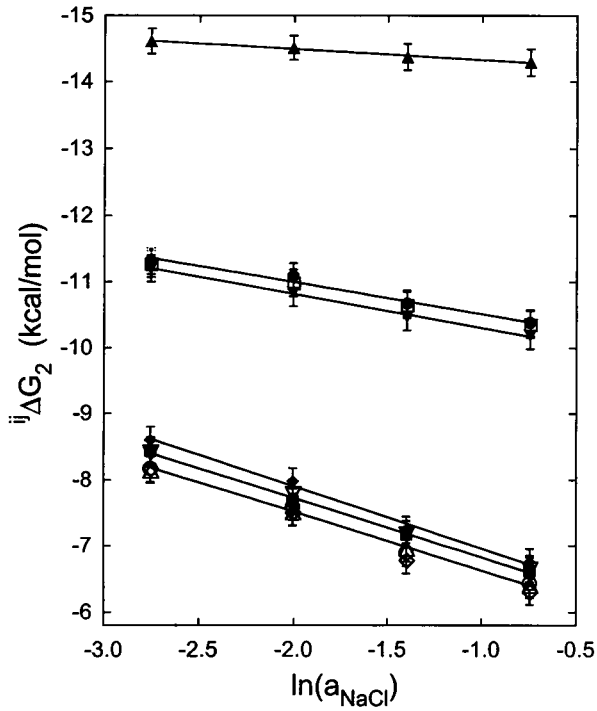

FIGURE 4 Assembly free energies versus logarithm of NaCl activity for Fe/FeCN species [01] (▲), [11] (▼), [12] (□), [21] (●), [22] (♦), [23] (△), [24] (▽), [31] (■), [32] (◇), and [41] (○). Curves are linear representation of the data for species [01], [12], [21], [24], [31], and [41]. Solution conditions are given in Fig. 3. Water activity was maintained constant at 1.3 osmol/kg water.

Assembly free energies of species [11], [12], [21], [22], [31], and [32]

Each of the six species, [11], [12], [21], [22], [31], and [32], must be studied in hybrid mixture with its appropriate parent species (see Fig. 2 for construction of these hybrids). Equilibrium fractions of each hybrid and its two parent species were determined by cryogenic isoelectric focusing (LiCata et al., 1990). The approach to equilibrium in formation of hybrid species was monitored by determining fractions of each tetrameric species in hybrid mixture versus the hybrid incubation time. Dimer-tetramer assembly free energies of the hybrid species were evaluated by Eqs. 4–5 using the determined $^{ij}\Delta G_2$ for species [01], [23], [24], and [41].

Standard Gibbs energies, $^{ij}\Delta G_2$, of dimer-tetramer assembly for the 10 ligation microstates of cyanomet hemoglobin at the various chloride activities under constant activity of water are given in Table 2 and shown graphically in Fig. 4. The values in Table 2 show the same dramatic and characteristic features that have previously been found for this system over a wide range of pH (Daugherty et al., 1994) and temperature (Huang and Ackers, 1995). Thus the doubly ligated species are always found in two of the three distinct free energy levels, with species 21 behaving differently from the other three. With water activity maintained at a constant value, the data of Table 2 show that this previously

