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Oxygen-linked CO_2 Binding to Isolated β Subunits of Human Hemoglobin

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CHRISTIAN BAUER AND ARMIN KURTZ

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

It is known that most of the oxygen-linked carbamate which is formed in normal adult human hemoglobin (Hb A) is confined to the β subunits rather than to the α subunits. In order to find out if similar differences exist in the isolated protomers of Hb A we have measured the effect of various pressures of carbon dioxide (p_{CO_2}) on the oxygen affinity in the following heme pigments: isolated α and β subunits with free —SH groups (α^{SH}, β^{SH}), mercurated β subunits (β^{PMB}), myoglobin (Mb), and β^{SH}_{PLP} in which the terminal α-amino group of β^{SH} was irreversibly blocked with pyridoxal phosphate (PLP). Similar measurements were done on Hb A and the fraction of oxygen-linked carbamate calculated from the effect of p_{CO_2} (at constant pH) on the oxygen half-saturation pressure (p_{50}). A distinct influence of CO_2 on p_{50} was observed in β^{SH} which was absent in β^{SH}_{PLP} and thus indicates that the terminal α-amino group mediates the oxygen-linked binding of CO_2 in β^{SH} as it does in the β subunits of Hb A. However, the fraction of oxygen-linked carbamate was much less dependent on pH and p_{CO_2} in β^{SH} than in Hb A. Neither α^{SH}, β^{PMB}, or Mb, all of which are known to exist largely or wholly as monomers but have free terminal α-amino groups, showed a shift of p_{50} upon addition of CO_2. As both β^{SH} and β^{SH}_{PLP} were shown to be tetrameric molecules, we conclude from this study that homotetramers composed of isolated β subunits do exhibit a reciprocal interaction between the binding of O_2 and CO_2.

The binding of CO_2 to the terminal α-amino groups of hemoglobin in the form of carbamino 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 α and β subunits of hemoglobin but rather 70 to 80% of the oxygen-linked carbamate is confined to the β subunits at physiological pH (1-3). In view of the fact that isolated β subunits of some abnormal hemoglobins with either a low oxygen affinity (Hb Kansas: β102 Asp → Thr) or a high oxygen affinity (Hb Abruzzo: β143 His → Arg) show qualitatively the same functional changes as the whole molecule (4, 5), it seems possible that the unequal distribution of oxygen-linked carbamate is also retained in the isolated subunits from normal adult hemoglobin (Hb A). However, this reasoning appears to be inconsistent with previous evidence, indicating that subunits isolated from normal Hb A are devoid of heterotropic interactions, *i.e.* do not show significant changes in oxygen affinity upon addition of 2,3-diphosphoglycerate or with changes in pH (6, 7). Nevertheless, recent experiments have shown that there is an interaction between the release of hydrogen ions and the binding of oxygen in isolated α and β subunits (8) and that furthermore inositol hexaphosphate decreases the oxygen affinity of non-α subunits from adult and fetal hemoglobin and hemoglobin Abruzzo (5). In view of these results we considered it worthwhile to examine the effect of CO_2 on the oxygen affinity of isolated α and β 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[1] (12) and less than 1% methemoglobin (13). The isolated α and β subunits were prepared by incubating carbon monoxide hemoglobin with sodium *p*-chloromercuribenzoate (PMB) (Merck-Schuchhardt, Hohenbrunn), followed by chromatography on Whatman CM52 (14). The α^{PMB} and β^{PMB} subunits were demercurated by using 2-mercaptoethanol (Merck, Darmstadt) on a Sephadex G-25 column as described by Tyuma *et al.* for β^{PMB} subunits (15). With this procedure we obtained α^{SH} and β^{SH} 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 β 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 α and β 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 β 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 β subunits (β^{SH}_{PLP}) was done in the same way as with the unmodified β subunits. In order to establish if the β^{SH}_{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 × 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_0). From V_0 and the peak elution volume, V_e, the apparent molecular weight of the various pigments was calculated using the relationship given by Determann

[1] The abbreviations used are: DPG, 2,3-diphosphoglycerate; bis-Tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitriloethanol; IHP, inositol hexaphosphate; PMB, *p*-chloromercuribenzoate; α^{SH} and β^{SH}, isolated α and β subunits with free —SH groups; β^{PMB}, mercurated β subunits; β^{SH}_{PLP}, terminal α-amino group of β^{SH} irreversibly blocked with pyridoxal phosphate.

and Michel (18). All chromatographic steps were done at 4° 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 (19).

