Oxygen-linked CO$_2$ Binding to Isolated $\beta$ Subunits of Human Hemoglobin

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CHRISTIAN BAUER AND ARMIN KURTZ

From the Physiologisches Institut der Universität Regensburg, 8400 Regensburg, Germany

The binding of CO$_2$ to the terminal $\alpha$-amino groups of hemoglobin in the form of carboximino compounds is linked to the binding of oxygen in such a way that there is less carbamate being formed in oxygenated than in deoxygenated hemoglobin. This fraction of oxygen-linked carbamate is linked to the binding of oxygen in such a way that there is less carbamate being formed in oxygenated than in deoxygenated hemoglobin. This fraction of oxygen-linked carbamate is linked to the binding of oxygen in such a way that there is less carbamate being formed in oxygenated than in deoxygenated hemoglobin.

In view of these results we considered it worthwhile to examine the effect of CO$_2$ on the oxygen affinity of isolated $\alpha$ and $\beta$ subunits of Hb A in order to find out if, indeed, the oxygen-linked CO$_2$ binding is different in the two types of subunits.

EXPERIMENTAL PROCEDURES

Preparation of Hemoglobin Derivatives—Hb A was purified from hemolysates of fresh human blood by chromatography on DEAE-Sephadex (Pharmacia, Uppsala) using either the procedure of Williams and Tsay (10) or that of Dozy et al. (11). The Hb A thus obtained was completely homogeneous on isoelectric focusing electrophoresis and contained no DPG (12) and less than 1% methemoglobin (13). The isolated $\alpha$ and $\beta$ subunits were prepared by incubating carbon monoxide hemoglobin with sodium $p$-chloromercuribenzoate (PMB) (Merck-Schuchhardt, Hohenbrunn), followed by chromatography on Whatman CM52 (14). The $\alpha$PMB and $\beta$PMB subunits were demercurated by using 2-mercaptoethanol (Merck, Darmstadt) on a Sephadex G-25 column as described by Tyuma et al. for $\alpha$- and $\beta$-subunits (15). With this procedure we obtained $\alpha$PMB and $\beta$PMB subunits with a 5-fold excess of dithiothreitol (Calbiochem, Luzern) for 12 h (8) resulted in a preparation with 2.01 (S.E. ± 0.03) free $-SH$ groups. As a last step both $\alpha$ and $\beta$ subunits were equilibrated with 0.1 m NaCl on a Sephadex G-25 column. Hemoglobin with pyridoxal phosphate (PLP) attached to the NH$_2$-terminal amino groups of the $\beta$ subunits was prepared by incubating deoxygenated Hb A with a 2-fold excess of PLP (Calbiochem, Luzern) and chromatographic separation on Whatman P11 as described by Benesch et al. (9). Separation and demercuration of the PLP-reacted $\beta$ subunits ($\beta$PLP) was done in the same way as with the unmodified $\beta$ subunits. In order to establish if the $\beta$PLP subunits assemble to form tetramers, 50 mg of the protein in 1.5 ml of bis-Tris buffer were subjected to gel chromatography on a Sephadex G-100 column (2 x 90 cm) operated at a flow rate of 20 ml/h. The result was compared with the elution behavior of other isolated subunits and Hb A using dextran 2000 for the determination of the void volume ($V_v$). From $V_v$ and the peak elution volume, the apparent molecular weight of the various pigments was calculated using the relationship given by Petermann.

The binding of CO$_2$ to the terminal $\alpha$-amino groups of hemoglobin is in the form of carboximino compounds is linked to the binding of oxygen in such a way that there is less carbamate being formed in oxygenated than in deoxygenated hemoglobin. This fraction of oxygen-linked carbamate is not equally distributed between the $\alpha$ and $\beta$ subunits of hemoglobin but rather 70-80% of the oxygen-linked carbamate is confined to the $\beta$ subunits at physiological pH (1-3). In view of the fact that isolated $\beta$ subunits with 1.02 (S.E. ± 0.02) and 1.80 (S.E. ± 0.04) free $-SH$ groups, respectively, upon titration with PMB (16, 17). Incubation of the $\beta$ subunits with a 5-fold excess of dithiothreitol (Calbiochem, Luzern) for 12 h (8) resulted in a preparation with 2.01 (S.E. ± 0.03) free $-SH$ groups. As a last step both $\alpha$ and $\beta$ subunits were equilibrated with 0.1 m NaCl on a Sephadex G-25 column. Hemoglobin with pyridoxal phosphate (PLP) attached to the NH$_2$-terminal amino groups of the $\beta$ subunits was prepared by incubating deoxygenated Hb A with a 2-fold excess of PLP (Calbiochem, Luzern) and chromatographic separation on Whatman P11 as described by Benesch et al. (9). Separation and demercuration of the PLP-reacted $\beta$ subunits ($\beta$PLP) was done in the same way as with the unmodified $\beta$ subunits. In order to establish if the $\beta$PLP subunits assemble to form tetramers, 50 mg of the protein in 1.5 ml of bis-Tris buffer were subjected to gel chromatography on a Sephadex G-100 column (2 x 90 cm) operated at a flow rate of 20 ml/h. The result was compared with the elution behavior of other isolated subunits and Hb A using dextran 2000 for the determination of the void volume ($V_v$). From $V_v$ and the peak elution volume, the apparent molecular weight of the various pigments was calculated using the relationship given by Petermann.
and Michel (18). All chromatographic steps were done at 4°C and solutions of the isolated components concentrated by ultrafiltration with carbon monoxide using Amicon UM10 membranes. Between all phases of the investigation, tetrameric hemoglobin as well as isolated subunits were kept in liquid nitrogen which leaves ligand-binding properties and the number of regenerated -SH groups completely unaltered. Myoglobin was a gift from Dr. Schwarzmann, Regensburg, who prepared it from ox hearts (18).

Estimation of Oxygen-linked Carbamate—Oxygen equilibrium curves of the various hemoglobin derivatives in the absence and presence of CO₂ were determined spectrophotometrically at 20°C in a tonometer attached to a 10-mm cuvette. Measured volumes of CO₂ were injected via a rubber sealed side arm to achieve the desired pCO₂, (1). From oxygen-binding curves obtained at a variety of pCO₂ and pH values, the fraction of oxygen-linked carbamate was calculated on the basis of Wyman’s linked function theory (20);

\[
\log \frac{\text{pC₅O₂}}{\text{pC₇₅O₂}} = \frac{\log \text{pC₅O₂}}{\log \text{pC₇₅O₂}}
\]

After suitable rearrangement this equation can be transformed into:

\[
-\Delta \text{HbCO₂/ΔHbO₂} = \frac{\log \text{pC₅O₂}}{\log \text{pC₇₅O₂}}
\]

-ΔHbCO₂/ΔHbO₂ is the fraction of oxygen-linked carbamate, i.e., the number of moles of CO₂ which are liberated per hemoglobin subunit upon oxygen binding and pCO₂ is the oxygen pressure necessary to attain half-saturation of the hemoglobin with oxygen. Thus, a plot of log pCO₂ against log pO₂ at various pH values yields a family of curves where the slope of the tangent at a given pH and pCO₂, i.e., the first derivative of the function, yields -ΔHbCO₂/ΔHbO₂ in a very convenient way.

Composition of Solutions and Buffers—Oxygen-binding curves in the absence of CO₂ as well as gel chromatography was done in 0.05 M bis-Tris buffer (Sigma, München) at pH 7.3, at 20°C and a total [Cl⁻] of 0.15. In both cases 0.1 mM EDTA (Serva, Heidelberg) was added. The concentration of hemoglobin was 120 μM (monomer) in all the ligand-binding experiments.

