Gas Exchange Properties of Goat Hemoglobins A and C*

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Hypoxic or anemic goats with the A hemoglobin genotype switch to the production of hemoglobin C, resulting in a reduced blood oxygen affinity. However, the physiologic consequences of this switch are not clear. We therefore studied the gas exchange properties of the two hemoglobin types.

We found that purified hemoglobins A and C have very similar oxygen affinities and H+ Bohr effects, but in the presence of CO₂, the affinity of hemoglobin C is substantially less than that of hemoglobin A. That this is not a nonspecific ionic effect is suggested by identical effects of NaCl on O₂ binding to the two proteins and by a 2-fold higher capacity of hemoglobin C to bind CO₂. The data can be explained by a class of CO₂ binding sites in the β chain whose affinity is much higher than that of either of the primary sites or of those in Hb A.

Our results suggest that in hemoglobin C-containing red cells CO₂ acts as a potent allosteric effector, analogous to the role played by 2,3-diphosphoglycerate in human red blood cells. Goat hemoglobin C may have advantages over hemoglobins A or B in O₂ transport under hypoxic conditions or in anemia.

The hemoglobin types, A and B, are commonly found in the red blood cells of sheep and goats. They share a common α chain, but their β chains (β⁰ and β₇) contain many amino acid differences and are produced by different genes (1). In addition to these hemoglobins, animals whose hemoglobin phenotype is either AA or AB are capable of producing still another hemoglobin, C, whose β chain (β₇) is the product of a third gene. Hemoglobin C is produced normally in newborn lambs and kids as production of fetal hemoglobin declines and is later replaced by adult hemoglobin A or B, or both.

This example of a molecular “switch” has been studied intensively over the past 20 years in the hope of elucidating possible mechanisms for reactivation of dormant genes (2). An understanding of the switch mechanism would be of obvious importance in hematological disorders such as thalassemia and sickle cell anemia. However, in spite of detailed knowledge of the structure of the involved genes and surrounding DNA regions (3), the possible advantage of the switch to the animal remains speculative.

Among the many structural differences between β⁰ and β₇ globin chains (4) is the absence of the first five amino acids in the β₇ globin chain (Table I) (5). Moreover, the amino terminus of the β₇ globin chain is proline, which does not have a free amino group. In human hemoglobin, the β₇ amino terminus (valine) is important in ligand binding since it contributes to the environment of the 2,3-diphosphoglycerate (DPG) “pocket” (6) and is a site for CO₂ binding (7). Thus, study of the hemoglobin switching mechanism would seem a good opportunity to correlate structural, functional, and physiologic properties.

Huisman and Kitchens (8) reported that the O₂ affinity of hemoglobin C of sheep and goats is substantially less than that of hemoglobin A and suggested the switch from A to C may be advantageous in anemia. However, in preliminary experiments using purified hemoglobins A and C in the absence of the allosteric effectors CO₂ and DPG, we were unable to confirm any significant difference in O₂ binding properties of the two purified hemoglobins. We therefore undertook a detailed study of their functional properties in order to elucidate their physiologic control mechanisms under cellular conditions.

MATERIALS AND METHODS

Blood Samples—Blood from domestic goats was screened for hemoglobin phenotype by cellulose acetate electrophoresis (see below). Two animals that demonstrated only hemoglobin A were selected. Five hundred ml of blood were removed from each goat every other day until the hematocrit reached 12%. Thereafter 200 ml of blood were removed every other day to maintain the hematocrit level near 12%. The amount of blood withdrawn was then adjusted to maintain this hematocrit, and the goats received supplemental iron and vitamins to sustain good health while they were kept anemic.

Hematology—All hematological determinations were carried out using routine clinical methods. In all cases, blood was collected from limb veins. Cellulose acetate electrophoresis was performed after each bleeding to monitor the switch from hemoglobin A to C. This was done at pH 8.4, according to standard methods (9) with the following modifications. (a) The 0.084 M Tris-EDTA-borate (TEB) electrophoresis buffer was diluted 1:1 with deionized water; (b) the samples constituted hemolysates of a 10 g/dl cell suspension and were diluted 1:2 with electrophoresis buffer; (c) electrophoresis was carried out at 4 °C for 30 min at 500 V.

