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Role of the α-Amino Groups of the α and β Chains of Human Hemoglobin in Oxygen-linked Binding of Carbon Dioxide*

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SUMMARY

Human hemoglobin has been reacted with potassium cyanate and purified to yield three species, $\alpha_2^c\beta_2^c$,$\alpha_2^c\beta_2$, and $\alpha_2\beta_2^c$, where superscript c denotes specific reaction of cyanate with the α-amino group of the particular chain. The effect of carbon dioxide on the oxygen affinity of these species in the presence and in the absence of 2,3-diphosphoglycerate has been measured. Carbon dioxide has no effect on the oxygen affinity of $\alpha_2^c\beta_2^c$, confirming that the usual lowering of the oxygen affinity of carbon dioxide in normal hemoglobin is mediated by the α-amino groups of the α and β chains. The lowering of the oxygen affinity of $\alpha_2\beta_2^c$ by carbon dioxide was not affected by the presence or absence of 2,3-diphosphoglycerate, showing that 2,3-diphosphoglycerate does not interfere with the oxygen-linked binding of carbon dioxide at the α chain α-amino group. In $\alpha_2^c\beta_2$ there was a much larger effect of carbon dioxide on the oxygen affinity in the absence of 2,3-diphosphoglycerate than in $\alpha_2\beta_2^c$; however, on addition of 2,3-diphosphoglycerate the effect of carbon dioxide on the oxygen affinity of $\alpha_2^c\beta_2$ was much smaller and similar to that occurring in $\alpha_2\beta_2^c$. This shows that there is a large difference in the carbon dioxide binding constants of the β chain α-amino group in the oxy and deoxy forms of human hemoglobin, and that 2,3-diphosphoglycerate suppresses this difference, probably by binding strongly to the β chain α-amino group of deoxyhemoglobin and displacing any bound carbon dioxide.

After specific modification of the α-amino groups of the α and β chains of horse hemoglobin by reaction with cyanate, Kilmartin and Rossi-Bernardi (1–3) showed that the binding of CO_2 to horse hemoglobin under physiological conditions takes place exclusively at the α-amino groups of the α and β chains. The individual contribution of either the β chain α-amino group or the α chain α-amino group by preparation of the species $\alpha_2\beta_2^c$ or $\alpha_2^c\beta_2$ was not possible due to shortage of material.[1]

Perrella *et al.* (4) have shown that in both the oxy and deoxy forms of bovine hemoglobin the CO_2 binding curve can only be fitted by having two groups per $\alpha\beta$ dimer having different affinity constants. In view of the work of Kilmartin and Rossi-Bernardi (1–3) and van Kempen and Kreuzer (5), these two groups in the dimer probably correspond to the bovine α and β chain α-amino groups.

Brenna *et al.* (6) have described the effect of DPG on the binding of CO_2 to human deoxyhemoglobin; in the absence of DPG the CO_2 binding curve, as in bovine hemoglobin (4), can only be fitted by two groups per $\alpha\beta$ dimer with different affinity constants for CO_2. In the presence of DPG, the CO_2 binding curve was found to coincide with the calculated binding curve for the high affinity binding site, suggesting that DPG displaces most of the CO_2 from the low affinity binding site. Since DPG is known to bind to the β chain α-amino group of deoxyhemoglobin (7–9), an obvious explanation is that the low affinity binding site for CO_2 is the β chain α-amino group and that DPG displaces the CO_2 from this site because it has a much higher affinity constant. A possible way of confirming this hypothesis is to examine the CO_2 binding properties of the singly blocked derivatives $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$. This, however, requires extensive modifications of the CO_2 binding method of Perrella *et al.* (4) due to the relatively small quantities of material available. Before attempting these modifications a careful examination of the functional properties of the carbamylated hemoglobin derivatives is necessary to ensure that no extensive structural alterations have been caused by the chemical modification.

The study of the functional properties of carbamylated human hemoglobin under physiological conditions is important also because of the possible therapeutic use of cyanate in the treatment of sickle cell anemia (10).