RESULTS

Effect of CO₂ on pCO₂ of Isolated Subunits—The effect of CO₂ on pCO₂ of β²H and β²Ⅲ, is shown in Fig. 1. It can be seen that there is a clear-cut decrease in pCO₂ of the β²H subunits with increasing pCO₂ at pH 7.3. Similar results were obtained at pH 7.5 and pH 7.75 (Fig. 3). In β²Ⅲ, on the other hand, where the terminal α-amino group is blocked, pCO₂ did not change upon addition of CO₂. Neither α²H nor β²Ⅲ or myoglobin had a different pCO₂ in the absence and presence of CO₂ (Fig. 2) which indicates that oxygen-linked formation of carbamate is negligible in these derivatives.

Note that the oxygen affinity of β²H and β²Ⅲ, in the absence of CO₂ are alike but that α²H has a significantly lower oxygen affinity than β²H. The pCO₂ values shown in Figs. 1 and 2 for α²H and β²H are in excellent agreement with those obtained by Riggs and Gibson (4) for α²H and β²H and also with the ones measured by Tynmaa et al. (15), when their figures are corrected from 30–20°C using a ΔH value of -13.5 kcal/mol as determined for isolated subunits (21). Similarly, the lack of effect of PLa with only one phosphate group on the oxygen affinity of β²H is in keeping with the observation of Benesch et al. (22) that DPG with two phosphate groups has no influence on the pCO₂ of β²H. It should be mentioned at this point that the Hill constant n was unity in all isolated subunits both in the absence and presence of CO₂.

In order to test the ability of the isolated subunits to form normally functioning heterotetramers, oxygen-binding curves were done on recombined products. α²H and β²H formed a pigment with a pCO₂ of 3.9 mm Hg and a Hill constant n of 2.9 (at 20°C and pH 7.3) which are exactly the figures found in Hb A under identical conditions. Likewise, the response of the recombination product toward CO₂ was indistinguishable from that of Hb A (Fig. 3). Combination of α²H and β²Ⅲ, yielded a product with a pCO₂ of 10 mm Hg and a Hill constant n of 2.5 at 20°C and pH 7.3. These figures correspond very well to the ones measured by Suzuki et al. (23) for the native derivative αβPⅢ,

Oxygen-linked Carbamate in Hb A and β²H

Fig. 3 shows a plot of log pCO₂ against log pO₂ for Hb A (upper panel) and β²H (lower panel) at various pH values. From the slopes of the tangents corresponding to pCO₂ 20, 40, and 60 mm Hg we estimated the fraction of oxygen-linked carbamate (ΔHbCO₂/ΔHbO₂). Fig. 4 shows a plot of -ΔHbCO₂/ΔHbO₂ against pH for Hb A and β²H against pH at three pCO₂ values. It can be seen that at pH 7.3 and at a pCO₂ of 20 and 40 mm Hg -ΔHbCO₂/ΔHbO₂ is about the same for Hb A and β²H but that there is little further increase in -ΔHbCO₂/ΔHbO₂ in the case of β²H with increasing pH, contrary to what is observed with

\[
-\Delta HbCO₂/\Delta HbO₂ = \frac{\log \text{pC₅O₂}}{\log \text{pC₇₅O₂}}
\]
Hb A. Also, the effect of increasing pCO₂ at constant pH on -ΔHbCO₂/ΔHbO₂ is much less pronounced in β''' than in Hb A, particularly at more alkaline pH values.

The experimental points obtained with Hb A can be compared with the fraction of oxygen-linked carbamate being calculable from the constants K; and K₂ published by Garber et al. (24) and Morrow et al. (3) for α and β subunits in liganded and unliganded Hb A. K is the ionization constant of the protonated terminal α-amino groups and K₂, the equilibrium constant of the reaction of CO₂ and the unprotonated NH₂ groups. Knowing K; and K₂, -ΔHbCO₂/ΔHbO₂, can be computed from the relationship derived by Rossi-Bernardi and Roughton (25):

\[ f = \frac{K; \cdot K₂ \cdot [CO₂]}{K; \cdot K₂ \cdot [CO₂] + K; \cdot [H⁺] + [H⁺]^2} \]  

where f is the fraction of α amino groups which have combined with CO₂ to form carbamate either in deoxy- or in oxyhemoglobin. K₂ was corrected from 26-20°C using ΔH = 14.5 kcal/mol (26, 27) and K; from 30-20°C using ΔH = -3.2 kcal/mol (27). [CO₂] was calculated using a solubility coefficient of 0.861 ml of CO₂ ml⁻¹ atm⁻¹ (28). It turned out (Fig. 3) that the agreement between -ΔHbCO₂/ΔHbO₂ obtained from the effect of CO₂ on pCO₂ and from NMR results using ¹³CO₂ which yields the carbamate equilibrium constant (3) is satisfactory.