Hemolysate Preparation—When hemoglobin was to be isolated, EDTA anticoagulant was used. The blood was refrigerated immediately and within 2 h it was centrifuged at 3,500 rpm at 4 °C for 15 min. The separated cells were lysed with deionized water; the ratio of packed cells to water was 1:2.5. After mixing, the solution was refrigerated at 4 °C for 1 h, and the cellular debris was sedimented by centrifugation at 18,000 rpm for an additional hour. The supernatant fluid was removed and frozen by adding dropwise to liquid
Partial amino acid sequences of goat and human \( \beta \) globin polypeptide chains

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<th>6</th>
<th>7</th>
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<td>Pro</td>
<td>Glu</td>
<td>Glu</td>
<td>Ser</td>
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<td>Thr</td>
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<td>Ala</td>
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Gas Exchange by Goat Hemoglobins

Purification of Hemoglobin Fractions—Hemoglobins A and C were purified on a 2.5 \( \times \) 30-cm column of DEAE-Sephadex A-50 (Pharmacia LKB Biotechnology Inc.) that had been equilibrated to pH 8.0 with 50 mM Tris/Cl buffer, pH 8.0. A linear pH gradient of from 8.0 to 7.0 was used, and the flow rate was 96 ml/h. Pooled fractions corresponding to hemoglobins A and C were concentrated using a stirred cell (Amicon) and were then centrifuged at 10,000 rpm for 20 min.

The spectrophotometer was a Cary 118C (Varian) equipped with a special reaction cell that allowed continuous monitoring of the PO\(_2\) with an O\(_2\) electrode (Beckman) and pH using a p\(H\) electrode (Radiometer). Continuous records of absorbance at 540 nm were recorded on an analog chart recorder. The curves were later digitized using a digitizing tablet (Houston Instruments). The curves were analyzed by fitting to the Adair equation and calculating \( P_0 \) and \( n_{max} \), the maximal slope of the Hill plot, as described previously (12). In the fitting procedure, the data were not weighted, and parameters were not constrained to positive values.

Determination of the CO\(_2\) Binding Data—The overall reaction of CO\(_2\) with hemoglobin is

\[
\text{Hb} + \text{CO}_2 \rightleftharpoons \text{HbCO}_2
\]

The equilibrium constant, \( k \), for this reaction is

\[
\lambda = \frac{[\text{HbCO}_2]}{[\text{Hb}][\text{CO}_2]}.
\]

The fractional saturation, \( z \), of hemoglobin with CO\(_2\) is

\[
z = \frac{[\text{HbCO}_2]}{[\text{Hb}]} + [\text{HbCO}_2]
\]

which, using Equation 2, simplifies to

\[
z = \frac{x[\text{CO}_2]}{1 + x[\text{CO}_2]}
\]

The carbamino hemoglobin values are expressed as fractional saturation (z) as a function of CO\(_2\) concentration. Since \( z \) is moles of bound CO\(_2\)/0.25 mol of hemoglobin, \( z = 1 \) for full binding.

The data were analyzed assuming that CO\(_2\) binds to the four terminal amino groups, as is known to occur in human hemoglobin at physiological \( p\)H (14, 17). In human hemoglobin, these four sites are grouped into two classes corresponding to the \( \alpha \)- and \( \beta \)-terminal amino groups (18). Curve fitting was performed using a least squares program (RS/1, Bolt, Beranek, and Newman, Cambridge, MA).

RESULTS

Whole Blood Oxygen Equilibria

Under standard conditions, PCO\(_2\) 40 torr, the oxygenation curve of fresh whole blood containing hemoglobin C is shifted to the right relative to that of blood containing hemoglobin A. The oxygenation parameters measured under a variety of conditions are shown in Table II (19). The difference in \( O_2 \) affinity between cells containing hemoglobin A and C is apparent over the entire physiologic \( p\)H range when the PCO\(_2\) is 40 torr (Fig. 1). Note that the cells containing hemoglobin A have a stronger Bohr effect than the cells containing...
Gas Exchange by Goat Hemoglobins

hemoglobin C. It is also of interest that as the OEC shifts to the right (lower affinity), the value of \( a_t \) tends toward negative values, particularly in hemoglobin A-containing cells. We have previously suggested that this tendency can have no physical meaning, since a negative equilibrium constant is clearly impossible (12).