Estimation of Oxygen-linked Carbamate – Oxygen equilibrium curves of the various hemoglobin derivatives in the absence and presence of CO_2 were determined spectrophotometrically at 20° in a tonometer attached to a 10-mm cuvette. Measured volumes of CO_2 were injected via a rubber sealed side arm to achieve the desired p_{CO_2} (1). From oxygen-binding curves obtained at a variety of p_{CO_2} and pH values, the fraction of oxygen-linked carbamate was calculated on the basis of Wyman's linked function theory (20);

$$(\delta HbO_2/\delta \log p_{CO_2})_{pH,p_{O_2}} = (\delta HbCO_2/\delta \log p_{O_2})_{pH,p_{CO_2}} \quad (1)$$

After suitable rearrangement this equation can be transformed into:

$$-\Delta HbCO_2/\Delta HbO_2 = (\Delta \log p_{50}/\Delta \log p_{CO_2})_{pH} \quad (2)$$

$-\Delta HbCO_2/\Delta HbO_2$ is the fraction of oxygen-linked carbamate, *i.e.* the number of moles of CO_2 which are liberated per hemoglobin subunit upon oxygen binding and p_{50} is the oxygen pressure necessary to attain half-saturation of the hemoglobin with oxygen. Thus, a plot of $\log p_{50}$ against $\log p_{CO_2}$ at various pH values yields a family of curves where the slope of the tangent at a given pH and p_{CO_2}, *i.e.* the first derivative of the function, yields $-\Delta HbCO_2/\Delta HbO_2$ in a very convenient way.

Composition of Solutions and Buffers – Oxygen-binding curves in the absence of CO_2 as well as gel chromatography was done in 0.05 M bis-Tris buffer (Sigma, München) at pH 7.3, at 20° and a total $[Cl^-]$ of 0.15 M. In the presence of CO_2, the desired pH was obtained by varying the concentration of $NaHCO_3$ and NaCl, keeping the ionic strength at 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 CO_2 on p_{50} of Isolated Subunits – The effect of CO_2 on p_{50} of β^{SH} and β^{SH}_{PLP}, is shown in Fig. 1. It can be seen that there is a clear-cut decrease in p_{50} of the β^{SH} subunits with increasing p_{CO_2} at pH 7.3. Similar results were obtained at pH 7.5 and pH 7.75 (Fig. 3). In β^{SH}_{PLP}, on the other hand, where the terminal α-amino group is blocked, p_{50} did not change upon addition of CO_2. Neither α^{SH} nor β^{PMB} or myoglobin had a different p_{50} in the absence and presence of CO_2 (Fig. 2) which indicates that oxygen-linked formation of carbamate is negligible in these derivatives.

Note that the oxygen affinity of β^{SH} and β^{SH}_{PLP} in the absence of CO_2 are alike but that α^{SH} has a significantly lower oxygen affinity than β^{SH}. The p_{50} values shown in Figs. 1 and 2 for α^{SH} and β^{SH} are in excellent agreement with those obtained by Riggs and Gibson (4) for α^{SH} and β^{SH} and also with the ones measured by Tyuma *et al.* (15), when their figures are corrected from 30-20° using a ΔH value of −13.5 kcal/mol as determined for isolated subunits (21). Similarly, the lack of effect of PLP with only one phosphate group on the oxygen affinity of β^{SH} is in keeping with the observation of Benesch *et al.* (22) that DPG with two phosphate groups has no influence on the p_{50} of β^{SH}. 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 CO_2.