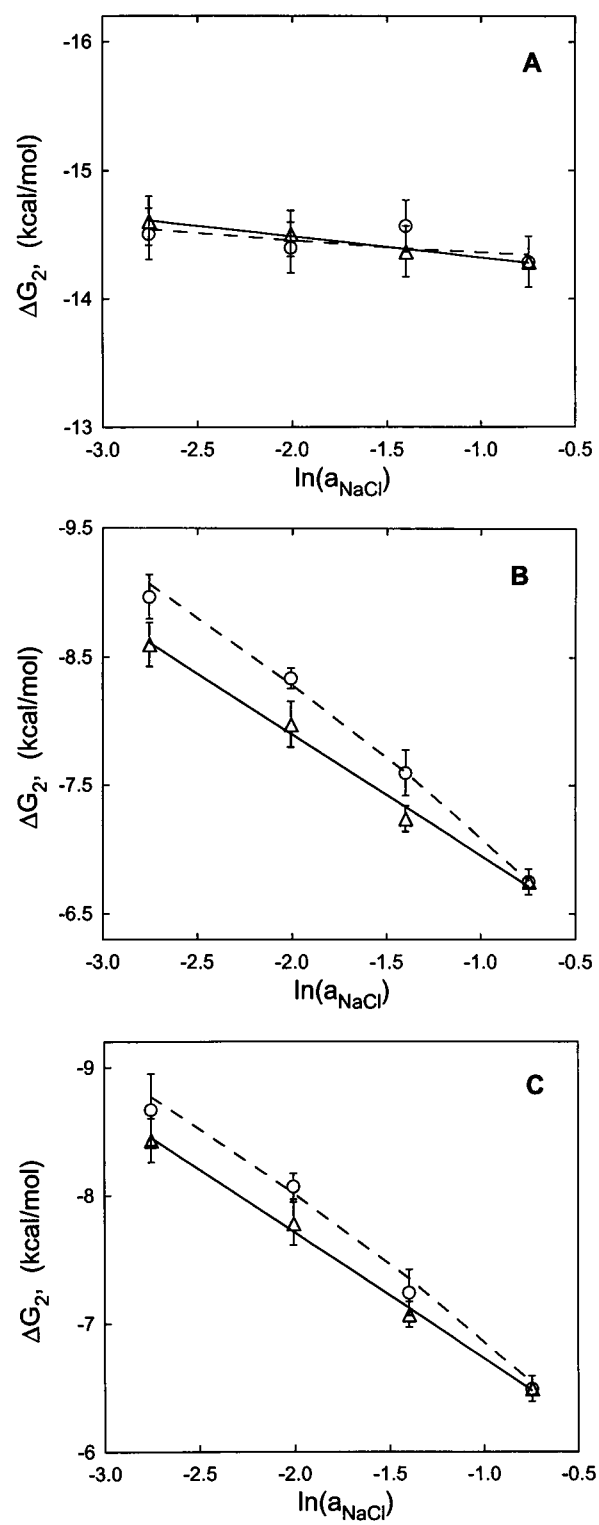

FIGURE 5 Comparison of the NaCl activity-dependent assembly free energies in the presence (△) and absence (○) of sucrose for deoxy Hb (*A*, triangles are species [01] data from Fig. 4), cyanomet Hb (*B*, triangles are species [41] data from Fig. 4), and oxy Hb (*C*). Solid and dashed lines were calculated using integrated forms of Eqs. 10a and 11a, respectively, and plotted as assembly free energies versus activity of NaCl. The parameter values used in these calculations were obtained from global analysis of the data in the presence and absence of sucrose, based on an equation combining the integrated forms of the Eqs. 10a and 11a.

TABLE 2 Effects of chloride on quaternary assembly free energies for the 10 ligation states of CN-met hemoglobin at constant activity of water

Tetrameric ligation state	0.08 M [Cl⁻]/ 0.84 M sucrose	0.18 M [Cl⁻]/ 0.74 M sucrose	0.35 M [Cl⁻]/ 0.53 M sucrose	0.70 M [Cl⁻]/ 0 M sucrose
[01]	−14.61 ± .19	−14.51 ± .18	−14.37 ± .20	−14.29 ± .20
[11]	−11.27 ± .17	−10.98 ± .17	−10.65 ± .15	−10.36 ± .15
[12]	−11.20 ± .17	−10.83 ± .17	−10.47 ± .15	−10.19 ± .15
[21]	−11.31 ± .17	−11.08 ± .17	−10.67 ± .15	−10.38 ± .15
[22]	−8.17 ± .17	−7.59 ± .17	−6.89 ± .12	−6.43 ± .15
[23]	−8.42 ± .20	−7.79 ± .20	−7.18 ± .10	−6.65 ± .10
[24]	−8.17 ± .20	−7.50 ± .20	−6.78 ± .10	−6.31 ± .20
[31]	−8.15 ± .17	−7.51 ± .17	−6.96 ± .12	−6.41 ± .15
[32]	−8.44 ± .17	−7.67 ± .17	−7.17 ± .12	−6.62 ± .12
[41]	−8.60 ± .20	−7.98 ± .20	−7.24 ± .10	−6.75 ± .10
$Hb(O_2)_4$	−8.43 ± .20	−7.78 ± .20	−7.07 ± .10	−6.57 ± .10

observed combinatorial feature does not arise from a compensation between the effects of salt versus water that masks a more fundamental distribution. It is seen (Fig. 4) that the assembly free energies of species [01] exhibits a negligible change with [NaCl]. By contrast, $^{ij}\Delta G_2$ values of the other nine ligated species become less negative with increasing [NaCl], indicating that chloride promotes the disassembly of tetramers. The linear dependence of ΔG_2 on $\ln(a_{NaCl})$ was found for all ligation species. The degree of the chloride concentration dependence of $^{ij}\Delta G_2$ differs for each species, with species [41] exhibiting the largest effect of chloride activity on the assembly free energy.

