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# Carbamoylated Hemoglobins A and S: Physical Properties†

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ABSTRACT: Dimer–tetramer association constants ($K_{2,4}$) of derivatives of CO-hemoglobins A and S specifically carbamoylated at the $NH_2$-terminal valine residues were measured. Reactivities of the $\beta$-93 sulfhydryls of the hemoglobin A derivatives were also investigated. As compared with the association constants of the parent molecules, the values of $K_{2,4}$ of both hemoglobin types are raised by carbamoylation of the $\alpha$-chain $NH_2$ terminus, lowered by carbamoylation of the $\beta$-chain $NH_2$ terminus, and raised by carbamoylation of both termini. The apparent second-order rate constant for reaction of *p*-mercuribenzoate (PMB) with the $\beta$-93 sulfhydryls is, however, unchanged by carbamoylation. These two observations are interpreted to indicate that in the liganded molecule structural changes are produced at the interface between dimers but not in the region of the $\beta$-93 sulfhydryls. From the combination of the $K_{2,4}$ measurements with ligand-binding data for the same derivatives (Kilmartin, J. V., et al. (1973), *J. Biol. Chem. 248,* 7039; Nigen, A. M., et al. (1974), *J. Biol. Chem. 249,* 6611) the carbamoylation-induced changes in the dimer–tetramer association constants of the unliganded derivatives were estimated to be of magnitude equal to or smaller than those in $K_{2,4}$. It is concluded that much of the change in oxygen affinity that occurs upon carbamoylation of hemoglobins A and S can be accounted for without invoking extensive structural changes in the unliganded molecule.

Hemoglobins specifically carbamoylated at the $NH_2$-terminal valine residues of the $\alpha$- or $\beta$-chains have been useful in the elucidation of a number of functional properties of the molecule. The carbamoyl group has been employed as a modifier in studies of the alkaline Bohr effect and of the binding of $CO_2$ and 2,3-diphosphoglycerate to hemoglobin (Kilmartin and Rossi-Bernardi, 1971; Kilmartin et al., 1973; Kilmartin, 1974). The rates of carbamoylation of the $NH_2$-terminal groups of hemoglobin have also been exploited (Garner et al., 1975) to measure the p*K*'s of those groups. Additional interest in the properties of these derivatives has been stimulated by the finding of Cerami and Manning (1971) that carbamoylation of hemoglobin S (HbS[1]) in the erythrocytes of sickle-cell homozygotes leads to an inhibition of sickling, and that this effect is directly related to carbamoylation of the $NH_2$ termini. This inhibition apparently results partly from an elevation of oxygen affinity (de Furia et al., 1972; May et al., 1972), partly from changes in the conformation of the partially or fully deoxygenated HbS molecule which are reflected in an increase in the minimum concentration at which the protein will gel (Williams, 1973; Nigen et al., 1974), and partly from other less well understood factors (Wagner et al., 1975).

This paper reports a study of the dimer–tetramer association behavior and sulfhydryl reactivity of the carbamoyl derivatives of HbA and HbS. The results are interpreted tentatively in terms of conformational differences which may be induced by carbamoylation.

## Materials and Methods

Reagent grade chemicals were used throughout, with the exceptions of Tris, which was "Ultra Pure" (Mann), and KNCO, which was recrystallized from the best available grade (initially 97% KNCO).

*Preparation of Specifically Carbamoylated Hemoglobins.* HbA, free of minor hemoglobin components and of organic phosphates, was prepared (as oxy-Hb) by the chromatographic method of Williams and Tsay (1973). HbS was prepared from the erythrocytes of an individual known to be homozygous for the sickle-cell gene by a modification of that method in which chromatography was performed at pH 7.8 instead of the pH 7.6 employed with HbA. Specifically carbamoylated hemoglobins were prepared and separated chromatographically (as CO-Hb) exactly as described by Williams et al. (1975). The three modified hemoglobins obtained are $\alpha\beta^c$, $\alpha^c\beta$, and $\alpha^c\beta^c$, where the superscript c implies the presence of a carbamoylated $NH_2$ terminus.