Molecular Weight Estimation – For β''''' and β'''''' we estimated an apparent molecular weight of 42,200 and 42,500, respectively, which is very similar to that found for Hb A under identical conditions (42,400). α'''' subunits on the other hand, are monomers under the present experimental conditions (Mₐ = 15,700) which is in agreement with their sedimentation behavior (29). The molecular weight of Hb A but not of α''''' is only about 70% of the value which can be calculated from the amino acid sequence of α and β subunits. Such an anomalously low molecular weight of tetrameric hemoglobin as obtained from molecular sieve experiments on Sephadex G-100 was also observed by other investigators (30-32). This phenomenon may be related to the finding that the elution volume of tetrameric hemoglobin is not proportional to its molecular weight but rather to the molecular Stokes radius (31) or due to reversible dissociation of the molecule in dimers (33). However, as Hb A, β''''' and β'''''' all elute in very similar volumes it can safely be assumed that β''''' has a tetrameric structure as has β'''''' (29). β'''''' subunits did not elute as a homogeneous fraction so that it was not possible to estimate their molecular weight. However, about 85% of β'''''' eluted in the same volume as the α'''' subunits, being preceded by a fraction of a higher apparent molecular weight. It can be concluded therefore that β'''''' exists largely as monomers under the present experimental conditions which, again, is in agreement with their sedimentation behavior (29).

**DISCUSSION**

It becomes clear from the present data that the oxygen affinity of β subunits with free —SH groups decreases upon addition of CO₂ which in turn implies that there must be a difference in carbamate formation between the liganded and the unliganded molecular structure of β''''' This liganded formation of carbamate in β''''' requires both the availability of a free terminal α-amino group and a tetrameric molecular structure. This can be concluded from the lack of effect of CO₂ on pCO₂ in β'''''' which is a tetramer but has a blocked terminal α-amino group α'''''' β'''''' and myoglobin on the other hand, have free terminal amino groups but are known to exist wholly or to large extent as monomers. In spite of some similarities between β''''' and Hb A with respect to the ligand-linked binding of CO₂, there are large quantitative differences. This can be seen from a comparison of the fraction of oxygen-linked carbamate being formed at the β subunits of Hb A (Fig. 5) and at the isolated homotetramer consisting of β subunits (Fig. 4). In the range between pH 7.3 and 7.7 the fraction of oxygen-linked carbamate at the β subunits of Hb A is about 2 to 3 times higher than that formed at the terminal α-amino groups of β'''''' the differences being larger at more alkaline pH. In order to get an idea which set of constants for K; and K₂ would describe the behavior of oxygen-linked carba-
In a preliminary x-ray analysis of hemoglobin H in βIII, Perutz and Mazzarello (35) have observed "small alterations in the conformation of the isolated subunits, which are of course not drastic as in Hb A, but sufficient to produce a reciprocal action between heme ligand and some allosteric effectors. While a number of these heterotropic interactions in isolated subunits do apparently not require a tetrameric molecular structure, it is a prerequisite for the binding of oxygen-linked carbamate to the tetrameric βIII. Homotropic interactions, however, i.e. the cooperative binding of oxygen, are not possible without having unlike subunits in a hemoglobin molecule. If it is true, that a tetramer consisting of β-like subunits preceded the heterotetramers of the type αββ in the evolution of hemoglobin (37), it follows that heterotropic interactions were present before cooperative oxygen binding fully evolved.

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REFERENCES