In order to test the ability of the isolated subunits to form normally functioning heterotetramers, oxygen-binding curves were done on recombined products. α^{SH} and β^{SH} formed a pigment with a p_{50} of 3.9 mm Hg and a Hill constant n of 2.9 (at 20° and pH 7.3) which are exactly the figures found in Hb A under identical conditions. Likewise, the response of the recombination product toward CO_2 was indistinguishable from

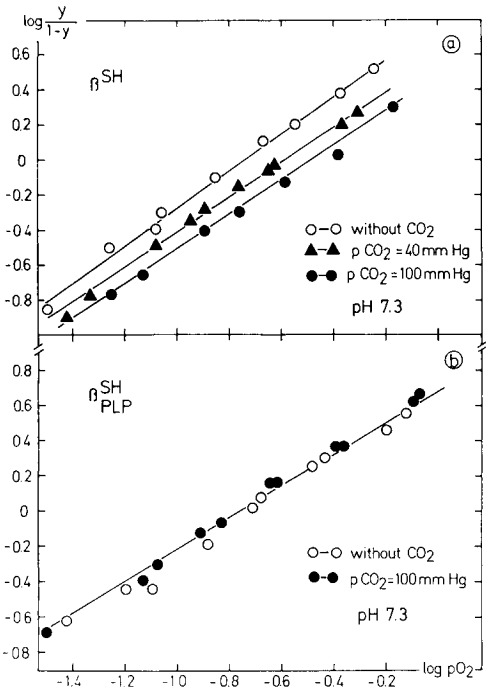

FIG. 1. Hill plot for β^{SH} and β^{SH}_{PLP} in the absence and presence of CO_2. Y is the saturation of hemoglobin with oxygen, p_{CO_2} is the partial pressure of CO_2. Temperature was 20° and pH 7.3.

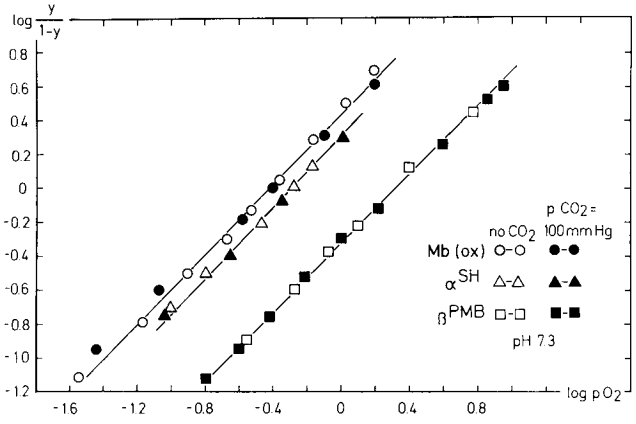

FIG. 2. Hill plot of α^{SH}, β^{SH}, and myoglobin (*Mb*) in the absence and presence of CO_2. Y is the saturation of hemoglobin with oxygen. *Open symbols*, no CO_2 added; *closed symbols*, p_{CO_2} = 100 mm Hg. pH was 7.3 and temperature 20°.

that of Hb A (Fig. 3). Combination of α^{SH} and β^{SH}_{PLP} yielded a product with a p_{50} of 10 mm Hg and a Hill constant n of 2.6 at 20° and pH 7.3. These figures correspond very well to the ones measured by Suzuki *et al.* (23) for the native derivative $\alpha_2\beta_2^{PLP}$.

Oxygen-linked Carbamate in Hb A and β^{SH} – Fig. 3 shows a plot of $\log p_{50}$ against $\log p_{CO_2}$ for Hb A (*upper panel*) and β^{SH} (lower panel) at various pH values. From the slopes of the tangents corresponding to p_{CO_2} 20, 40, and 60 mm Hg we estimated the fraction of oxygen-linked carbamate ($-\Delta HbCO_2/\Delta HbO_2$). Fig. 4 shows a plot of $-\Delta HbCO_2/\Delta HbO_2$ against pH for Hb A and β^{SH} against pH at three p_{CO_2} values. It can be seen that at pH 7.3 and at a p_{CO_2} of 20 and 40 mm Hg $-\Delta HbCO_2/\Delta HbO_2$ is about the same for Hb A and β^{SH} but that there is little further increase in $-\Delta HbCO_2/\Delta HbO_2$ in the case of β^{SH} with increasing pH, contrary to what is observed with