*Measurement of Association Constants.* Dimer–tetramer association of the CO-hemoglobins was measured by equilibrium centrifugation in a Beckman Model E ultracentrifuge. The instrument employs a modified absorption scanner that operates in conjunction with an on-line computer (Williams, 1976). The scanner was calibrated both for linearity and absolute absorbance against a Gilford spectrophotometer at 419 nm. Absorbance measurements were converted to molarity of heme by means of this calibration and the extinction coefficient ($1.91 \times 10^5$ $M^{-1}$) of CO-Hb at 419 nm (Rossi-Fanelli et al., 1959). Centrifuge cells of 30-mm optical path of the type described by Ansevin et al. (1970) were employed, together with

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[1] Abbreviations used: PMB, *p*-mercuribenzoate; HbS, hemoglobin S; HbA, hemoglobin A; oxy-Hb, oxyhemoglobin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

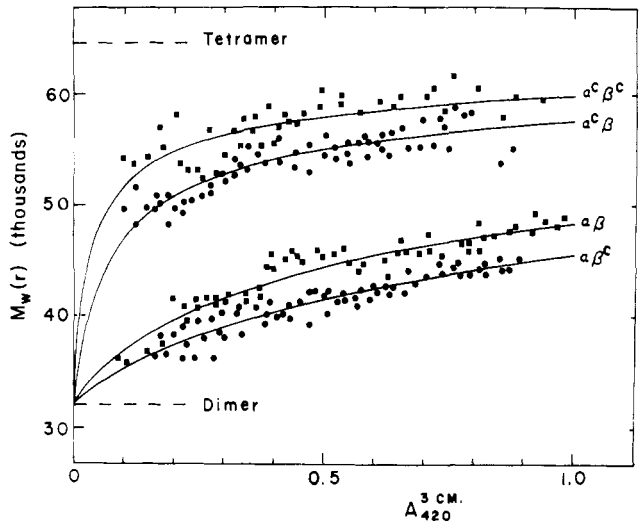


FIGURE 1: Weight-average molecular weight of normal and carbamoylated CO-HbA in 0.1 M NaCl + 0.05 M Tris + 0.001 M EDTA, pH 7.00, ionic strength 0.15. The carbamoylated derivatives are as indicated. For each derivative, results are shown from three initial concentrations: approximately $3 \times 10^{-6}$, $1.5 \times 10^{-6}$, and $7.5 \times 10^{-7}$ M (as heme). Not every point is shown. Lines were calculated from the best-fitting values of $K_{2,4}$. Conditions: 20 °C; 20 000 rpm; height of liquid column approximately 3 mm; scans taken 20–24 h after start of run.

an An-E rotor. The cells were loaded in a CO-filled glove bag. Buffer was prepared by titrating a solution of 0.1 M NaCl, 0.05 M HCl, and 0.001 M EDTA to pH 7.00 by the addition of solid Tris base. The ionic strength of this buffer was approximately 0.15, and its density, measured pycnometrically, was 1.006 at 20 °C. The presence of EDTA was a precaution against the possible formation of ferrihemoglobin during the course of the experiments (Kellett, 1971). Apparent absorption spectra in the Soret region were measured at the beginning and end of each centrifuge run. Slight changes in some of these spectra were observed, but the fraction of ferrihemoglobin and oxyhemoglobin together did not exceed 5% in any of the experiments.