Linkage of chloride to the dimer-tetramer assembly and cooperative ligation

The dependence of assembly free energy on chloride activity indicates that chloride is coupled to the dimer-tetramer assembly reaction. Because the chemical potential of water was kept constant in these experiments, changes in bound chloride ($^{ij}\Delta\bar{\nu}_2$) upon assembly can be rigorously evaluated from analyzing the data in Fig. 4, based on the linkage relation of Wyman (1964):

$$\frac{-\mathrm{d}^{ij}\Delta G_2}{RT\,\mathrm{d}\ln a_{NaCl}} = {}^{ij}\Delta\bar{\nu}_2. \qquad (8)$$

Here R is the gas constant and T is temperature. Thermodynamic activities of NaCl (a_{NaCl}) were calculated at each concentration, using activity coefficients from Hamer and Wu (1972). In the fitting, each data point was weighted based on the standard deviation of $^{ij}\Delta G_2$ obtained in this work. For all Hb species studied, $^{ij}\Delta G_2$ was found to be linearly dependent on $\ln(a_{NaCl})$ within the [NaCl] range studied. Resulting values of $^{ij}\Delta\bar{\nu}_2$ for all 10 cyanomet species and for oxy Hb are given in Table 3. The negative sign for $^{ij}\Delta\bar{\nu}_2$ indicates chloride release. Because compensating sucrose was used to maintain constant water activity, application of Eq. 8 assumes that there is no difference in the interactions of sucrose with Hb dimer and tetramer. It is also assumed that the difference between numbers of solute-excluding water molecules for dimeric and tetrameric species is the same for NaCl and sucrose.

Because dimer-tetramer assembly reactions of deoxy Hb and of the ligated Hb species are thermodynamically linked to ligand binding (Smith and Ackers, 1985), one can evaluate the cooperative chloride binding ($^{ij}\Delta\bar{\nu}_c = {}^{ij}\Delta\bar{\nu}_4 - {}^{ij}\Delta\bar{\nu}_2$) by the difference in chloride release upon assembly between species [ij] and [01], as given in Eq. 9:

$$^{ij}\Delta\bar{\nu}_c = {}^{ij}\Delta\bar{\nu}_2 - {}^{01}\Delta\bar{\nu}_2. \qquad (9)$$

$^{ij}\Delta\bar{\nu}_c$ represents the change in mean number of chlorides bound to tetramers relative to dimers upon heme-site ligation. From the resulting $^{ij}\Delta\bar{\nu}_c$ value (Table 3), it is noted that the values of $^{41}\Delta\bar{\nu}_2$ and $^{41}\Delta\bar{\nu}_c$ for oxy Hb are identical within statistical error to those for cyanomet Hb. This excellent agreement between extents of chloride linkage by the two ligation systems argues that the cyanomet ligation system follows the same overall mechanism with respect to chloride modulation as the oxygen ligation system.

A recent study has determined oxygenation-linked chloride effects on the dissociated dimers ($^{D}\Delta\bar{\nu}_x$) to be 0.18 ± 0.10 mol of chloride bound per mol of dimer upon oxygenation of both sites (Doyle et al., manuscript submitted for

TABLE 3 Change in mean number of bound chloride upon dimer-tetramer assembly ($^{ij}\Delta\bar{\nu}_2$) and cooperative ligation ($^{ij}\Delta\bar{\nu}_c$) of hemoglobin

Ligation species	$^{ij}\Delta\bar{\nu}_2$	$^{ij}\Delta\bar{\nu}_c$
Cyanomet species		
[01]	−0.28	0
[11]	−0.79	−0.51
[12]	−0.87	−0.59
[21]	−0.82	−0.54
[22]	−1.52	−1.24
[23]	−1.52	−1.24
[24]	−1.62	−1.34
[31]	−1.49	−1.21
[32]	−1.54	−1.26
[41]	−1.62	−1.34
OxyHb	−1.62	−1.34

Standard errors on $^{ij}\Delta\bar{\nu}_2$ and $^{ij}\Delta\bar{\nu}_c$ are ±0.10.

publication). Assuming the variation in water activity due to increasing concentration of NaCl to have little effect on the value of ${}^{D}\Delta\bar{\nu}_x$, the moles of chloride released from the Hb tetramer upon oxygenation (${}^{T}\Delta\bar{\nu}_x$) may be estimated from the experimental value of ${}^{41}\Delta\bar{\nu}_c$ to be -1.0 ± 0.15 mol [${}^{T}\Delta\bar{\nu}_x = {}^{41}\Delta\bar{\nu}_c + 2({}^{D}\Delta\bar{\nu}_x) = -1.34 + 2(0.18)$]. This result is in agreement with the value of -1.0 chloride reported by Colombo et al. (1994) for chloride coupling to complete oxygenation during the deoxy-to-oxy transition.