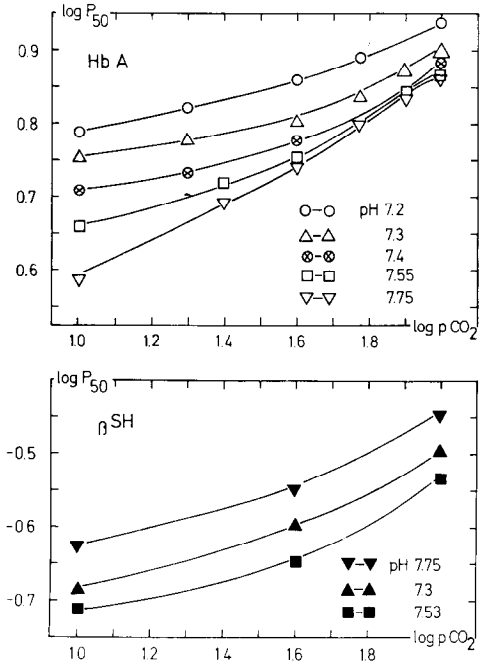

FIG. 3. Plot of log p_{50} against log p_{CO_2} at various pH values for Hb A (*upper panel*) and β^{SH} (*lower panel*). The values obtained at pH 7.4 for Hb A (⊗) were obtained from an equimolar mixture of α^{SH} and β^{SH}. Temperature was 20°.

Hb A. Also, the effect of increasing p_{CO_2} at constant pH on $-\Delta HbCO_2/\Delta HbO_2$ is much less pronounced in β^{SH} 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_z and K_c published by Garner *et al.* (24) and Morrow *et al.* (3) for α and β subunits in liganded and unliganded Hb A. K_z is the ionization constant of the protonated terminal α-amino groups and K_c the equilibrium constant of the reaction of CO_2 and the unprotonated NH_2 groups. Knowing K_z and K_c, $-\Delta HbCO_2/\Delta HbO_2$, can be computed from the relationship derived by Rossi-Bernardi and Roughton (25):

$$f = \frac{K_c \cdot K_z \cdot [CO_2]}{K_c \cdot K_z \cdot [CO_2] + K_z \cdot [H^+] + [H^+]^2} \quad (3)$$

where f is the fraction of α amino groups which have combined with CO_2 to form carbamate either in deoxy- or in oxyhemoglobin. K_z was corrected from 26–20° using ΔH = 14.5 kcal/mol (26, 27) and K_c from 30–20° using ΔH = −3.2 kcal/mol (27). $[CO_2]$ was calculated using a solubility coefficient of 0.861 ml of CO_2 ml^{-1} atm^{-1} (28). It turned out (Fig. 3) that the agreement between $-\Delta HbCo_2/\Delta HbO_2$ obtained from the effect of CO_2 on p_{50} and from NMR results using $^{13}CO_2$ which yields the carbamate equilibrium constant (3) is satisfactory.

Molecular Weight Estimation—For β^{SH}_{PLP} and β^{SH} 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^{SH} subunits on the other hand, are monomers under the present experimental conditions (M_r = 15,700) which is in agreement with their sedimentation behavior (29). The molecular weight of Hb A but not of α^{SH}, 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

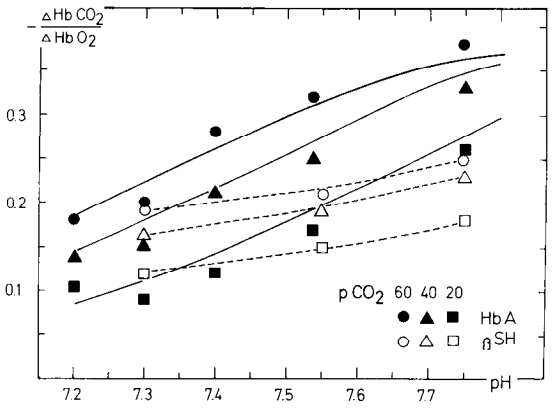

FIG. 4. Plot of the fraction of oxygen-linked carbamate ($-\Delta HbCO_2/\Delta HbO_2$) against pH for Hb A (*closed symbols*) and β^{SH} (*open symbols*) at various pressures of CO_2. *Circles*, p_{CO_2} = 60 mm Hg; *triangles*, p_{CO_2} = 40 mm Hg; *squares*, p_{CO_2} = 20 mm Hg. Experimental points were obtained from the plot of log p_{50} against log p_{CO_2} (Fig. 3). The *solid lines* were calculated using published constants (3, 24) as described in the text. The *dotted lines* were drawn by inspection. Temperature 20°.

