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Direct observation of two distinct affinity conformations in the T state human deoxyhemoglobin

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Abstract The main features of cooperative oxygenation of human hemoglobin have been described by assuming the equilibrium between two affinity conformations of the entire molecule, T and R. However, the molecular basis for explaining the wide variation in the O_2 affinities of the deoxy T state has remained obscure. We address this long-standing issue by trapping the conformational states of deoxyhemoglobin molecules within wet porous transparent silicate sol-gels. The equilibrium O_2 binding measurements of the encapsulated deoxyhemoglobin samples showed that deoxyhemoglobin free of anions coexists in two conformations that differ in O_2 affinity by 40 times or more, and addition of inositol hexaphosphate to this anion-free deoxyhemoglobin brings about a very slow redistribution of these affinity conformations. These results are the first, direct demonstration of the existence of equilibrium between two (at least two) functionally distinguishable conformational states in the T state deoxyhemoglobin.

Key words: Hemoglobin; Allostery; Cooperative effect; Monod–Wyman–Changeux model; Sol-gel

1. Introduction

The Monod–Wyman–Changeux (MWC) two-state allosteric model [1] has made the most significant impact in our understanding of structure–function relationships in human hemoglobin and other allosteric proteins. The principal idea of MWC was that there is rapid equilibrium between two affinity conformations of the entire molecule, T and R. In this model cooperativity arises simply from the displacement of the conformational equilibrium. The crystal structures of fully unliganded and fully liganded forms of human adult hemoglobin are widely accepted to represent the structures of the T and R states, respectively [2]. With regard to the function of the two states, however, the following problem still exists. The oxygen equilibrium constant of the T state (K_T) varies depending on solution conditions, although that of the R state (K_R) remains essentially constant [3]. In fact, the K_T values of human adult hemoglobin vary with solution conditions by at least 40 times, corresponding to approximately one-half the entire affinity change of hemoglobin in terms of free energy [3]. Therefore, it would be inappropriate to claim that the variation in deoxy T state affinities may be simply explained as minor perturbations of the T state.

Perhaps the simplest way of explaining the wide range of the T state affinities is to assume an equilibrium between two distinct affinity conformations in the T state, as was done by several investigators [3–6], but without direct evidence. To test for the existence of such an equilibrium, we herein apply a recently developed method of trapping particular conformations of proteins by encapsulation in a wet transparent porous silicate sol-gel [7–9]. This gel matrix provides a means of slowing large-scale protein motions [8–11] while the encapsulated hemoglobin molecules are solvated and retain their properties in solution [7–11]. As a result, the protein conformations in solution can be effectively trapped. It has been shown that the wet sol-gel is capable of maintaining the affinity states of fully oxy- and deoxyhemoglobin molecules during the O_2 equilibrium measurements [7,12]. In more recent studies, we succeeded in trapping a mixture of the T and R states that are populated in doubly liganded hemoglobin molecules in 0.1 M potassium phosphate, pH 7.0, and we could obtain reasonable O_2 affinities of both allosteric states through analysis of the observed biphasic O_2 equilibrium curve [8]. Another key observation in our earlier work was that the deoxy T state-locked hemoglobin in the presence of 0.1 M potassium phosphate, pH 7.0, shows an O_2 equilibrium curve with a Hill coefficient of 0.86, suggesting a slight heterogeneity of the O_2 affinity [7]. However, the origin of this heterogeneity was not pursued further. In the present work, attempts have been made to trap the conformational states of deoxyhemoglobin at neutral pH under low-salt conditions, where the putative high-affinity T conformation would be significantly populated (where the deoxyhemoglobin exhibits a moderate O_2 affinity).

2. Materials and methods

2.1. Hemoglobin

Human adult hemoglobin was prepared as described previously [13]. The purified hemoglobin was deionized by passage through a column of Amberlite MB-3 for the experiments under low-salt conditions.