Dimer–tetramer association constants ($K_{2,4}$) were calculated from the observed concentration distributions at sedimentation equilibrium by a nonlinear least-squares fitting technique. All experimental data for a given modified hemoglobin were pooled together and treated simultaneously to find a single value of $K_{2,4}$. The least-squares best fit to the expressions

$$c_j(r_i) = c_{2,j} \exp[\sigma_2(r_i^2/2 - r_0^2/2)] + (K_{2,4}c_{2,j}^2) \exp[2\sigma_2(r_i^2/2 - r_0^2/2)] \quad (1)$$

was calculated, where the subscripts indicate the $i$th point in the $j$th experiment. The quantity

$$\sum_i \sum_j w_{i,j}[c_{i,j}^{obsd} - c_j(r_i)]^2$$

was minimized. (Here $K_{2,4}$ is the association constant; $c_{2,j}$ is the concentration in units of molar heme of the hemoglobin dimer at the top of the liquid column in the $j$th experiment; $\sigma_2$ is the reduced molecular weight of the hemoglobin dimer: $\sigma_2 = M_2(1 - \bar{V}\rho)\omega^2/RT$; $r_i$ is the radius of the $i$th point; $r_0$ is the radius corresponding to $c_{2,j}$; $c_j$ is the calculated and $c_{i,j}^{obsd}$ the observed value of concentration at the $i$th radial point in the $j$th experiment.) The minimization was carried out by an algorithm of Marquardt (1963), with $\sigma_2$ held fixed. The statistical weight ($w_{i,j}$) for each point was taken to be the reciprocal of the square of the standard deviation (SD) of the concentration at that point. The standard deviations were estimated by the relationship

$$(SD)^2 = (\beta/\epsilon^2)(1 + 10^{\epsilon c})$$

where $c$ is the concentration in molar heme, $\epsilon$ the molar extinction coefficient, and $\beta$ is a constant approximately equal to $1.1 \times 10^{-5}$. This relationship is in accord with experimental measurement of the variation in standard deviation of absorbance with concentration (Williams, 1976). The range of weights was approximately sixfold for the reported data. To facilitate graphical presentation of results, weight-average molecular weights were also calculated at each radial point in the cell by a running-fit method similar to that described by Roark and Yphantis (1969) and by Teller (1973). Less smoothing was applied to the data in our calculations than in theirs.

*Rates of Reaction with PMB.* Reaction of PMB with the modified CO-hemoglobins was measured at 250 nm with a Durrum-Gibson stopped-flow apparatus equipped with a storage oscilloscope. The observed transmittances were converted to absorbances by means of a calibration of the instrument with unmixed reagents and with reacted hemoglobin. The "baseline" transmittance was taken to be the value of transmittance of the mixed reagents from the preceding measurement and was observed just before each measurement. No attempt was made to calibrate the dead time of the apparatus; rather, times were reckoned from the beginning of the oscilloscope trace. The total absorbance change was 0.076, as measured in the 2-cm optical path employed. The same buffer was used in these measurements as in the measurements of $K_{2,4}$, except that EDTA was omitted. Initial concentrations of Hb and PMB were determined spectrophotometrically at 539 and 232 nm, respectively (Rossi-Fanelli et al., 1959; Boyer, 1954). To facilitate intercomparison of results, the concentrations of Hb and of PMB were the same in all experiments. Apparent second-order rate constants were calculated by standard methods, and initial rates of reaction were estimated from the first 50% of reaction as described by Antonini and Brunori (1969).

## Results

Results of the equilibrium sedimentation experiments with HbA are shown in Figure 1. The weight-average molecular weights ($M_w(r)$) from three experiments performed at three different initial concentrations are shown for each of the modified CO-hemoglobins and for unmodified HbA. Although for purposes of clarity no distinction is shown between the points in the figure, the data from the three experiments for each of the derivatives overlap within experimental error, indicating that homogeneous, labile equilibria are being observed (Roark and Yphantis, 1969; Teller, 1973). It is clear, furthermore, that differences in association behavior between the derivatives are visible.