Oxygen Equilibria of Hemoglobin Fractions

The \( O_2 \) binding properties of the purified hemoglobin fractions were measured over a wide range of conditions. The \( P_{o_2} \) values were indistinguishable when 0.05 M bis-tris, 0.1 M NaCl was used and the temperature was 30 °C (Fig. 2). This surprising result, if it is to be reconciled with the whole blood experiments shown in Fig. 1, implies that some allosteric effector must operate within the cell to lower the oxygen affinity of hemoglobin C with respect to hemoglobin A.

CO2 Effect on Oxygen Affinity

Oxygen binding curves were measured using purified hemoglobin fractions after a DEAE-Sephadex separation. In these experiments, PCO\(_2\) was either 0 or 40 torr and the pH was titrated to the desired value by the addition of NaHCO\(_3\). The total ionic strength was held constant by the addition of NaCl. Addition of CO\(_2\) had a more pronounced effect on the hemoglobin C curve than it did on the position of the hemoglobin A curve. Moreover, the shape of the two curves is different as illustrated by the values of Hill’s parameter, \( n_{max} \) (Table III).

NaCl Effect

To evaluate the possibility that the right shift of the hemoglobin oxygenation curve seen with hemoglobin C may be a nonspecific ionic effect (or the effect of Cl\(^-\) binding), a series of experiments was carried out varying the NaCl concentration but with 0.05 M bis-tris buffer (Fig. 3). The two hemoglobins behaved the same in response to changing NaCl concentration, suggesting that the CO\(_2\) observations were not simply a manifestation of differing salt effects.

Analysis of Hbs A and C Mixture

Fig. 4 is a densitometric scan of the low temperature separation of a 1:1 mixture of Hbs A and C. It is clear that a large part of the material is the hybrid molecule. The actual quantitation showed about 43% hybrid and about 28% each of Hbs A and C. Since this is close to the expected binomial distribution and since the accuracy of the integration procedure cannot distinguish it from the ideal binomial distribution, 50% hybrid and 25% each of Hbs A and C were used in the subsequent calculations.

<table>
<thead>
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<th>Abbreviations are as in Table II.</th>
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<td></td>
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<tr>
<td>pH</td>
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<tr>
<td>P(_{O_2}), torr</td>
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<tr>
<td>( n_{max} )</td>
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<tr>
<td>( P_a ), torr</td>
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<tr>
<td>( a_t ) ((x10^{-4}))</td>
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<td>( a_t ) ((x10^{-5}))</td>
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<td>( a_t ) ((x10^{-6}))</td>
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<td>( a_t ) ((x10^{-7}))</td>
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Fig. 1. H\(^+\) Bohr effect, fresh whole goat blood. All \( P_{o_2} \) values were determined at PCO\(_2\) = 40 torr, 37 °C. The slopes of the log \( P_{o_2} \) versus pH lines are -0.327 for hemoglobin A and -0.205 for hemoglobin C blood.

Fig. 2. The H\(^+\) Bohr effect of purified hemoglobins A (open circles) and C (closed circles). Experiments were performed in the absence of CO\(_2\), 30 °C, 0.05 M bis-tris, 0.1 M NaCl.

Fig. 3. The effect of NaCl concentration on the \( P_{o_2} \) of purified fractions of hemoglobin A (open circles) and C (closed circles). No difference in the salt effect was observed. Temperature 30 °C, 0.05 M bis-tris buffer, pH 7.4.
Gas Exchange by Goat Hemoglobins

**CO₂ Binding**

The CO₂ binding data are given in Table IV, and their evaluation according to two models is shown in Table V. Reasoning by analogy with human hemoglobin A, we can assume there are four CO₂ binding sites/tetramer, two on each of the α and β subunits. Perrella et al. [20] showed that the β sites have slightly higher CO₂ affinity than the α sites (λ₁(CO₂) = 0.100, λ₂(CO₂) = 0.120) and that both sites have higher affinity in deoxy (λ₁(deoxy) = 0.190, λ₂(deoxy) = 0.579). This difference between the binding constants of the CO and deoxy forms is the basis for the allosteric effect of CO₂ on O₂ binding.