DISCUSSION

Correspondence with the symmetry rule mechanism

By maintaining constant water activity, the differential change in moles of chloride bound that accompany ligand binding were explicitly determined in the present study for all ligation microstates of cyanomet Hb. These ${}^{ij}\Delta\bar{\nu}_c$ values (Table 3) were found to distribute into three distinct levels with a combinatorial feature (i.e., dependency on the number of ligands bound and the configuration of ligated subunits within the tetramer). At every condition, species [21] exhibits a ${}^{ij}\Delta\bar{\nu}_c$ value significantly different from those of the other three doubly ligated species. From the ${}^{ij}\Delta\bar{\nu}_c$ results, changes (${}^{i}\Delta\bar{\nu}_c^s$) in bound chloride were evaluated for each of the 16 stepwise ligation steps (Fig. 1) in tetramer relative to corresponding ligation of the same sites on dissociated dimers. It was found that the 16 stepwise chloride couplings were synchronized into three groups, identically with those of the Bohr protons (Daugherty et al., 1994), enthalpies, and entropies (Huang and Ackers, 1995), as shown in Table 4. The initial heme-site ligation step (reactions 1 and 2 of Fig. 1) couples with 0.5 moles of chloride release, along with the previously found Bohr proton release, positive enthalpy, and entropy components. At each of the six T-R switchpoint transitions (reactions 3, 5, 7, 8, 10, 11 in Fig. 1), a second chloride effect (0.7 moles chloride releases) is manifested along with the corresponding (T→R) Bohr effect, enthalpy effect, and large positive entropy change. The other stepwise ligation reactions (4, 6, 9, 12–16) exhibit little or no chloride and negligible enthalpic, entropic, and Bohr effects. This dramatic synchronization of functional parameters is consistent with specific predictions of the symmetry rule mechanism previously proposed (Ackers et al., 1992). The combinatorial distribution found previously (Huang et al., manuscript submitted for publication) for the apparent salt release values (without controlling water activity) is thus not the resultant of water-salt compensation that is masking a more fundamental distribution.

Mechanistic origins of the chloride effects

Cooperative oxygen binding to Hb has long been known to be modulated by chloride ions. The chloride effects are generally considered to arise directly from specific chloride binding interactions and general ionic strength effects and indirectly from alterations of water activity. In this study the water activity was maintained at a constant value while concentrations of chloride varied from 0.08 to 0.7 M, allowing explicit evaluation of the effects of chloride-protein interaction. Comparison of the present results with a previous study of chloride effects in the absence of sucrose (Huang et al., manuscript submitted for publication) then provides an evaluation of the indirect effects arising when high chloride concentration alters water activity and consequently the hydration of Hb.

The direct effects of chloride on the equilibrium between any two structural forms of Hb can be quantitatively characterized (Eq. 8) as the change ($\Delta\bar{\nu}$) in mean number of chlorides that are thermodynamically associated with the protein. This term, determined at constant water activity, includes contributions from the change in the number of chloride ions that are bound chemically to the protein and from the effect of ionic strength on the relative stability of the two structural forms. Structural transitions or subunit assembly could also change the accessibility of ionizable groups for chloride interaction and their structural distribution, resulting in altered chloride-protein interactions. In the case of dimer-tetramer assembly of Hb in the R quaternary form (such as oxy Hb and cyanomet Hb), a mean value of 1.6 moles of chloride release was found. One possibility is

TABLE 4 Synchronized clusters of cooperative response parameters for CN-met Hb, pH 7.4