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[1] The abbreviations used are: $\alpha_2\beta_2^c$, human hemoglobin reacted specifically with cyanate at the β chain α-amino group; $\alpha_2^c\beta_2$, human hemoglobin reacted specifically with cyanate at the α chain α-amino group; DPG, 2,3-diphosphoglycerate; $\alpha_2^c\beta_2^c$, human hemoglobin reacted specifically with cyanate at the α and β chain α-amino groups; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitriloethanol.

EXPERIMENTAL PROCEDURE

Preparation of Human $\alpha_2^c\beta_2^c$—The basic procedure for the carbamylation of horse hemoglobin (3) was followed; however, certain modifications were introduced to facilitate the handling of larger amounts of material. Only these modifications will be described in detail.

Human CO-hemoglobin (21 g prepared as described for horse hemoglobin (3)) was reacted with cystamine. After filtration through a Sephadex G-25 (fine) column (8 × 40 cm) equilibrated with 0.2 M sodium phosphate, pH 6.0, the hemoglobin was diluted to 40 mg per ml with 0.2 M sodium phosphate, pH 6.0. Recrystallized potassium cyanate was added (115 mg per g of hemoglobin) and allowed to react for 2 hours at 25°, in an atmosphere of CO. After removal of the cystamine with dithiothreitol the hemoglobin sample was concentrated to about 150 ml in a 400-ml Amicon ultrafilter using a UM-10 membrane. After filtration through a Sephadex G-25 (fine) column (7 × 23 cm) equilibrated with 0.05 M sodium phosphate, pH 6.7 (2.20 g of Na_2HPO_4, 2.96 g of $NaH_2PO_4 \cdot 2H_2O$ per liter), the sample was applied to a column (8 × 50 cm) of Bio-Rex 70 (200 to 400 mesh, Bio-Rad Laboratories, Richmond, California) equilibrated with the same buffer. The air at the top of the column (between the buffer and the bung) was replaced with CO to ensure that the buffer always contained a small amount of dissolved CO. The column was eluted at 250 ml per hour until the first two peaks were off and the optical density at 540 nm was less than 0.3; then the molarity of the buffer was changed to 0.1 M to elute $\alpha_2^c\beta_2^c$ which can be seen as the main peak approaching the bottom of the column. A typical elution pattern is shown in Fig. 1. After concentration by ultrafiltration the yield of $\alpha_2^c\beta_2^c$ was about 9 g. No attempt was made to isolate the derivatives $\alpha_2^c\beta_2$ and $\alpha_2\beta_2^c$ off the Bio-Rex 70 column since it was thought that these could be more easily prepared by chain separation of $\alpha_2^c\beta_2^c$; hence the extent of carbamylation was chosen such that the yield of $\alpha_2^c\beta_2^c$ was maximized.

Preparation of Mercury-free Human α and β Chains—The α and β chains of either normal human hemoglobin or $\alpha_2^c\beta_2^c$ were separated by a convenient modification of the method of Geraci *et al.* (11). First, 21 g of human CO-hemoglobin were reacted with *p*-mercuribenzoate (Sigma) and applied to a DE-52 column (8 × 28 cm) (Whatman Biochemicals Ltd.), equilibrated with 0.01 M potassium phosphate, pH 8.0, and CO was added to the air at the top of the column. After elution of the α chains, the β chains were washed off with 4 column-volumes of buffer containing 0.5 M mercaptoethanol. The presence of CO protects the β chains against denaturation by mercaptoethanol and allows the use of a much higher concentration of mercaptoethanol to ensure complete removal of the mercuribenzoate. Similarly, the α chains were first treated with 0.05 M mercaptoethanol for 30 min at 0° under CO. Then the pH was adjusted to 6.6 by careful addition of phosphoric acid and they were applied to a CM-23 (Whatman Biochemicals Ltd.) column. After addition of CO to the top of the column they were eluted with 8 column-volumes of buffer containing 0.03 M mercaptoethanol.

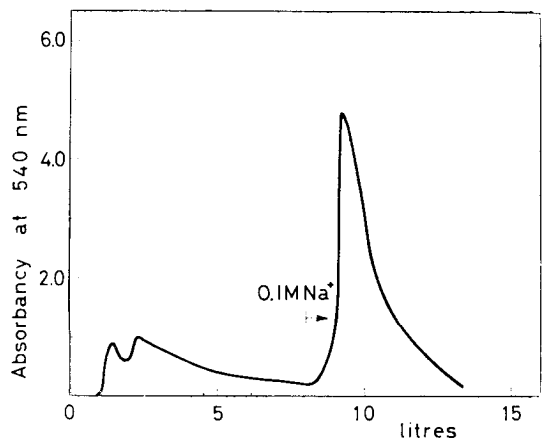

FIG. 1. Elution pattern of 21 g of carbamylated hemoglobin off a column (8 × 50 cm) of Bio-Rex 70.

Recombination of Chains—It was noticed that the free sulfhydryl groups of the chains tended to oxidize on storage, hence the chains were recombined in the presence of 60 mM dithiothreitol at pH 8.5 for 1 hour.

Chemical Characterization of $\alpha_2^c\beta_2^c$—Human $\alpha_2^c\beta_2^c$ was made radioactive using [^{14}C]cyanate and chemically characterized using all the methods described by Kilmartin and Rossi-Bernardi (3). The only exception was that the hemoglobin was heat denatured (12) and digested with trypsin without separation of the α and β chains.

Preparation of Hemoglobin Solutions for Functional Studies—The CO-hemoglobin solution was adjusted to pH 8.5 by addition of solid Tris and the CO removed (3). The oxyhemoglobin was stripped of DPG as described by Berman *et al.* (13). Enzymatic assay (14) of the stripped hemoglobin showed less than 0.05 mole of DPG per mole of hemoglobin tetramer. For measurement of the Bohr effect by differential titration the hemoglobin solutions were passed through a Dintzis column (3).

Methods Used for Functional Studies—Oxygen equilibrium curves of dilute solutions (1 mg per ml) were measured as described previously (3) in 0.05 M bis-tris, 0.1 M KCl, pH 7.30, at 25°. Measurement of the p_{50} values (the oxygen affinity at half-saturation) of concentrated hemoglobin solutions (100 mg per ml) was carried out as described by Brenna *et al.* (6). For measuring the alkaline and acid Bohr effects the pH of a solution of deoxyhemoglobin (10 mg per ml in 0.1 M KCl) was measured using a G202C glass electrode and a K4112 calomel electrode connected to a Radiometer pH meter 26. Then, after oxygenation, the amount of acid or alkali necessary to restore the pH of the solution to its original value was recorded, from which the value of the Bohr effect at that particular pH could be calculated. The methemoglobin at the end of any of these measurements was always less than 10%. DPG (Sigma) was prepared as described by Benesch *et al.* (15).

RESULTS

Chemical Characterization of $\alpha_2^c\beta_2^c$—When human hemoglobin was carbamylated with [^{14}C]cyanate, the purified $\alpha_2^c\beta_2^c$ was found to have 1.9 moles of cyanate per mole of $\alpha\beta$ dimer. If the $\alpha_2^c\beta_2^c$ was treated as described by Stark and Smyth (16), then 76% of the counts present in $\alpha_2^c\beta_2^c$ were released as valine hydantoin. This shows that at least 76% of the 1.9 moles of cyanate per mole of $\alpha\beta$ dimer represented cyanate which had reacted exclusively with the α-amino groups. This conclusion was checked further by tryptic digestion of the hemoglobin; 95% of the counts were soluble after digestion and only three radioactive peptides were found and purified (3); amino acid analysis of these peptides showed that they corresponded to residues 1 to 8 of the β chain, and 1 to 7 and 1 to 11 of the α chain, which are the NH_2-terminal tryptic peptides. (There are two α chain NH_2-terminal peptides because trypsin only partially splits the bond between residues 7 and 8.) The fact that only the NH_2-

terminal tryptic peptides are radioactive therefore confirms the earlier deduction that cyanate had only reacted with the NH_2-terminal α-amino groups.

Oxygen Equilibrium Parameters of Carbamylated Hemoglobin—The values of Hill's constant n of the carbamylated hemoglobins in 0.05 M bis-tris, 0.1 M KCl, pH 7.30, at 25° are shown in Table I. These are higher and closer to normal hemoglobin than the results obtained for the equivalent horse hemoglobin derivatives (2), except for the higher than normal value of n obtained with human $\alpha_2{}^c\beta_2$, which might be due to the large error (about ± 0.3) associated with the measurement of values of n around 3.0. The high values of n observed in these derivatives make it unlikely that any extensive structural changes have occurred.

Bohr Effect of $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$—The values of both the acid and alkaline Bohr effects are shown for $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$ in Table I. The reduced alkaline Bohr effect in $\alpha_2{}^c\beta_2$ is in agreement with the previous work done on the equivalent horse hemoglobin derivative (2). The reason for the large acid Bohr effect in $\alpha_2\beta_2{}^c$ is not known probably because that effect is not yet understood in stereochemical terms.

Effect of CO_2 and DPG on Oxygen Affinity of Carbamylated Hemoglobins—The effect of CO_2 and DPG on the oxygen affinity of $\alpha_2{}^c\beta_2{}^c$ is shown in Fig. 2; CO_2 has no effect on the oxygen affinity of $\alpha_2{}^c\beta_2{}^c$ either in the presence or absence of DPG, thus confirming the earlier conclusions (2) that the α-amino groups are solely responsible for oxygen-linked CO_2 binding under physiological conditions. The reduced effect of DPG on the oxygen affinity of the doubly blocked derivative compared with normal hemoglobin (*Lines A* and *C* in Fig. 5) is due to the removal of the beta chain α-amino group, which has been shown to be involved in DPG binding (7–9); thus, there is a similar reduced effect in $\alpha_2\beta_2{}^c$ (Fig. 3) and a normal effect in $\alpha_2{}^c\beta_2$ (Fig. 4).

The effect of CO_2 on the oxygen affinity of $\alpha_2\beta_2{}^c$ where only the α chain α-amino group is free to react is shown in Fig. 3. Here the change in log p_{50} caused by CO_2 is the same in the presence and absence of DPG, showing that DPG does not influence the binding of CO_2 to the α chain α-amino group because it does not interact with this group. The oxygen affinity of stripped $\alpha_2\beta_2{}^c$ is identical to that of stripped normal hemoglobin, as shown by Fig. 5.

The effect of CO_2 on the oxygen affinity of $\alpha_2{}^c\beta_2$ is shown in Fig. 4. In the absence of DPG there is a large effect of CO_2 on the oxygen affinity showing that there is a large difference in the affinity constants for CO_2 of the β chain α-amino group in the oxy and deoxy forms of human hemoglobin. In the presence of DPG, however, this difference is suppressed so that there is now a much smaller effect of CO_2 on the oxygen affinity.

The method described here only measures the oxygen affinity of hemoglobin at a single saturation, 50 ± 5%. Hence, the effect of CO_2 and DPG on Hill's constant of the derivatives

TABLE I

Oxygen equilibrium parameters and Bohr effect at 25°

Hemoglobin derivative	n	Protons/heme released on oxygenation	
		Acid Bohr effect at pH 5.2	Alkaline Bohr effect at pH 7.4
Normal	2.9	−0.27	0.48
$\alpha_2\beta_2{}^c$	2.8	−0.43	0.50
$\alpha_2{}^c\beta_2$	3.3	−0.24	0.35
$\alpha_2{}^c\beta_2{}^c$	3.0		

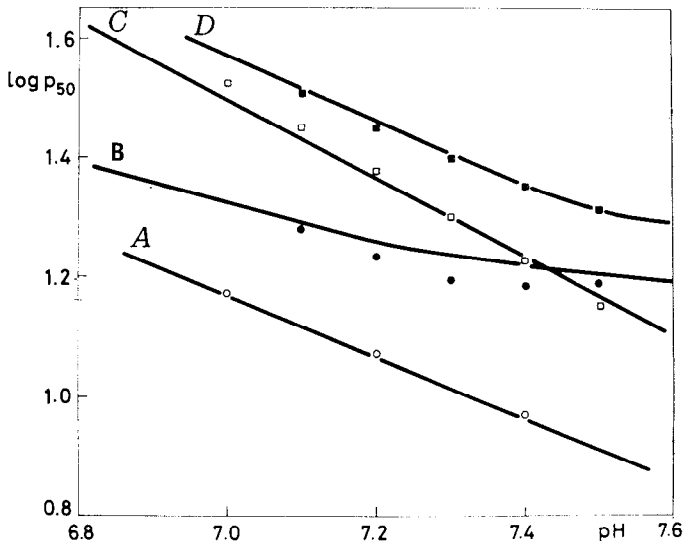

FIG. 5. The lines show the values of log p_{50} of normal hemoglobin (100 mg per ml) in 0.1 M KCl at 37° as determined by Dahms *et al.* (23). *A*, stripped hemoglobin; *B*, p_{CO_2} 42 mm Hg; *C*, 1.2 moles of DPG per mole of hemoglobin tetramer; *D*, 1.2 moles of DPG per mole of hemoglobin tetramer and p_{CO_2} 42 mm Hg; ○, values of log p_{50} of stripped $\alpha_2\beta_2{}^c$ taken from Fig. 3; ●, change in log p_{50} on addition of p_{CO_2} 42 mm Hg to stripped $\alpha_2\beta_2{}^c$ and $\alpha_2{}^c\beta_2$ taken from Figs. 3 and 4 added to *Line A*; □, change in log p_{50} on addition of DPG to stripped $\alpha_2{}^c\beta_2$ taken from Fig. 4 added to *Line A*; ■, change in log p_{50} on addition of p_{CO_2} 42 mm Hg to $\alpha_2\beta_2{}^c$ and $\alpha_2{}^c\beta_2$ in the presence of DPG taken from Figs. 3 and 4 added to *Line C*.

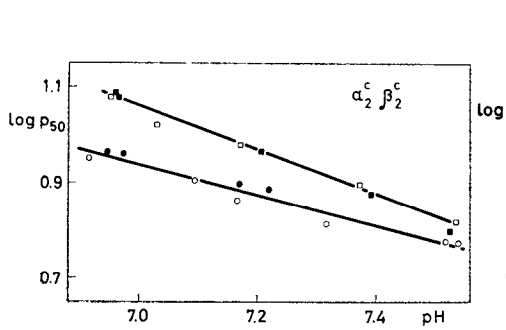

FIG. 2 (*left*). Values of log p_{50} of $\alpha_2{}^c\beta_2{}^c$ (90 mg per ml) in 0.1 M KCl at 37°. ○——○, no additions; ●——●, p_{CO_2} 42 mm Hg; □——□, 1.0 mole of DPG per mole of hemoglobin tetramer; ■——■, 1.0 mole of DPG per mole of hemoglobin tetramer and p_{CO_2} 42 mm Hg.

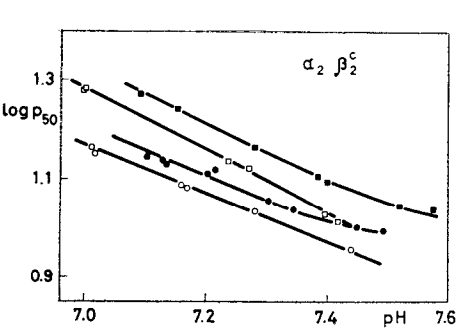

FIG. 3 (*center*). Values of log p_{50} of $\alpha_2\beta_2{}^c$ (90 mg per ml) in 0.1 M KCl at 37°. Symbols are as in Fig. 2.

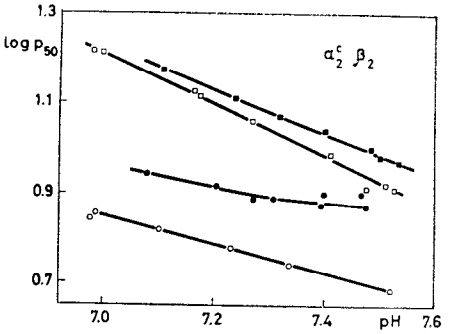

FIG. 4 (*right*). Values of log p_{50} of $\alpha_2{}^c\beta_2$ (90 mg per ml) in 0.1 M KCl at 37°. Symbols are as in Fig. 2.

could not be determined. It would probably be small since CO_2 was without effect on Hill's constant of the horse hemoglobin derivatives in dilute solution (2), and DPG is also without effect on Hill's constant of dilute solutions of normal hemoglobin in 0.1 M NaCl (17).

The comparative values of the alkaline Bohr effect calculated from these data as $\Delta \log p_{50}/\Delta$ pH at 37° are in good relative agreement with the values measured from the differential titration curves at 25°.

DISCUSSION

The set of experiments described in this paper had two main aims. The first was a general investigation of the response of specifically carbamylated human hemoglobin to physiological ligands under conditions approximating those occurring *in vivo*. This is particularly relevant in view of the possible therapeutic use of cyanate in sickle-cell anemia (10). The second aim was to test the suitability of the derivatives $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$ for measuring the affinity constants for the combination of CO_2 with the β chain α-amino group and the α chain α-amino group, respectively. This is a way of distinguishing which of the two affinity constants for CO_2 combination found for normal human hemoglobin (6) correspond to the α or β chain α-amino groups.

Two sets of measurements on the specifically carbamylated derivatives are reported in this paper. The first set of measurements of Hill's constant n and the Bohr effect was carried out in dilute solutions at 25° to show that these derivatives resembled closely the previously described horse hemoglobin derivatives (2). In particular, carbamylation of the α chain α-amino group of both horse and human hemoglobin leads to a similar reduction of the Bohr effect. The high values of n observed in these derivatives make it unlikely that carbamylation causes any extensive structural alterations in hemoglobin. The small variations in the values of n amongst the derivatives are probably due to the relatively large experimental error associated with measuring high values of n.

After these experiments had shown that the carbamylated human hemoglobin had similar functional properties to normal hemoglobin, the second set of measurements was carried out. This was the effect of the combination of CO_2 with either the α chain α-amino group in $\alpha_2\beta_2{}^c$ or the β chain α-amino group in $\alpha_2{}^c\beta_2$ on the oxygen affinity in the presence and absence of DPG. These experiments were done with higher hemoglobin concentration and higher temperature (37°), than those described above for two reasons. First, the changes in oxygen affinity caused by CO_2 in some of the derivatives were likely to be rather small and might not be convincingly demonstrated using the methods appropriate for dilute hemoglobin solutions. It was therefore decided to use the accurate tonometer described by Brenna *et al.* (6) which requires the use of concentrated hemoglobin solutions at 37°. Secondly, since these derivatives could be used to measure the binding constants of CO_2 to the α and β chain α-amino groups by methods which absolutely require the use of high hemoglobin concentration (4, 6), then, for comparative purposes, measurements of the effect of CO_2 on the oxygen affinities of the derivatives should be carried out under similar conditions.

The use of the derivatives $\alpha_2\beta_2{}^c$ and $\alpha_2{}^c\beta_2$ to determine the binding constants for CO_2 to the α-amino groups of the α and β chains requires that they be good model compounds for normal hemoglobin in the sense that carbamylation of the α-amino group of one chain should not affect the properties of the free α-amino group of the other chain, so that this group would have identical properties to the equivalent group in normal hemoglobin. An indication that this is the case comes from the fact that the change in log p_{50}, which is proportional to the change in free energy per mole of ligand bound (18), caused by carbamylation in $\alpha_2{}^c\beta_2{}^c$ is equal to the sum of the change in log p_{50} in $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$. This suggests that carbamylation of either the α or β chain α-amino group has independent effects on the structure of hemoglobin which do not include any effect on the unreacted α-amino group.