**Model 1**—We have no structural basis to assign different CO₂ affinities to various sites in the goat hemoglobins A and C or the mixed hybrid, AC. Therefore, model 1 is a first approximation to the analysis in which we determine single values of λ for each hemoglobin tetramer by least squares determinations. For these fits, Equation 4 is used for hemoglobins A and C. Using the distribution of hemoglobins in the 1:1 mixture (Fig. 4), the binding of CO₂ to the mixed hybrid hemoglobin AC is

\[ Z_{AC} = (0.25 Z_A + 0.25 Z_C) + 0.25 \frac{\lambda_{AC}[CO₂]}{1 + \lambda_{AC}[CO₂]} \]

The results of the evaluation (Table V, model 1) indicate that the fit for the hemoglobin C and AC data is not as close as the fit to the hemoglobin A data. Note the values for the sum of residuals in Table V.

**Model 2**—As a second approximation, we assume that, like human hemoglobin A, the CO₂ affinities will vary in the goat hemoglobins A and C or the mixed hybrid, AC. Therefore, model 2 is a first approximation to the analysis in which we determine single values of λ for each hemoglobin tetramer by least squares determinations. For these fits, Equation 4 is used for hemoglobins A and C. Using the distribution of hemoglobins in the 1:1 mixture (Fig. 4), the binding of CO₂ to the mixed hybrid hemoglobin AC is

\[ Z_{AC} = (0.25 Z_A + 0.25 Z_C) + 0.25 \frac{\lambda_{AC}[CO₂]}{1 + \lambda_{AC}[CO₂]} \]

The results of the evaluation (Table V, model 2) indicate that the fit for the hemoglobin C and AC data is not as close as the fit to the hemoglobin A data. Note the values for the sum of residuals in Table V.

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**Table IV**

CO₂ binding data

<table>
<thead>
<tr>
<th>PO₂ (torr)</th>
<th>[CO₂] (mM)</th>
<th>Z</th>
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<tbody>
<tr>
<td>20</td>
<td>0.884</td>
<td>0.085</td>
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<tr>
<td>40</td>
<td>1.585</td>
<td>0.130</td>
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<tr>
<td>5.6</td>
<td>1.583</td>
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<tr>
<td>74.1</td>
<td>2.283</td>
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<td>101.7</td>
<td>3.128</td>
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<td>128.5</td>
<td>3.874</td>
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<tr>
<td>238</td>
<td>2.294</td>
<td>0.333</td>
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<tr>
<td>101.7</td>
<td>3.152</td>
<td>0.386</td>
</tr>
<tr>
<td>115.5</td>
<td>3.649</td>
<td>0.414</td>
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**Table V**

Equilibrium constants for CO₂ binding

For explanation of the models, see text. n denotes the number of data points used for each fit, and the residuals are the sums of the differences between the observed and fit values. Units for λ are torr⁻¹.

<table>
<thead>
<tr>
<th>Model 1</th>
<th>CO₂</th>
<th>Deoxy</th>
<th>Hb A</th>
<th>n</th>
<th>Residuals</th>
<th>Hb C</th>
<th>n</th>
<th>Residuals</th>
<th>Hb AC</th>
<th>n</th>
<th>Residuals</th>
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<tr>
<td></td>
<td>λ₁</td>
<td>λ₂</td>
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<tr>
<td>Hb A</td>
<td>0.1122</td>
<td>0.1128</td>
<td>0.1052</td>
<td>0.3487</td>
<td></td>
<td></td>
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<td>n</td>
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<td></td>
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<tr>
<td>Residuals</td>
<td>-0.0084</td>
<td>0.0013</td>
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<tr>
<td>Hb C</td>
<td>0.0752</td>
<td>1.861</td>
<td>0.1662</td>
<td>4.287</td>
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<tr>
<td>Residuals</td>
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<td>0.0007</td>
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<tr>
<td>Hb AC</td>
<td>-0.0075</td>
<td>0.7226</td>
<td>0.0598</td>
<td>0.5911</td>
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Gas Exchange by Goat Hemoglobins

In CO₂ saturation, \( dZ \), is \( Z_{\text{deoxy}} - Z_{\text{CO}_2} \), where we assume CO₂ binds to oxyhemoglobin with the same affinity as it does to CO hemoglobin. The function \( dZ(C) \) is much greater than \( dZ(A) \) in the physiological \( PCO_2 \) range, 20–100 torr (Fig. 6). It is noteworthy that the maximal difference in CO₂ binding between \( \alpha \) and deoxyhemoglobin C is at a \( PCO_2 \) of about 20 torr. This value would correspond to a rather severe respiratory alkalosis as would be found, for example, at high altitudes or in severe anemia.

Our data do not permit us to assign specific CO₂ binding sites to the two hemoglobins, A and C. By analogy with human hemoglobin, we suppose there are four sites on each hemoglobin tetramer, two corresponding to the \( \alpha \) and two to the \( \beta \) subunits. In human hemoglobin, the \( \beta \) sites were found to have a higher CO₂ affinity than the \( \alpha \) sites (20). Thus, we tentatively assign two classes of binding sites to the goat hemoglobins also, the \( \beta \) sites having higher affinity than the \( \alpha \) sites.

We cannot suggest why the \( \beta \) subunits should have higher CO₂ affinity than the \( \alpha \) subunits. There is no reason, however, not to assume that the imino group of the NH₂-terminal proline (see Table I) cannot bind CO₂ as a carbamino group. Resolution of this issue and definitive assignments of the CO₂ binding sites would require extensive studies, including x-ray crystallographic analysis.

Table V shows that this model describes the data much more closely. In fact, the sums of the residuals for the various data sets show no regular pattern of difference, and the \( A, C, \) and AC fits are about equally good (Fig. 5). There is also no difference between the CO and deoxy data in terms of the ability of this model to describe CO₂ binding.

Note that the various \( \lambda_1 \) values are not very similar: 0.1122, 0.0752, and −0.0075. The latter value, of course, is impossible and should be regarded as 0. This finding suggests that the CO₂ affinity of the \( \alpha \) subunit may depend on which \( \beta \) subunit is present. Note also that model 2 predicts that the \( \beta \) subunit has very high affinity for CO₂ in deoxyhemoglobin C.

Other models could be devised and tested, but, as shown in Fig. 5, model 2 fits our data very well. Therefore, there is little value in trying additional hypotheses without further data.

**DISCUSSION**

The CO₂ binding results appear to explain the right shift of the oxygen equilibrium curves of hemoglobin C-containing red cells relative to that of hemoglobin A-containing red cells. The explanation lies in the difference in the CO₂ binding curves between the CO and deoxyhemoglobins. The difference

Fig. 5. CO₂ binding to CO (open circles) and deoxy (closed circles) derivatives of purified fractions of goat hemoglobin A. The solid lines are fits of the data to theoretical binding equations (see text).

Fig. 6. Difference in CO₂ binding (dZ) between CO and deoxy forms of hemoglobins A (single line) and C (double line). Note that in the physiological range of \( PCO_2 \) (20–100 torr) the effect of CO₂ is much greater on hemoglobin C.
except that the allosteric effector in this case is CO₂, not DPG.

It is tempting to speculate on the question of the physiological advantages of the various β chains. Observations on the efficiency of lamb production in sheep suggest that the β² gene may have some advantages but that later in life this may not be the case (21). Recent analysis of the structures of the globin genes indicates that the β² gene represents a relatively recent divergence from the β¹ gene (3). If its appearance is recent enough, perhaps there need not be an advantage if natural selection has had too little time to operate. Nevertheless, it is intriguing that β² appears as an “adolescent” globin gene product and appears only in conditions of anemia or hypoxia.

Acknowledgment—We wish to thank Vincenzo Russo for technical assistance.

REFERENCES