	Stepwise parameter	Generation of tertiary constraint (1, 2)	Null reactions (4, 6, 9, 12–16)	Switchpoint reactions (3, 5, 7, 8, 10, 11)	Overall transitions (T → R)	
					CN-met	O_2
Free energy*	ΔG_c^s	3.2 ± 0.2	−0.1 ± 0.3	3.1 ± 0.3	6.2 ± 0.3	6.3 ± 0.2
Enthalpy#	ΔH_c^s	14 ± 3	1 ± 3	14 ± 3	29 ± 3	34 ± 3.0
Entropy§	$-T\Delta S_c^s$	−11 ± 3	−1 ± 3	−11 ± 3	−23 ± 3	−27.7 ± 2.7
Bohr protons¶	$\Delta\bar{\nu}_c^s$ (H^+)	0.7 ± 0.2	0.0 ± 0.2	0.8 ± 0.2	1.5 ± 0.3	1.2 ± 0.2
Chloride effect‖	$\Delta\bar{\nu}_c^s$ (Cl^-)	0.5 ± 0.1	0.0 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	1.3 ± 0.1

* Smith and Ackers, 1985; Perrela et al., 1990; Huang and Ackers, 1995.
# Huang and Ackers, 1995.
§ Huang and Ackers, 1995.
¶ Moles of Bohr protons released (Daugherty et al., 1994).
‖ Moles of chloride released (this study), for reactions defined in Fig. 1.

that assembly of the R tetramer decreases the accessibility of ionizable amino acid residues within the subunit interfaces and alters their interactions with chloride ions. For assembly of the deoxy (quaternary T) tetramer, only 0.3 moles of chloride were released, suggesting that accessibility of the ionizable groups that interact with chloride ions is greater in quaternary T than in quaternary R. It follows that more chloride ions are participating in interactions with cation groups in quaternary T than quaternary R. In the heme-site binding-induced quaternary T to R structural transition, chloride ions are released from the protein because of structural alterations of the chloride-binding sites.

An important question regarding the coupling of chloride effects to specific ligand-induced structural transitions is whether the observed chloride effects arise from the tertiary or quaternary structural transitions. To address this question, we have evaluated the cooperative chloride effects for all 16 stepwise ligation steps and have considered their correlation with those from a second heterotropic allosteric effector, Bohr protons, and with the separate cooperative enthalpy and entropy contributions. The finding (Table 4) of only two chloride effects that correlate with ligation steps that generate tertiary constraint (reactions 1 and 2, Fig. 1) and with those steps that trigger quaternary switching (reactions 3, 5, 7, 8, 10, and 11, Fig. 1) indicates that cooperative chloride release arises from both the tertiary structural transition within quaternary T and from the quaternary T-R switching that accompanies ligation. Given the revealed complexity of the chloride effects on dimer-tetramer assembly and cooperative ligation, and the specific distribution of the chloride effects among the 16 stepwise ligations, it is unlikely that a single specific chloride-protein interaction generates the entire allosteric chloride effect. This conclusion is consistent with recent conclusions of Perutz et al. (1994).

The indirect effect of chloride on cooperative oxygen binding was suggested by Colombo et al. (1994) to arise from the alteration of water activity by the presence of high concentrations of NaCl. It is based on the finding that approximately 60 water molecules bind to the Hb tetramer during transition from the deoxy T structure to fully oxygenated R structure (Colombo et al., 1992). The presence of NaCl within the physiological concentration range significantly changes water activity and consequently modulates the oxygenation of Hb. To what extent and how does the NaCl concentration contribute to cooperative free energy through changing water activity? Can this contribution be assigned to a specific ligation step or ligand-induced structural transition? To address these questions, we first compared salt-dependent assembly free energies determined in the present study with controlled water activity and the corresponding values and without such control (Huang et al., manuscript submitted for publication) (Fig. 5). It is seen that at a given chloride activity, the assembly free energies of deoxy Hb are insensitive to changes in the osmotic pressure of sucrose. However, assembly free energy of fully ligated Hb is slightly reduced with the addition of sucrose, which decreases the chemical potential of water. For example, at 0.08 M NaCl the addition of 0.84 M sucrose reduces the assembly free energy of cyanomet Hb by 0.4 kcal. Therefore, the overall contribution to cooperative free energy by the osmotic stress of 0.84 M sucrose (equivalent to osmotic stress generated by 0.62 M NaCl) is only −0.4 kcal of the 6.2 kcal total cooperative free energy (Fig. 6 *A*). A similar result was found for cooperative oxygen binding to Hb (Fig. 6 *B*). Even smaller contributions from the osmotic effect were found for the eight intermediate species of the

