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Interaction between cytochrome b_5 and hemoglobin: Involvement of β66 (E10) and β95 (FG2) lysyl residues of hemoglobin

(methemoglobin/reduction/hemoglobin variants/titration curve/binding domain)

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ABSTRACT **In erythrocytes the reduction of oxidized hemoglobin (methemoglobin) is dependent upon an electron transport reaction between cytochrome b_5 and methemoglobin. These two proteins are believed to form a complex whose bonding is principally determined by complementary charge interactions between acidic groups of cytochrome b_5 and basic groups of hemoglobin. In order to refine this model, three surface lysyl hemoglobin variants—namely Hb N Baltimore β95 (FG2) Lys → Glu, Hb I Toulouse β66 (E10) Lys → Glu, and Hb I Philadelphia α16 (A14) Lys → Glu—have been studied with respect to their reducibility and ability to bind cytochrome b_5. In the two former variants, the substituted amino acids are located near the heme crevice; in the third one the substitution lies far from it. Substitutions of lysine for glutamic acid in positions β66 and β95 perturb the formation of the cytochrome b_5–hemoglobin complex and result in a dramatic impairment of the cytochrome b_5–mediated reduction, whereas the same mutation in position α16 has no effect. We conclude that the lysine residues in positions β66 and β95 are directly involved in the binding of cytochrome b_5. The three-dimensional structure of hemoglobin suggests that the cytochrome b_5-binding domain of hemoglobin is constituted by four lysine residues surrounding the heme crevice in both α and β chains. Similarities with other interacting hemoproteins are discussed.**

Interactions between hemoproteins are involved in several important pathways of electron transport, such as the respiratory chain in mitochondria (1) and the cytochrome *P*-450 monooxygenase pathway of the endoplasmic reticulum (2, 3). In the erythrocyte, as shown by Hultquist and Passon (4), the reduction of oxidized hemoglobin (methemoglobin) is accomplished by soluble reduced cytochrome b_5 (5). In turn oxidized cytochrome b_5 is continuously reduced by a soluble form of NADH:cytochrome b_5 reductase (6). Hemoglobin and cytochrome b_5 are well-characterized proteins and their amino acid sequences are known (7–9). Their three-dimensional structures in both redox states have been determined by x-ray crystallography studies (10–13). Recent studies suggested to us that cytochrome b_5 and methemoglobin can form a complex whose bonding is determined mainly by complementary charge interactions between acidic groups of cytochrome b_5 and basic groups of hemoglobin (14). Theoretical (15) and experimental (16) studies of the cytochrome b_5–cytochrome *c* complex have led to similar conclusions. In order to refine this model we have investigated the consequences of substitutions of basic surface residues of hemoglobin. Three variants have been used for these studies—namely Hb I Toulouse β66 (E10) Lys → Glu, Hb N Baltimore β95 (FG2) Lys → Glu, and Hb I Philadelphia α16 (A14) Lys → