Kinetics of p-Mercuribenzoate Binding to Sulfhydryl Groups on the Isolated Cytoplasmic Fragment of Band 3 Protein

EFFECT OF HEMOGLOBIN BINDING ON THE CONFORMATION

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Hemoglobin binds to the cytoplasmic domain of band 3 protein (CDB3) at physiologic pH and ionic strength in an oxygen-linked fashion, with deoxyhemoglobin having the higher affinity. The evidence in the literature suggests functional communication between the hemoglobin-binding site on CDB3 and the anion transport sites within the membrane-bound domain of band 3. Since the hemoglobin-binding site is estimated to be over 200 Å from the transport domain, the functional communication hypothesis would require the existence of long-range, global changes in the CDB3 dimeric quaternary structure consequent to hemoglobin binding. In this report sulfhydryl reactivity toward p-mercuribenzoate is studied in an attempt to identify such long-range conformational changes. Formation of stoichiometric hemoglobin/CDB3 complexes is shown to produce major changes in sulfhydryl reactivity. Since the sulfhydryl pocket of CDB3 is known to lie at the dimeric interface over 100 Å from the hemoglobin-binding site, the observed changes in reactivity suggest that hemoglobin complexation induces a global change in quaternary structure of the CDB3 dimer. This change offers a mechanism to explain functional connections between CDB3-binding sites and the anion transport sites on band 3. The existence of such long-range conformational changes would imply that the CDB3 dimer is poised to function as a cytosolic arm or lever in order to modulate the global structure of the porter.

Band 3 is a multifunctional transmembrane protein found in the red blood cell (1, 2). The integral domain functions to exchange anions while a 45-kDa cytosolic extension (known as CDB3) offers binding sites for cytoskeletal proteins (3–6), hemoglobin (7, 8), and certain glycolytic enzymes (9, 10 for reviews). Oxygen-linked hemoglobin binding to CDB3 (11–13) has been observed under physiological conditions of pH and ionic strength (13), while hemoglobin binding has been shown to increase the rate of anion exchange (14). Another interaction at the hemoglobin site involves hemichrome binding which appears to somehow disrupt the naturally occurring band 3 connections with the cytoskeleton (10). In order to rationalize such linkages between band 3 subdomains, a conformational change in CDB3 of global consequence needs to be postulated.

Early studies seeking conformational connections between CDB3 and the anion transport domain failed to demonstrate linkages (10, 15). However, newer evidence supports such connections. Stoichiometric coverage of the band 3 transport site with 4,4’diisothiocyanostilbene-2,2’disulfonate lowers hemoglobin affinity for CDB3 sites (8, 14, 16). Stilbene disulfonate binding to band 3 diminishes spectrin and ankyrin extractability (17) and specifically changes the conformation of CDB3 within the intact molecule (18). A new mutant form of band 3 shows altered connections between the ankyrin-binding site on CDB3 and the anion transport sites such that the transport rate is higher and the ankyrin binding capacity is lower compared to normal (19). Removal of CDB3 using genetic engineering technology causes a diminution of anion transport (20). Phosphorylation of CDB3 significantly enhances anion exchange (21). Hemoglobin binding to CDB3 causes several significant changes in the kinetic parameters involved in reversible stilbene disulfonate binding (22). Cellular deoxygenation to 50% saturation reduces macromolecule binding to exofacial band 3 sites involved in cellular aggregation (23). Finally, Racker and his co-workers (14) have very recently shown that hemoglobin potentiates phosphate transport in a reconstituted band 3-phospholipid system. With this evidence favoring functional connections between various band 3 sites, what is the structural basis for communication?