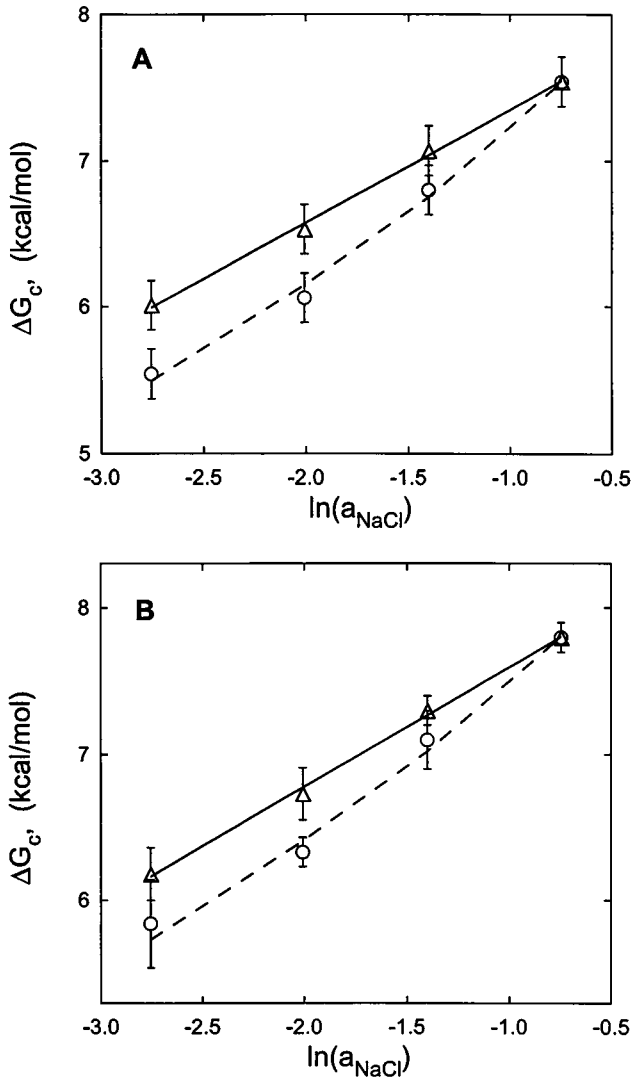

FIGURE 6 Comparison of the NaCl activity-dependent cooperative free energies in the presence (△) and absence (○) of sucrose for cyanomet ligation (*A*) and oxygen ligation (*B*) systems. The cooperative free energies for oxygenation and cyanomet ligation were calculated by subtracting corresponding data in Fig. 5 *A* from Fig. 5 *C* and Fig. 5 *B*, respectively. Solid and dashed lines were calculated using integrated forms of Eqs. 10b and 11b, respectively, and plotted as cooperative free energies versus activity of NaCl. The parameter values used in these calculations were obtained from global analysis of the data in the presence and absence of sucrose, based on an equation combining integrated forms of Eqs. 10b and 11b. The resulting values of cooperative ligation-linked chloride and water are −1.28 moles chloride and 41 moles water for cyanomet ligation, and −1.36 moles chloride and 36 moles water for oxygenation.

Fe/FeCN system (data not shown), and it is not statistically possible to assign the contributions to specific molecular events (such as stepwise ligation and structural transitions). From these results it was estimated that, under physiological concentrations of NaCl about 0.1–0.2 M, the contribution of the salt-induced osmotic effect to cooperative free energy is less than −0.2 kcal. Therefore, it seems unlikely that the osmotic effect of NaCl plays a significant role in modulating cooperative oxygenation of Hb. The direct interactions between chloride and Hb are thus responsible for the allosteric "chloride" effect. Nevertheless, a rigorous analysis of the chloride-protein interactions does require correcting the osmotic effect in high concentrations of NaCl according to the methodology that has been developed by Colombo and Parsegian.

Allosteric effects of water

In analyzing NaCl-dependent energetics of Hb cooperativity in the presence and absence of sucrose, a possibility arose for resolving the linkage effects of water on cooperative free energies and correlating these effects with stepwise ligation to probe ligand-induced structural transitions. We have explored this possibility in the present study. When water activity is constant, assembly free energy (ΔG_2) and cooperative free energy (ΔG_c) are related to the concentration (molality) of NaCl (m_{NaCl}) according to the Wyman linkage relations:

$$\frac{d^{ij}\Delta G_2}{d\ln(m_{NaCl})} = -RT\,\Gamma^{ij}\Delta\bar{\nu}_2, \tag{10a}$$

$$\frac{d^{ij}\Delta G_c}{d\ln(m_{NaCl})} = -RT\,\Gamma^{ij}\Delta\bar{\nu}_c, \tag{10b}$$