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^{SH} and β^{SH}_{PLP} all elute in very similar volumes it can safely be assumed that β^{SH}_{PLP} has a tetrameric structure as has β^{SH} (29). β^{PMB} subunits did not elute as a homogeneous fraction so that it was not possible to estimate their molecular weight. However, about 85% of β^{PMB} eluted in the same volume as the α^{SH} subunits, being preceded by a fraction of a higher apparent molecular weight. It can be concluded therefore that β^{PMB} 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 CO_2 which in turn implies that there must be a difference in carbamate formation between the liganded and the unliganded molecular structure of β^{SH}. This ligand-linked formation of carbamate in β^{SH} 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 CO_2 on p_{50} in β^{SH}_{PLP} which is a tetramer but has a blocked terminal α-amino group. α^{SH}, β^{PMB}, 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^{SH} and Hb A with respect to the ligand-linked binding of CO_2, 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^{SH}, the differences being larger at more alkaline pH. In order to get an idea which set of constants for K_c and K_z would describe the behavior of oxygen-linked carba-

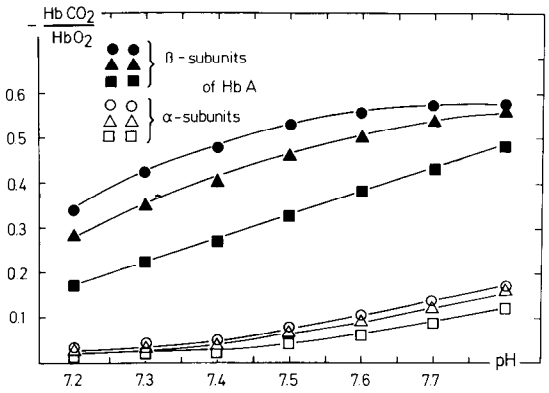

FIG. 5. Plot of the fraction of oxygen-linked carbamate. $(-\Delta HbCO_2/\Delta HbO_2)$ for the α and β subunits within Hb A. *Open* and *closed symbols* stand for α and β subunits, respectively. Values of $-\Delta HbCO_2/\Delta HbO_2$ were calculated for p_{CO_2} = 60 mm Hg (●, ○), p_{CO_2} = 40 mm Hg (▲, △), and 20 mm Hg (■, □) from published constants (3, 24) as described in the text. Temperature 20°.

mate in β^{SH} we have used Equation 3 in the following way to get estimates on these quantities: $-\Delta HbCO_2/\Delta HbO_2 = f$ (deoxy-Hb) $- f$ (oxy-Hb). As K_c and K_z for the deoxygenated and oxygenated form of β^{SH} are unknown, one has to have four equations in which p_{CO_2}, pH, and $-\Delta HbCO_2/\Delta HbO_2$ are the known variables. Such a set of equations can then be solved for K_c and K_z for deoxygenated and oxygenated β^{SH}. We have taken $-\Delta HbCO_2/\Delta HbO_2$ at a number of pH and p_{CO_2} values from Fig. 4 and obtained estimates for K_z and K_c. It turned out that with the following pK values the oxygen-linked carbamate reaction can be described within the limits of experimental error: $pK_z = 7.0$ for both the liganded and unliganded form of β^{SH}; $pK_c = 4.83$ for deoxygenated β^{SH}; and $pK_c = 5.25$ for oxygenated β^{SH}. It thus appears as if only the negatively charged carbamate groups, but not the terminal α-amino groups, experience a different environment when going from the liganded to the unliganded form of β^{SH}. This is in contrast to Hb A, where the large conformational changes accompanying removal of ligand seem to fix sterically the terminal α-amino group at least of the α subunits (24). From crystallographic studies at 2.5 Å resolution it appears that the linkage between the NH_2 and COOH termini of opposite α subunits in human deoxyhemoglobin is mediated via a water molecule rather than by a salt bridge (34).

In a preliminary x-ray analysis of hemoglobin H (β^{SH}) Perutz and Mazzarella (35) have observed "small alterations in the intensities of certain reflexions" when reducing the heme iron in a crystal of β^{SH}. If these small changes have a bearing on the heterotropic interactions in isolated β subunits which are observed not only with CO_2 but also with IHP (5) and protons (8, 36) remains to be seen. In any event, it follows from the functional studies that there must be changes in the conformation of the isolated subunits which are of course not as 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 β^{SH}. 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 $\alpha_2\beta_2$ in the evolution of hemoglobin (37), it follows that heterotropic interactions were present before cooperative oxygen binding fully evolved.

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