This report presents results identifying hemoglobin-induced changes in CDB3 quaternary structure which can explain how connections between otherwise distant sites may occur. The effect of stoichiometric addition of hemoglobin on the PMB reactivity of CDB3 sulfhydryl groups is studied. The use of PMB reaction kinetics to detect protein conformational changes is well established (24, 25). Its usefulness in the present setting lies in the fact that the CDB3 dimer is a highly elongated structure (about 250 Å in length (10)). The sulfhydryl groups are clustered in the COOH-terminal half of the monomer, perhaps over 100 Å from the acidic NH2-terminal hemoglobin-binding site (10), while there are no sulfhydryl groups in the NH2-terminal 23-kDa half of the fragment where hemoglobin binds.
MATERIALS AND METHODS

The chemicals and enzymes used in this study came from Sigma. Recently out-dated human red cells were obtained from the Omaha Chapter of the American Red Cross. Column materials were from Pharmacia LKB Biotechnology Inc.

The basic methods for the preparation of isolated CDB3 have been described (8, 26, 27) and generally follow the procedures given by Bennett and Stenbuck (28) and Appell and Low (29). CDB3 concentration was determined by its optical density at 280 nm using the previously established extinction coefficient at 280 nm of 27 mM\(^{-1}\) cm\(^{-1}\) (26).

Purified hemoglobin was prepared by standard methods in this laboratory (30) and converted to the CO form. HbCO was dialyzed in 200 mM phosphate, pH 7.15, and reacted with NEM in the same buffer for 1 h (final concentrations were 1 mM heme and 3 mM NEM). This was followed by extensive dialysis in 5P6. The reaction was considered to be complete when addition of PMB showed no change in absorbance at 255 nm.

Stock solutions of 100 and 200 \(\mu\)M PMB solutions were prepared in 5P6 as follows. A 10 mM PMB stock was prepared in 0.1 N NaOH. An appropriate amount of this was added to 5 mM monobasic phosphate so that the above concentration would be obtained when brought to volume. Before being brought to volume, the solutions were adjusted to pH 6.0 with 5 mM monobasic phosphate to which a few drops of HCl were added (about 2 drops of 1 M HCl in 150 ml of 5\(\times\) phosphate). Once adjusted to pH 6, the PMB stocks were used to make volumetric dilutions in 5P6 for the kinetic studies. One problem which can occur in working with high concentrations of PMB is the development of a whitish, colloidal-like solution in acidic conditions. Since reactions were to be studied as a function of PMB concentration, it was necessary to ensure that no precipitate developed. This was always checked through both visual and spectrophotometric inspection and was found not to be a problem over the concentration range studied.

The kinetic studies were performed in a Gibson-Durrum stopped-flow apparatus equipped with a Xenon lamp and interfaced to a Northstar computer with software provided by On-line-Instrument System (OLIS, Athens, GA). Stopped-flow data collection was standardized as follows. All reactions were measured at 255 nm in a 3-cm path length cuvette. Slits were set at 0.4 mm. The drive syringes and the reaction cell were in a regulated water bath at 25 °C. Any given reaction at each PMB concentration was the sum of five reactions with each containing 300 data points.

Curve fitting was accomplished using a program capable of performing weighted nonlinear fits (31).

Static titrations of CDB3 were performed in a Hitachi spectrophotometer.

RESULTS

Numbers of Reactive Sulfhydryl Groups on CDB3—Five reactive sulfhydryl groups exist on band 3 (32–34). Two are located on the CH35 (the 35-kDa integral subdomain of band 3 generated by extracellular chymotrypsin digestion) integral subdomain while three are located on CH65 (the 65-kDa fragment of band 3 generated by extracellular chymotrypsin digestion) (Fig. 1). A static titration of the CDB3 sulfhydryls is shown in Fig. 2. There was 10 \(\mu\)M of band 3 monomer present. The saturation point was found to be 18 \(\pm\) 2 \(\mu\)M. The results indicate the presence of 2 mol of sulfhydryl groups/mol of CDB3, in agreement with one estimate of Rao and Reithmeier (33) and the value determined by Appell and Low (29).

Kinetcs of PMB Binding to CDB3 Sulfhydryl Groups—Fig. 3 shows time courses for the CDB3/PMB reaction at several PMB concentrations. In Fig. 3A, short time data are given while Fig. 3B shows the reaction’s progress over a longer time period. Two well-defined phases are observed. The lines represent computer fits to the double exponential equation given in the figure legend.

Mean values of apparent rate constants from several determinations are plotted versus PMB concentration in Fig. 4. The data follow a hyperbolic pattern rather than a linear relationship. A simple physical mechanism which could explain hyperbolic patterns would involve a reversible pre-equilibrium step at each sulfhydryl (Equation 1, of Fig. 5).

\[
\frac{dN}{dt} = k_{+1}(N) [\text{PMB}] - k_{-1}(N) [\text{PMB}] \cdot N
\]

Equation 2 of Fig. 5 gives the formula for the dependence of \(k_{\text{obs}}\) on PMB concentration based on the mechanism. Equation 2 was used to fit the data shown in Fig. 4. The lines come from the fits which satisfactorily represent the data. The values of \(k_{1}\) and \(k_{-1}\) for each phase are given in Table I. The fast phase has a 4-fold higher affinity and 16-fold larger intrinsic rate constant than the slow phase.

Kinetcs of PMB Binding to Stoichiometric CDB3/NEM-HbCO Complexes—The difference in PMB reactivity between CDB3 and the hemoglobin-bound complex is shown in Fig. 6. Both PMB reaction rates of the complex slowed. Graphical comparison of apparent rates in double reciprocal form (Fig. 7) shows that hyperbolic kinetics are followed and that the complex has a considerably lower PMB affinity at each sulfhydryl. The fast-phase sulfhydryl of the complex has a 6-fold lower PMB affinity while the affinity of the slow phase is lowered 9-fold (Table I). The change in \(k_{1}\) corresponds to a 1.1 kcal/mol difference in free energy for the fast phase and a 1.3 kcal/mol difference for the slow phase. These changes represent the predominant reason for the slowing of apparent reaction.
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**FIG. 3.** Time courses of PMB binding to the sulfhydryl groups on CDB3. The final protein concentration was 5 μM in CDB3 monomer. The final PMB concentrations for each curve are shown in the figure. The samples were in 5 mM phosphate buffer, pH 6. The temperature was 25 °C. The reaction was followed at 255 nm in a 2-cm cell. A shows data at low PMB for the initial time periods. The lines drawn in both A and B are based on computer fits to an equation representing the weighted sum of two exponentials:

\[ \Delta A = \Delta A_1 \exp(-k_{1}t) + \Delta A_2 \exp(-k_{2}t) \]

where \( \Delta A \) is the observed absorbance change and the \( k \) values are apparent rate constants with \( f \) and \( s \) meaning fast and slow, respectively.

**FIG. 4.** Plot of computer-extracted rate constants from kinetic progress curves at various PMB concentrations. The kinetic constants from double exponential fits like those shown in Fig. 3, were plotted versus their respective PMB concentrations. The data were fit to Equation 2 of Fig. 5. The lines drawn come from the fits and the constants used are shown in Table I.

rates since maximal intrinsic chemical reaction rate constants increase 2-fold.

**DISCUSSION**

Physical-chemical and hydrodynamic studies indicate an elongated CDB3 dimeric structure of perhaps 250 Å (10). The 23 amino acid, acidic NH₂-terminus of each monomer has been shown to constitute the exclusive hemoglobin-binding site (Fig. 1) (35). The NH₂-terminal 23-kDa piece which contains that site is devoid of sulphydryl groups, while the COOH-terminal 22-kDa piece contains the two reactive groups examined here (Fig. 1) (36, 37). Although the two sulphydryl groups on one monomer are separated by about 15-kDa of amino acid residues (36, 37), each group can participate in interdomain (34) and intradomain (38) disulfide bond formation indicating close spatial proximity within the folded dimeric structure. It has been proposed that the tertiary folding of the polypeptide may form an interfacial "pocket"

1. \( P + S \rightleftharpoons (PS) \)
2. \( k_{obs} = \frac{k_{2}([P]_0)/K_{-1} + ([P]_0)}{K_f} \)

where \( K_f \) is the equilibrium constant of the fast phase.

**TABLE I**

| Pre-equilibrium step \( (K_f \) of Fig. 5) | Slow phase | Fast phase |
|------------------------------------------|------------|------------|
| **No HbCO**                             | 80 ± 20    | 20 ± 3     |
| **Stoichiometric NEM-HbCO**             | 750 ± 300  | 150 ± 80   |

**Maximal rate constant \( (k_{+1} \) of Fig. 5)****

| Slow phase | Fast phase |
|------------|------------|
| **No HbCO** | 3.1 ± 0.4  | 50 ± 5     |
| **Stoichiometric NEM-HbCO** | 7 ± 3     | 110 ± 50   |

**FIG. 5.** Mechanism of PMB binding to a single CDB3 sulphydryl group. This mechanism proposes a reversible pre-equilibrium of PMB with the protein followed by a chemical reaction step (Equation 1) where \( P = [\text{PMB}] ; S = [\text{sulphydryl groups}] ; (PS)^* \) is the initial complex and \( (PS) \) the final covalent complex. The scheme can be described by the second equation with the terms defined as shown and with \( [P]_0 \) being the total concentration of PMB after mixing. Equation 2 was used to fit the data shown in Fig. 4 measured in the absence of NEM-HbCO and for data on PMB binding to the stoichiometric CDB3/NEM-HbCO complex (Table I).
FIG. 6. Reaction time courses of PMB with CDB3 and with the stoichiometric CDB3/NEM-HbCO complex. The left-hand panel shows the early phase of the time course to illustrate that formation of a stoichiometric complex with NEM-HbCO decreases the apparent rate of reaction. The right-hand panel shows the time course over longer periods to illustrate that the slow phase is also diminished in apparent rate. The final concentration of PMB was 5 μM. The conditions were otherwise as in Fig. 3. See the text, Table I, and Fig. 7 for further quantitative details.

containing the cysteine cluster (10). The kinetic results of this paper are consistent with an interfacial "pocket of sulphydryls" hypothesis. PMB would first pre-equilibrate with the protein at the entrance to the pocket before reacting chemically with either sulphydryl. This physical picture would be expected to reflect a mechanism like that in Fig. 5. If PMB can bind to each group without steric hindrance, then the pocket must be large enough to accommodate two PMB molecules. The inherent reactivity of each group would then reflect microstructural differences within the pocket.

The large reduction in initial PMB affinity with hemoglobin complexation (Fig. 7 and Table I) would indicate a partial occlusion of the interfacial pocket of sulphydryls according to the physical picture just discussed. This proposal is illustrated schematically in Fig. 8. The four sulphydryls are shown to be more readily accessible to PMB in the absence of hemoglobin. Formation of the complex is shown to change the quaternary structure, thereby occluding the entrance to the sulphydryl pocket at the interface. In this model hemoglobin would not physically block the sulphydryls. Although it is not possible to distinguish between direct steric and allosteric competition, the allosteric hypothesis is favored since the available evidence strongly suggests that CDB3 has a very elongated structure (10). The inability to cross-link hemoglobin and CDB3 sulphydryls when the two form a complex in solution (27), may support the allosteric linkage hypothesis.

The view that an interfacial sulphydryl pocket becomes occluded to a small molecule like PMB consequent to changes in quaternary structure may be supported by a thermodynamic comparison to a well-known example of interfacial pocket closure to a small molecule. The example chosen for this comparison is the difference in the binding of 2,3-diphosphoglycerate to oxy versus deoxyhemoglobin. The binding site between the β chains in oxyhemoglobin is occluded to organic phosphates while in deoxyhemoglobin the pocket is open (39). The various binding data have been reviewed by Bunn and Forget (40). Binding to oxyhemoglobin occurs with a Kd of 3 × 10^-3 M, while the same constant for deoxyhemoglobin is 3 × 10^-4 M, under comparable physiological conditions (40). The difference in binding free energy for the closure of the hemoglobin pocket with oxygenation would then be about 2.6 kcal/mol. The magnitude of this change is favorably comparable with 1.1–1.3 kcal/mol change determined for PMB binding to the interfacial sulphydryl pocket of the CDB3 dimer consequent to complex formation.

FIG. 7. Double reciprocal plots of the observed, computer-extracted rate constants for the fast and slow phases of the PMB reaction with CDB3 and with the stoichiometric CDB3/NEM-HbCO complex. This figure illustrates that both data sets are saturable and follow hyperbolic patterns. Second, the figure shows that formation of the CDB3-hemoglobin complex significantly lowers the apparent affinity of PMB in its pre-equilibrium binding to CDB3. The results of the quantitative analysis based on computer fits to Equation 2 of Fig. 5 are shown in Table I. The conditions were as in Fig. 3.
One pernicious question in this field concerns the absence or presence of a linkage between CDB3 sites and the anion transport sites on band 3. If the band 3 transport mechanism involves a rigid protein channel, there would be little reason to look for hemoglobin modulation of anion exchange. On the other hand, if each band 3 monomer contained an anion transport domain whose function was sensitive to global conformational changes, then modulation of anion exchange by hemoglobin binding would become a more likely possibility. Work with covalent 4,4'-diisothiocyanostilbene-2,2'-disulfonate binding has shown linear activity-labeling correlation plots indicating an absence of intersubunit interactions (1, 2). Yet, linear inhibition does not preclude interprotomeric allosterism if 4,4'-diisothiocyanostilbene-2,2'-disulfonate binding were to uncouple naturally occurring interactions.

Direct evidence for “half-of-the-sites” inhibition by pyrophosphol 5'-phosphate (a substrate and affinity probe) has been recently presented by Salhany and co-workers (41). Evidence was also presented that reversible 4,4'-dinitrostilbene-2,2'-disulfonate binding eliminated the half-of-the-sites pattern, yielding linear correlation plots. This shows that stilbene disulfonates can uncouple the site-site interactions for a transported anion. Since transport kinetics for physiologic anions show allosteric patterns in the form of partial substrate inhibition (1, 2), allosteric coupling between band 3 protomers seems probable. Evidence that allosteric coupling may explain the partial substrate inhibition effect, comes from the fact that this type of inhibition can be relieved by addition of an external cross-linking agent to band 3 (42). Since the substrate inhibition effect is dominated by an intracellular chloride site (43), the transformation to hyperbolic kinetic patterns resulting from the addition of an extracellular agent offers strong support for the view that anion exchange is indeed modulated by allosteric events.

If interprotomeric allosterism exists for the band 3 porter, then hemoglobin may serve as a heterotropic allosteric affector of the transport activity through modulation of quaternary structure. This modulation, since it would be linked to the state of hemoglobin oxygenation (13), could provide a direct connection between the oxygen transport and the CO2 transport systems of the red cell. The asymmetrical structural disposition of band 3 in the membrane speaks of such a direct connection. Only about 10% of the copies of band 3 are directly bound by red cell ankyrin (3). The remaining copies of the porter may form a looser association, so explaining the surprisingly rapid exchange of ankyrin-free for ankyrin-bound band 3 (44). Dynamic band 3 exchange has the potential to be modulated by the state of cellular oxygenation if deoxy-hemoglobin binds to ankyrin-free band 3 and stabilizes that quaternary structure of CDB3.

Linkage between the hemoglobin-binding site and the anion transport sites may be needed during extreme exercise, when bicarbonate transport through band 3 is thought to become rate limiting to CO2 transport (45). Increased steady state levels of deoxyhemoglobin during extreme exercise could favor greater fractional hemoglobin binding to band 3 (13). The attendant increase in the rate of anion exchange (14) afforded by increased binding could relieve the putative rate limitation imposed by an otherwise immutable anion exchange rate (45). Even as small a change in rate as a factor of two could make a significant difference in CO2 transport during exercise, owing to the steepness of the curve relating anion exchange to CO2 efflux at the lung (45).

In summary, the hemoglobin-induced conformational change in CDB3 identified here offers a basis upon which to explain an otherwise vague functional connection between the NH2-terminal domain of band 3 and the COOH-terminal domain containing the transport sites. The physiological significance of this mechanism for site communication on band 3 may become more apparent as band 3's emerging function as an allosterically modulated porter (41) becomes better defined.

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