A further indication that carbamylation of the α-amino group of one chain does not affect the same group in the unreacted chain comes from the changes in oxygen affinity caused by CO_2 combination with the α-amino groups of the derivatives, if it can be assumed that CO_2 combination with either α-amino group in normal hemoglobin has independent effects on the structure of hemoglobin. Then the addition of the changes in log p_{50} caused by CO_2 in stripped $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$ should be equal to the same change in normal hemoglobin if the free α-amino groups in $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$ have the same properties as the equivalent groups in normal hemoglobin. Fig. 5 shows that the addition of the changes in log p_{50} caused by CO_2 in stripped $\alpha_2{}^c\beta_2$ and $\alpha_2\beta_2{}^c$ corresponds closely to the same changes in stripped normal hemoglobin. The changes in the presence of DPG are also additive, as shown by Fig. 5; however, here the situation is complicated by the competitive binding of both CO_2 and DPG to the β chain α-amino group.

Thus, the two sets of experiments described above suggest that carbamylation of the α-amino group of one chain does not affect the properties of the α-amino group of the other chain, and hence $\alpha_2\beta_2{}^c$ and $\alpha_2{}^c\beta_2$ are suitable hemoglobin derivatives for measuring the affinity constants for CO_2 combination with the α and β chain α-amino groups.

The effect of DPG on the oxygen affinity of $\alpha_2{}^c\beta_2$ is shown to be the same as that on normal hemoglobin, since the Δ log p_{50} in $\alpha_2{}^c\beta_2$ due to DPG when added to the values of log p_{50} for stripped hemoglobin now coincides with the values of log p_{50} for normal hemoglobin in the presence of DPG.

The much larger effect of CO_2 on the oxygen affinity of stripped $\alpha_2{}^c\beta_2$ compared with $\alpha_2\beta_2{}^c$ shows that there is a large difference in affinity constants for CO_2 combination with the β chain α-amino group between the oxy and deoxy forms. In the presence of DPG there is a much smaller effect of CO_2 on the oxygen affinity which is about the same for both the α and β chain α-amino groups. It should be noted that Perrella *et al.* (4) find the greatest change in affinity constant on oxygenation to occur in the low affinity site of bovine hemoglobin which would lead to a correspondingly large change in oxygen affinity caused by CO_2. If human hemoglobin behaved similarly to bovine hemoglobin, then the low affinity CO_2 binding site could be tentatively assigned to the β chain α-amino group, thus confirming the findings of Brenna *et al.* (6), who tentatively assigned the low affinity CO_2 binding site to the β chain α-amino group.

It might seem surprising that the α chain α-amino group could have a high affinity for CO_2, particularly in the deoxyhemoglobin structure where this group forms a salt bridge with the COOH-terminal α-carboxyl group of the opposite α chain (19–22); one would expect the salt bridge to oppose the binding of carbamino CO_2 by the α chain α-amino group in deoxyhemoglobin. However, the binding of CO_2 to an α-amino group is dependent on two parameters: the pK of the group and the equilibrium constant for the formation of the hemoglobin-carbamino CO_2 complex. Since CO_2 lowers the oxygen affinity of $\alpha_2\beta_2{}^c$, it follows that the α chain α-amino group must bind more CO_2 in the

deoxy form than in the oxy form. Since the pK of this group is higher in deoxyhemoglobin compared with oxyhemoglobin (3), this increased binding of CO_2 can only be explained by a large change in the equilibrium constant for CO_2 binding in deoxyhemoglobin. In structural terms this might mean that the negatively-charged carbamate forms a strong salt bridge with a neighboring positively charged group, thus further increasing the stability of the deoxyhemoglobin structure and causing a lower oxygen affinity.

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