where R is the gas constant and T is temperature. Over the range of [NaCl] in this study, d ln γ/d ln m_{NaCl} remains nearly constant (Hamer and Wu, 1972), with $\Gamma = \{1 + d\ln\gamma/d\ln m_{NaCl}\} = 0.937$, where γ is the activity coefficient of NaCl in aqueous solution. Equation 10 assumes that there is no energetic difference in "binding" of sucrose to the reactant and product Hb species. When both water and NaCl activities are varied, the free energy of dimer-tetramer assembly and the cooperative free energy are related to changes in both bound chloride and water ($^{ij}\Delta\bar{\nu}_{2w}$ and $^{ij}\Delta\bar{\nu}_{cw}$) by Eq. 11, which is based on the relation of Tanford (1969):

$$\frac{d^{ij}\Delta G_2}{d\ln(m_{NaCl})} = -RT\,\Gamma\left(^{ij}\Delta\bar{\nu}_2 - \frac{2m_{NaCl}{}^{ij}\Delta\bar{\nu}_{2w}}{55.5}\right). \tag{11a}$$

$$\frac{d^{ij}\Delta G_c}{d\ln(m_{NaCl})} = -RT\,\Gamma\left(^{ij}\Delta\bar{\nu}_c - \frac{2m_{NaCl}{}^{ij}\Delta\bar{\nu}_{cw}}{55.5}\right). \tag{11b}$$

The assembly free energies for deoxy Hb were found to be totally insensitive to the presence of sucrose (Fig. 5 *A*). For the fully liganded state, assembly free energies in the absence (Huang et al., manuscript submitted for publication) and presence (this study) of sucrose were fitted simultaneously to an equation that combines integrated forms of both Eqs. 10a and 11a. The resolved changes in the mean number of chloride and water bound are −1.59 ± 0.13 mole chloride and 32 ± 16 mole water for cyanomet Hb, −1.56 ± 0.17 mole and 29 ± 17 mole for oxy Hb. The resolved values of chloride release agree with the values determined by Eq. 8 from the data at constant water activity (Table 3). It is noted that in these analyses both $^{ij}\Delta\bar{\nu}_2$ and $^{ij}\Delta\bar{\nu}_{2w}$ were assumed to be constant over the salt concentration range examined. The observed small energetic magnitude of the osmotic effect in these experiments resulted in large uncertainties for the determined $^{41}\Delta\bar{\nu}_{2w}$ values. Nevertheless, the finding that assembly is less favorable with increasing osmotic pressure does suggest that water molecules are bound upon assembly of ligated Hb.

The cooperative free energies in the presence and absence of sucrose (Fig. 6) were analyzed using an equation that combines integrated forms of both Eqs. 10b and 11b to assess the linkage effects of water on ligand-induced cooperative transitions. The resolved $^{ij}\Delta\bar{\nu}_{cw}$ values represent the difference between change in number of preferentially bound water on the tetramer and that on the dissociated dimers upon heme-site binding. The evaluated $^{41}\Delta\bar{\nu}_c$ and $^{41}\Delta\bar{\nu}_{cw}$ are −1.28 ± 0.10 and 41 ± 12 for cyanomet ligation, and −1.36 ± 0.11 and 36 ± 13 for oxygen binding to Hb. The $^{41}\Delta\bar{\nu}_c$ values for chloride release agree with those from the data at constant water activity (Table 3). Taking into consideration the linked effects of water on the assembly of deoxy and liganded Hbs, the positive $^{41}\Delta\bar{\nu}_{cw}$ values suggest that upon oxygenation or cyanomet ligation, more water molecules bind to liganded Hb tetramers than to dimers. However, it is unlikely that the additional water molecules associated with the tetramer result solely from increased solvent-accessible surface area upon quaternary switching from T to R, because two dissociated dimers would have a larger expected surface area than the tetramer. It is possible that water molecules trapped in the subunit interface contribute, at least in part, to the ligation-linked water effect. Nevertheless, the finding of water uptake upon cooperative ligation is qualitatively consistent with the finding by Colombo et al. (1992) that approximately 60 additional water molecules are thermodynamically bound to hemoglobin upon quaternary transition from T to R. The modest energetic magnitude of osmotic effects observed within the concentration range of osmolytes used in this study limits the accuracy in determination of the number of ligation-linked water molecules, as reflected by the large errors associated with $^{41}\Delta\bar{\nu}_{cw}$. For the same reason, accurate evaluation of the $^{ij}\Delta\bar{\nu}_{cw}$ values for the eight intermediate ligation species are statistically unrealistic from the present data base. Assigning the ligation-linked water molecules to stepwise ligation to probe ligand-induced structural transitions in the level of the 16 stepwise ligation would require a significantly wider range of osmolyte concentrations at a constant activity of NaCl.