The association constants obtained from the nonlinear fitting procedure are given in Table I for both HbA and HbS. (The lines in Figure 1 are drawn from these values of $K_{2,4}$.) It can be seen that the effects of carbamoylation are qualitatively similar in HbA and HbS. Carbamoylation of a $\beta$ chain lowers $K_{2,4}$; carbamoylation of an $\alpha$ chain raises it. In both proteins, the doubly carbamoylated material shows a stronger tendency to associate than does the parent protein, and this effect is less pronounced in HbS than in HbA.

Results of the measurement of rate of reaction of PMB with the CO-HbA derivatives are shown in Figure 2. Apparent second-order rate constants and initial rates of reaction are

Table I: Dimer–Tetramer Association Constants ($K_{2,4}$) of HbA, HbS, and Their Carbamoyl Derivatives.[a]

| Derivative | $K_{2,4}$ (M heme)$^{-1}$ HbA | HbS |
|---|---|---|
| $\alpha\beta$ | 0.88 (±0.09) × $10^6$ | 1.64 (±0.2) × $10^6$ |
| $\alpha\beta^c$ | 0.52 (±0.05) × $10^6$ | 0.24 (±0.02) × $10^6$ |
| $\alpha^c\beta$ | 7.0 (±1) × $10^6$ | 8.0 (±1) × $10^6$ |
| $\alpha^c\beta^c$ | 17 (±4) × $10^6$ | 5.1 (±0.8) × $10^6$ |

[a] Constants were obtained from three or four experiments analyzed simultaneously as described in Methods. Experimental uncertainties represent the range of values obtained when each of the experiments for a given derivative was analyzed separately, and they probably overestimate the true uncertainties. The standard deviations obtained from the diagonals of the covariance matrices were uniformly in the vicinity of 5% of the measured constants and, because of the nonlinear nature of the fitting problem, they probably underestimate the true experimental uncertainties. Conditions of measurement are given in the caption to Figure 1.

Table II: Apparent Second-Order Rate Constants and Initial Reaction Rates for Reaction of PMB with Carbamoyl Derivatives of HbA.[a]

| Derivative | $k$ ($M^{-1}$ $s^{-1}$) | Initial Rate ($s^{-1}$) |
|---|---|---|
| $\alpha\beta$ | 5.9 (±0.5) × $10^5$ | 4.4 (±0.2) |
| $\alpha\beta^c$ | 6.0 (±0.5) × $10^5$ | 4.4 (±0.3) |
| $\alpha^c\beta$ | 6.4 (±1) × $10^5$ | 4.4 (±0.7) |
| $\alpha^c\beta^c$ | 6.2 (±1) × $10^5$ | 4.3 (±0.3) |

[a] Apparent second-order rate constants ($k$) were calculated from the first 80% of reaction (see Figure 3) for each of three reactions for each derivative. The mean value is reported, and the experimental uncertainty represents the range of separate values about the mean. Initial rates were calculated from the first 50% of the reaction. Reaction conditions are given in caption to Figure 2.

given in Table II. It is clear, both from the figure and from the values of these constants, that carbamoylation brings about no large differences in the rate of reaction of PMB with hemoglobin.

## Discussion

The value of $K_{2,4}$ observed for HbA is in agreement with the generally accepted range (Ackers and Thompson, 1955; Chiancone et al., 1968; Edelstein et al., 1970; Kellett, 1971), although it differs by a factor of 2.5 from the most directly comparable value ($0.34 \times 10^6$ $M^{-1}$), which was measured for oxy-HbA in 0.09 M NaCl, 0.10 M Tris, 0.001 M EDTA, pH 7.0, ionic strength approximately 0.18 (Kellett, 1971). Much of this difference may be accounted for by the higher ionic strength and approximately twofold higher Tris concentration employed in the earlier study. The observed second-order rate constant for reaction with PMB is in good agreement with that obtained by Gibson (1973) in Tris buffer. That paper reports a 2.4-fold difference between values obtained in Tris and in phosphate buffers. After correction for this effect, the present observations also agree satisfactorily with the data of Antonini and Brunori (1969), although after the same correction an apparent 3.5-fold difference exists between our results and those of Geraci and Parkhurst (1973). These comparatively small differences in absolute values are not important in the interpretation offered below.