2.2. Sol-gel encapsulation

The silica sol was synthesized from tetramethylorthosilicate (Tokyo Kasei Company) according to the method of Ellerby et al. [14]. The sol was mixed with 1.5 volumes of 3.1% (w/v) deoxyhemoglobin in anion-free 50 mM *N*-[2-hydroxyethyl]piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), pH 7.2, under N_2 atmosphere in a rotating sealed

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Abbreviations: MWC, Monod–Wyman–Changeux; IHP, inositol hexaphosphate; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-(2-ethanesulfonic acid)

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glass tube (1 cm diameter) [7–9]. Gelation occurred within 4 min at 0°C. The resultant deoxyhemoglobin-doped thin film (i.e. 0.02–0.05 mm thickness), which adhered to the inner surface of the glass tube, was soaked in Ar-saturated 0.1 M HEPES with 2 mM sodium dithionite, pH 7.0, and was allowed to stand at 0°C for 1 h. Then the buffer was replaced by 10 mM HEPES with 5 mM homocysteine (Aldrich) or with 5.7 mM sodium dithionite, pH 7.0, followed by anaerobic equilibration at 35°C for 2 days. Because the allosteric transitions of hemoglobin are markedly accelerated as the sol-gel is warmed to 35°C [8,10,11], conformation equilibrium in the sol-gel can be reached within 2 days at 35°C (then the conformations are effectively locked upon re-cooling to 20°C and used for functional analysis [8]). The encapsulation of deoxyhemoglobin in the presence of inositol hexaphosphate (IHP) was carried out by the same procedures as mentioned above, except that 50 mM potassium phosphate, pH 7.1, with 5 mM IHP was used for the encapsulation and 20 mM HEPES, pH 7.0, with 5 mM IHP and 5.7 mM sodium dithionite was used for the succeeding equilibration.

2.3. O_2 equilibrium curves

For the O_2 equilibrium measurement, the deoxyhemoglobin sample was washed with Ar-saturated reductant-free buffer to remove excess dithionite or homocysteine. Then, the buffer was replaced by Ar gas and the O_2 association curve was determined at 20°C as previously described [7,8]. The O_2 pressure was detected using a galvanic O_2 sensor, Iijima MC-7G-L (Aichi, Japan). The fractional saturation with O_2 (Y) was calculated from the absorbance values at 430 nm. Although there was a slow conformational change of hemoglobin in the sol-gel, the accompanying slow absorption change was well separated from the initial phase for O_2 equilibration, as long as hemoglobin-doped sol-gel thin films were used. The increment of the methemoglobin contents during the oxygenation measurements was always less than 10%. The obtained O_2 equilibrium curves were fitted by an equation: $Y(P) = LP/\{(1+L)(K_L+P)\}+P/\{(1+L)(K_H+P)\}$, where P is the partial O_2 pressure, L is the concentration ratio of the low- and high-affinity T conformations, and K_L and K_H are the O_2 dissociation equilibrium constants of the low- and high-affinity T conformations, respectively.

3. Results and discussion

Fig. 1A shows the Hill plots of the O_2 association curve (closed circles) and the subsequently determined dissociation curve (open circles) for anion-free deoxy state-locked hemoglobin in sol-gel containing 10 mM HEPES, pH 7.0, at 20°C. The O_2 pressures at 50% saturation (P_{50}) for the O_2 association and dissociation curves are 33 mm Hg and 14 mm Hg, respectively, and the Hill coefficients at 50% saturation (n_{50}) for them are 0.7 and 0.5, respectively. The Hill coefficients that are less than unity imply the presence of at least two affinity components. Despite the lack of precise reversibility, it can be safely concluded that both curves are markedly biphasic.

To obtain more quantitative information, each of the O_2 association and dissociation curves in Fig. 1A was independently fitted assuming the presence of two non-cooperative components with different O_2 dissociation equilibrium constants. The O_2 dissociation equilibrium constants for the two affinity components obtained from the association curve are essentially identical to those obtained from the dissociation curve (see Fig. 1, legend). However, the fractional population of the high-affinity component in the dissociation curve appears to be significantly larger than that in the association curve, suggesting the occurrence of O_2 binding-induced conformational change during the determination of the association curve (~2.5 h). Such a slow redistribution of the affinity components in sol-gel is the major cause of the disagreement between the association and dissociation curves. It is important to mention here that the observed O_2 dissociation equilibrium constant for the high-affinity component (1.1–2.2 mm Hg) is too large to be regarded as that for the normal R state. In fact, the reported O_2 equilibrium con-