Preferential hydration of the dimer-dimer interface

Chothia and collaborators have calculated solvent-accessible surface area for both deoxy Hb (Chothia et al., 1976) and oxy Hb (Lesk et al., 1985) based on the respective crystal structures. They found that the pairwise subunit interfaces ($\alpha_1\beta_2$, $\alpha_2\beta_1$, and $\alpha_1\alpha_2$) of deoxy and oxy Hb bury approximately 3090 and 2630 Å^2, respectively. (The interfaces of oxy Hb also include a loosely packed $\beta_1\beta_2$ interface. Inclusion of this interface results in a buried surface area of 2950 Å^2 for oxy Hb, so that only 140 Å^2 additional surface area is exposed on switching from T to R.) Switching from deoxy T to oxy R structures results in a net exposure of some 460 Å^2 surface area. The dimer-dimer interfaces of deoxy and oxy Hb are composed of 40% and 35% polar fractions, respectively (Chothia et al., 1976). ("Polar fraction" refers to surface area contributed by nitrogen, oxygen, and sulfur atoms.)

Timasheff and his collaborators have pioneered a series of elegant studies of interactions between proteins and solvent components in sucrose/water (Lee and Timasheff, 1981), and in other systems of sugar/water (Arakawa and Timasheff, 1982a) and salt/water (Arakawa and Timasheff, 1982b). They showed that the preferential interaction parameter $(\partial\mu_2/\partial m_3)_{T,P,m2}$ is linearly dependent on protein surface area (s_2). Here μ_2 is protein chemical potential, and m_3 and m_2 are molality of osmolyte and protein, respectively. A value of approximately 1 cal/mol protein per molal sucrose per Å^2 surface area was found for the slope of $(\partial\mu_2/\partial m_3)_{T,P,m2}$ versus s_2 in the sucrose/water system (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982a). This value indicates that the addition of 1 molal sucrose would increase the chemical potential of the protein by 1 cal/Å^2 surface area of the protein. If we assume that the buried surface area within the dimer-dimer interface of Hb represents a loss of surface area that accompanies dimer-tetramer assembly, the expected change in assembly free energy (ΔG_2) resulting from the preferential exclusion effect of 1 molal sucrose would be on the order of -3.1 and -2.6 kcal for deoxy and oxy Hb, respectively. The preferential exclusion mechanism (Timasheff, 1993) thus predicts a stabilization of the tetramer relative to dimers by the addition of sucrose. However, in the present study we have found no significant change in assembly free energy of deoxyhemoglobin and a slight decrease in stability of the oxyhemoglobin tetramer when sucrose concentration was raised from 0 to 1 molal (Fig. 5). Possible explanations for these results are 1) the Hb subunit surface that forms the dimer-dimer interface within the tetramer may have characteristics that are very different from surfaces of the proteins studied by Timasheff et al., giving rise to a very low value of the preferential interaction parameter for the sucrose/water system; 2) dimer-dimer interactions (e.g., hydrogen bonds, salt bridges) within the interface might be, to a certain degree, osmotic pressure dependent. The overall effect of sucrose on assembly entails contributions from perturbation to the interactions between interfacial residues of the two dimers and from the preferential exclusion effect of sucrose. A possible origin of such perturbations to the dimer-dimer interactions is that some water molecules are participating weakly in the interfacial hydrogen-bond/salt-bridge network that stabilizes the tetramer. Reducing the activity of water by the addition of sucrose would reduce the contribution of these water molecules to the stability of the dimer-dimer interface. (Water molecules that are tightly bound (such as those seen by x-ray diffraction) within the dimer-dimer interface are unlikely to be affected by the osmotic pressure of sucrose: because 1 molal sucrose reduces water activity by only 2%, these tight binding sites will remain fully saturated with water in the presence of sucrose.) The observed insensitivity of ΔG_2 to osmotic pressure might have resulted from compensation between this destabilization effect and the stabilization effect of preferential exclusion.

This work is an outgrowth of discussions with Adrian Parsegian and Marcio Colombo that stimulated us to search for the connections between their germinal findings and the detailed mechanism of hemoglobin cooperativity.

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