The changes in free energy of association of CO-hemoglobin brought about by carbamoylation of either of the chain $NH_2$ termini are small (−1.1 to +1.0 kcal/mol heme) as compared with the change of about 8 kcal/mol (Kellett, 1971; Thomas and Edelstein, 1972, 1973) that accompanies the binding of four ligands. Similarly, the change in reactivity toward PMB of the $\beta$-93 sulfhydryls (too small to detect) is small in comparison to the 50- to 80-fold change (Antonini and Brunori, 1969; Geraci and Parkhurst, 1973) that occurs when ligand is bound. The changes in $K_{2,4}$ are, however, larger by about an order of magnitude than those which would be expected from a simple difference in electrostatic charge unaccompanied by structural perturbation. In addition, the direction of change is inappropriate in the case of $\alpha\beta^c$. One must suppose, then, that small conformational perturbations underlie the observed functional changes, at least in the liganded molecule.

Nigen et al. (1974) and Kilmartin et al. (1973) have measured the oxygen affinities of specifically $NH_2$-terminal carbamoylated derivatives of HbA and HbS. Their data can be combined with the present results to give an indication of the probable magnitude of the carbamoylation-induced change in the dimer–tetramer association constant of the deoxyhemoglobins. Following a scheme introduced by Noble (1969) and further developed by Thomas and Edelstein (1972) one may write an overall cycle involving dimer–tetramer association and ligand binding:

$$\begin{array}{ccc} 2H_2 + 4L & \overset{K_1}{\rightleftharpoons} & 2(H_2L_2) \\ \Big\downarrow\uparrow K_4 & & \Big\downarrow\uparrow K_{2,4} \\ H_4 + 4L & \underset{K_3}{\rightleftharpoons} & H_4L_4 \end{array} \qquad (2)$$

Here, a ligand molecule is represented by L and Hb dimers and tetramers by $H_2$ and $H_4$, respectively. The four constants are association constants. The free energies corresponding to the four constants are related by

$$\Delta G_4^\circ = \Delta G_{2,4}^\circ + \Delta G_1^\circ - \Delta G_3^\circ \qquad (3)$$

Introducing subscripts c and u to identify carbamoylated and uncarbamoylated hemoglobins, one can write the difference between two such cycles:

$$\delta\Delta G_4^\circ = \Delta G_{4,c}^\circ - \Delta G_{4,u}^\circ = \delta\Delta G_{2,4}^\circ + \delta\Delta G_1^\circ - \delta\Delta G_3^\circ \qquad (4)$$

In order to estimate the value of $\delta\Delta G_4^\circ$, the overall tetrameric ligand binding constant ($K_3$) can be considered to be equal to the inverse fourth power of the median ligand activity (Wyman, 1964) and approximately proportional to $(p_{50})^{-4}$, where $p_{50}$ is the partial pressure of ligand at 50% saturation. Then

$$\delta\Delta G_3^\circ = \Delta G_{3,c}^\circ - \Delta G_{3,u}^\circ \simeq 4RT \ln (p_{50,c}/p_{50,u}) \qquad (5)$$

The magnitude of change of the overall dimeric ligand binding constant ($K_1$) also needs to be estimated. In the absence of information about the change, two simple extreme possibilities can be considered, although other intermediate situations can also be imagined. For the purposes of calculation consider that either (1) the free energy of binding ligand to a dimer heme is unchanged by carbamoylation; i.e., $\delta\Delta G_1^\circ = 0$; or (2) the free energy is changed by an amount equal to the change in free energy of ligand binding observed for the tetramer, i.e., $\delta\Delta G_1^\circ = \delta\Delta G_3^\circ$. The first possibility assigns all the change in ligand affinity to interactions between dimers; the second assigns the change to the dimers themselves. Possibility 1 can be expressed