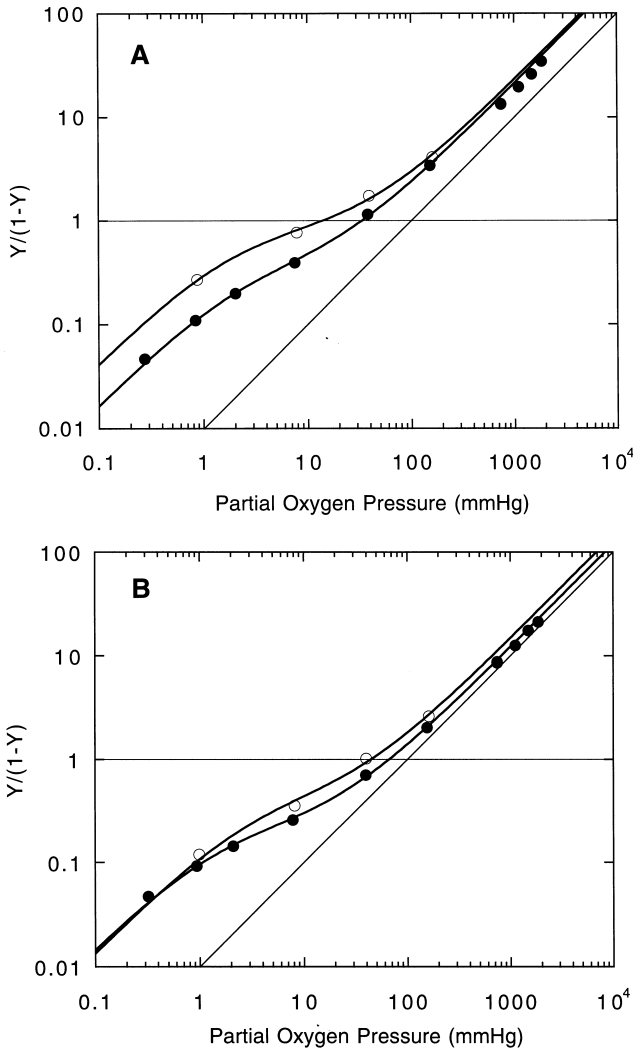

Fig. 1. Hill plots for O_2 equilibrium curves of sol-gel encapsulated human hemoglobin locked in (A), anion-free deoxy state at pH 7.0 (10 mM HEPES) and in (B), anion-free deoxy state at pH 7.0 but IHP (5 mM) was added before measurement (see text). The fractional saturation with O_2 (Y) was calculated from the absorbance values at 430 nm at 20°C. Closed circles represent the O_2 association curves and open circles the succeeding O_2 dissociation curves measured after the O_2 association measurements. The solid lines joining the circles are the results of analysis assuming the presence of two independent non-cooperative components with different O_2 dissociation equilibrium constants. A: The calculated O_2 dissociation equilibrium constants for the high- and low-affinity components of the association curve are 1.6 ± 0.3 mm Hg (26 ± 2%) and 64 ± 4 mm Hg (74 ± 2%), respectively, and those of the dissociation curve are 1.1 ± 0.3 mm Hg (45 ± 4%) and 81 ± 16 mm Hg (55 ± 4%), respectively (the fractional populations are shown in the parentheses). B: The calculated O_2 dissociation equilibrium constants for the high- and low-affinity components of the association curve are 1.2 ± 0.2 mm Hg (18 ± 1%) and 101 ± 4 mm Hg (82 ± 1%), respectively, and those of the dissociation curve are 2.2 ± 1.5 mm Hg (29 ± 8%) and 96 ± 24 mm Hg (71 ± 8%), respectively. In both panels, a straight line corresponding to $P_{50} = 100$ mm Hg ($n = 1$; n is Hill's exponent) is drawn as a reference of O_2 affinity.

stant of the oxy R-state locked hemoglobin in sol-gel at 20°C was approximately 0.16 mm Hg [7].

Fig. 1B demonstrates that the reversibility problem of the O_2 equilibrium curve, as discussed above, could be overcome by adding IHP to the gel-encapsulated deoxyhemoglobin which had been equilibrated in the absence of anions at 35°C for 2 days beforehand. Comparison of Fig. 1A with 1B shows that the reversibility of the O_2 equilibrium curve of the IHP-added sample (left at 0°C for 90 min in the presence of 5 mM IHP before the O_2 equilibrium measurement) was improved significantly, while keeping the basic features of the equilibrium curve of the IHP-free sample. This finding indicates that O_2 binding-induced conformational changes of hemoglobin in sol-gels are highly constrained in the presence of IHP, consistent with the recent resonance Raman spectroscopic measurements of the CO binding-induced conformational changes in the sol-gel encapsulated deoxyhemoglobin with IHP [15]. Another effect of the addition of IHP was seen in the slightly right-shifted upper asymptote of the Hill plot. This effect can be explained as follows. In the absence of IHP, the gel matrices cannot fully prevent the O_2 binding-induced conformational changes especially at high saturation levels, which would shift the upper asymptote of the Hill plot to the left.

In general, biphasic O_2 equilibrium curves can arise from either (i) microscopic heterogeneity of the sol-gel, (ii) intramolecular functional heterogeneity of the α and β subunits, or (iii) the presence of two (at least two) distinct affinity conformations that do not interconvert appreciably in the time scale of the O_2 equilibrium measurement. We must, therefore, exclude the first and second possibilities before reaching our major conclusion (iii).

To determine whether the observed two affinity components are an artifact of microscopic heterogeneity of the gel interior, the IHP-added sample (which had been equilibrated in the absence anions at 35°C for 2 days before adding IHP) was warmed to 35°C to facilitate conformational mobility [8,10,11], and then allowed to stand at 35°C for 2 days in the presence of 5 mM IHP (re-equilibration). The O_2 association curves of this sample before and after the re-equilibration were shown in Fig. 2, along with the association curve of the hemoglobin originally encapsulated anaerobically in the presence of IHP. After the re-equilibration, the O_2 association curve of the IHP-added sample became much similar to that of the hemoglobin originally encapsulated in the presence of IHP (Fig. 2), indicating that microscopic heterogeneity of the sol-gel, if any, is not essential determinant of the affinity states of hemoglobin. We would like to point out here that the same conclusion was drawn from the recent kinetic data on doubly liganded Ni(II)–Fe(II) hybrid hemoglobin in sol-gels, showing that the time course of the allosteric transition in the hybrid is approximately monophasic even in the sol-gel [8].

Another possibility of the functional heterogeneity of the α and β subunits, possibility (ii) mentioned above, can be excluded by the following reasons. Firstly, the observed distribution of the two affinity components is not 1:1 (see Fig. 1, legend). Secondly, the observed affinity difference between the two components is 40 times or more, whereas the generally accepted values of the α and β difference is less than five times.

Taken together, most likely interpretation of the observed biphasic O_2 equilibrium curves is that two distinct affinity conformations coexist in the T state deoxyhemoglobin and their interconversion is impeded by the gel matrices. If the states we trapped do indeed correspond to the functionally significant T conformations in solution, their O_2 affinities should agree with the two-end affinity values for the solution T state. This prediction is confirmed by available data on the O_2 affinities of the T state deoxyhemoglobin in solution (see below).

Earlier work has shown that the O_2 affinities of human adult hemoglobin in the deoxy T state (K_T) range from 5.9 to 250 mm Hg, depending on solution conditions [3]. Also, our independent studies on unmodified and chemically modified Ni(II)–Fe(II) hybrid hemoglobins, in which Ni(II) does not bind O_2, has shown that the O_2 affinities of the solution T state range from 4 to 220 mm Hg at 25°C under the conditions where the hybrids remain in the T state [16]. Our current O_2 affinities obtained for the trapped high- and low-affinity T conformations at 20°C are 1.1–2.2 and 64–101 mm Hg, respectively (see Fig. 1, legend). Although these affinity values are somewhat higher than the corresponding two-end affinities of the T state in solution, this discrepancy is, at least in part, due to the difference in temperature: The current measurements on the gel-encapsulated hemoglobins were carried out at 20°C (to trap the protein conformations) while the previous measurements for solution were at 25°C [3,16].

One of the possible ways to explain the wide range of the T state affinities in solution has been to allow some ligation-induced tertiary structural changes within the T state. Mozzarelli and coworkers [5] modified the MWC two-state model so as to include the basic feature of the Perutz stereochemical mechanism [2] in which the salt-bridges play a dominant role.

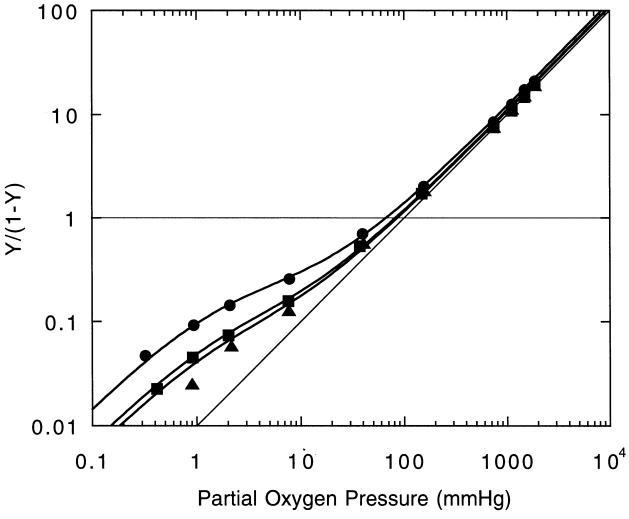

Fig. 2. Hill plots for O_2 association curves of sol-gel encapsulated human hemoglobin before and after the re-equilibration in the presence of 5 mM IHP (see text). The closed circles are the same data as in Fig. 1B. The closed squares represent the data determined after re-equilibration in the presence of 5 mM IHP at 35°C for 2 days under anaerobic conditions. The closed triangles indicate the data for another hemoglobin sample in sol-gel, which was originally encapsulated anaerobically in the presence of 5 mM IHP followed by equilibration in the presence of 5 mM IHP at 35°C for 2 days under anaerobic conditions. The solid lines joining the respective symbols are the results of analysis assuming a variable fraction of the two components with fixed O_2 dissociation equilibrium constants of 1.5 and 100 mm Hg, respectively. Others are as described in Fig. 1.

In their modified two-state model, both the high- and low-affinity tertiary conformations, with broken and unbroken salt-bridges, respectively, are populated in the T quaternary structure [5]. Our present findings are consistent with this proposal, if the sol-gels were able to trap the two T tertiary conformations.

Our results are also consistent with the three-state allosteric model of Minton and Imai [4], which put forward the hypothesis that hemoglobin is in equilibrium between three distinct affinity conformations of the entire molecule. In the framework of the three-state model, our observed high-affinity T conformation may correspond to the postulated intermediate affinity state. As pointed out, the intermediate affinity state (corresponding to the high-affinity T state) would be stabilized under anion-free deoxy conditions at pH 7.4 (see figure 3 in [4]) and would reach maximum thermodynamic stability under anion-free deoxy conditions at pH 9.1 [3]. However, the latter prediction cannot be immediately tested by our experimental technique, because chemical stability of sol-gels would be lowered at high pHs.

It is important to correlate the current observations with the previous experimental data on the T state molecules in solution. Miyazaki et al. [17] studied the O_2 equilibrium properties of normal hemoglobin, low-affinity mutants, and metal substituted hybrids in various solution conditions. They concluded that there is a stable thermodynamic state which determines the lowest O_2 affinity of human hemoglobin. Moreover, excellent agreement between the lowest O_2 affinity in solution and the O_2 affinity of deoxyhemoglobin crystals led them to propose that the classical T structure of deoxyhemoglobin in the crystals represents the lowest affinity state in solution [17]. Clearly, this lowest affinity state corresponds to our observed low-affinity T conformation.

Colombo and Seixas [6] reported that the binding of anions, such as Cl^- or 2,3-diphosphoglycerate (DPG), to deoxyhemoglobin at pH 7.2 is linked to the release of about 45–50 hydration water molecules. Therefore, our observed high-affinity T conformation, which is stabilized under anion-free conditions, is likely to correspond to the deoxyhemoglobin in its more hydrated state. Anion-dependent thermodynamic stability of the high-affinity T conformation is also evident from our previous data which showed no indication of the presence of the high-affinity T conformation in the sol-gel encapsulated hemoglobin in 0.1 M potassium phosphate, pH 7.0 [7,8]. This buffer composition would destabilize the high-affinity T conformation relative to the low-affinity T conformation [3,4,6]. Since the increase in hemoglobin hydration is expected to occur with the rupture of the salt-bridges within the deoxyhemoglobin, the more hydrated T state should be an unconstrained T state with a relatively high affinity for O_2. This consideration provides an important structural aspect of our observed high-affinity T state. It is noteworthy that recent kinetic studies of hemoglobin in the sol-gels [11] suggest the existence of kinetically distinguishable two T conformations: One exhibits a slow bimolecular CO recombination kinetic phase, and the other shows a faster phase (but still slower than the standard R kinetics), presumably corresponding to our proposed high-affinity T state.

In conclusion, the most significant conclusion to be drawn from the present findings is the coexistence of the two distinct affinity conformations in the T state deoxyhemoglobin. Although no detailed structural information can be safely drawn from the present findings, the ability of the sol-gel to impede the interconversion between these two affinity states suggests that global conformational changes, rather than minor perturbations of a single quaternary structure, should contribute to the affinity change. These results are the first, direct demonstration of the existence of equilibrium between two (at least two) functionally distinguishable conformational states in the T state deoxyhemoglobin.

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