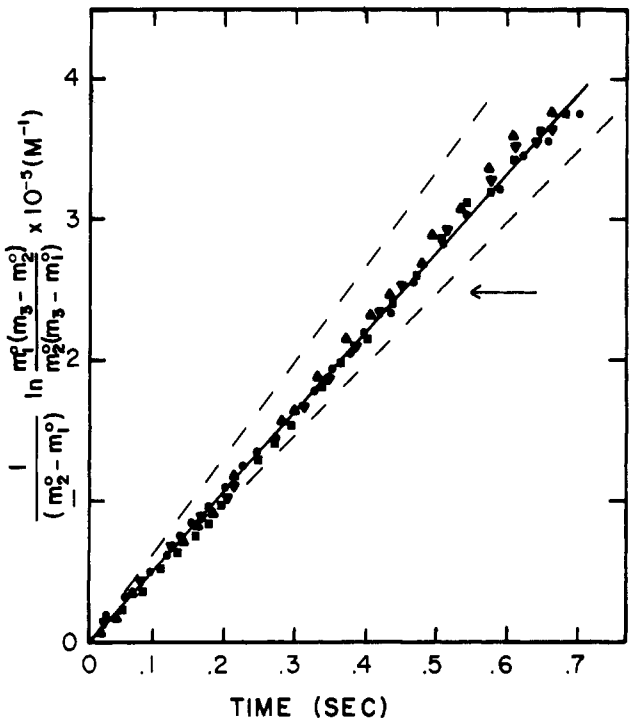


FIGURE 2: Second-order plot of reaction of PMB with HbA and its carbamoyl derivatives. One reaction course is shown for each derivative; the spread of all values obtained is shown by the dashed lines. Symbols: $\alpha\beta$, (▲); $\alpha\beta^c$, (●); $\alpha^c\beta$, (■); $\alpha^c\beta^c$, (▼). The quantities on the vertical axis are: $m_1^\circ$ = initial concentration of PMB; $m_2^\circ$ = initial concentration of $\beta$-93 sulfhydryl; $m_3 = (A_t - A_0)/\Delta\epsilon$, where $A_t$ is the absorbance at a given time, $A_0$ the absorbance at zero time, and $\Delta\epsilon$ is the change in molar extinction produced by the reaction of a PMB (Boyer, 1954). Initial concentrations: $1.0 \times 10^{-5}$ M PMB; $0.98 \times 10^{-5}$ M heme (after mixing). Temperature: 20 °C. The arrow indicates the point at which 80% of the reaction is complete.

as

$$\delta\Delta G_4^\circ \simeq -RT \ln (K_{2,4,c}/K_{2,4,u}) - 4RT \ln (p_{50,c}/p_{50,u}) \quad (6)$$

and possibility 2, which effectively decouples change in association constant from change in oxygen affinity, as

$$\delta\Delta G_4^\circ = \delta\Delta G_2^\circ = -RT \ln (K_{2,4,c}/K_{2,4,u}) \quad (7)$$

The values of $\delta\Delta G_4^\circ$ estimated from these two possibilities are shown in Table III. Clearly, the values of $\delta\Delta G_4^\circ$ are no farther from zero or closer to zero than the values of $\delta\Delta G_{2,4}^\circ$. In other words, the observed changes in the dimer-tetramer association constant of the liganded hemoglobins are in the appropriate direction and of roughly sufficient magnitude to account for the observed change in ligand affinity. It is thus unlikely that a large change in association constant of the deoxy-HbS needs to be invoked to explain the observed changes in oxygen affinity.

The conformational alteration (Perutz, 1970) induced by ligand binding encompasses structural rearrangements in the region of contact between the ($\alpha_1\beta_1$) and ($\alpha_2\beta_2$) dimers. These rearrangements give rise to a change of approximately six orders of magnitude in the dimer-tetramer association constant. Other alterations, notably the binding of organic phosphates and oxidation of heme iron, produce smaller changes in association constant (Hensley et al., 1975) which are accompanied by smaller changes in conformation (Arnone, 1972; E. J. Heidner, quoted by Perutz et al., 1974). The dimer-tetramer association constant of hemoglobin is thus a sensitive indicator of conformational alteration at the dimer-dimer interface and a qualitative guide to its magnitude. From the results in Table I, then, it seems reasonable to suppose that in liganded hemoglobin, carbamoylation of an $\alpha$ chain produces a strengthening of interdimer contacts while carbamoylation of a $\beta$-chain produces a weakening of these contacts. Since the carbamoylation-induced changes in free energy of subunit association of both the liganded and unliganded hemoglobins appear to be small, it seems likely that the structural changes accompanying carbamoylation will be found to be small. This inference is consistent with the small changes in minimum gelling concentration induced by carbamoylation (Nigen et al., 1974). Combination of the association data with the kinetic data may serve to delimit the regions of conformational alteration within the molecule, since it appears that the alterations at the interdimer contact region are *not* accompanied by changes affecting the accessibility of the $\beta$-93 sulfhydryls.

Table III: Calculated Changes in Dimer-Tetramer Association Constant of Unliganded Hemoglobin upon Carbamoylation.[a]

| Material | Log ($p_{50}$) | $\delta\Delta G_{2,4}^\circ$ (kcal/mol) | $\delta\Delta G_4^\circ$ (kcal/mol) (eq 6) | $\delta\Delta G_4^\circ$ (kcal/mol) (eq 7) |
|---|---|---|---|---|
| HbA[b] | | | | |
| $\alpha\beta$ | 1.16 | | | |
| $\alpha\beta^c$ | 1.17 | +0.3 (±0.1) | +0.3 (±0.1) | +0.3 (±0.1) |
| $\alpha^c\beta$ | 0.85 | −1.2 (±0.1) | +0.6 (±0.1) | −1.2 (±0.1) |
| $\alpha^c\beta^c$ | 0.94 | −1.7 (±0.2) | −0.4 (±0.2) | −1.7 (±0.1) |
| HbS[c] | | | | |
| $\alpha\beta$ | 0.60 | | | |
| $\alpha\beta^c$ | 0.68 | +1.1 (±0.1) | +0.7 (±0.1) | +1.1 (±0.1) |
| $\alpha^c\beta$ | 0.35 | −0.9 (±0.1) | +0.4 (±0.1) | −0.9 (±0.1) |
| $\alpha^c\beta^c$ | 0.41 | −0.6 (±0.2) | +0.4 (±0.2) | −0.6 (±0.2) |

[a] Calculations as in text. The experimental uncertainties in the calculated values of $\delta\Delta G_4^\circ$ reflect only the uncertainty in the $K_{2,4}$ value. The true uncertainty, after consideration of experimental error in the $p_{50}$ determinations, may be several fold larger. [b] Kilmartin et al. (1973). The quantity $\delta\Delta G_3^\circ$ was calculated at the temperature of the $p_{50}$ determination, 37 °C. [c] Nigen et al. (1974).

The reasons for the *directions* of the observed changes are not clear. The observed increase in association constant is not specific to the carbamoyl moiety, but is also observed in HbA specifically dinitrophenylated at the $NH_2$ terminus of the $\alpha$ chain (Neer and Konigsberg, 1968). Whether or not an increased $K_{2,4}$ is the usual consequence of modification of that site is unknown. Although removed from the main point of this paper, the observed difference in $K_{2,4}$ between HbA and HbS shown in Table I has been confirmed in several experiments and deserves comment. The effect appears to be buffer dependent since it is not seen in a $NaPO_4$-NaCl-EDTA system (Williams and Kim, 1975), nor in an ammonium phosphate buffer (Crepeau et al., 1974). The difference thus seems to reflect preferential binding of some solvent component. It is not possible with the present data to assign the binding to any given buffer component.

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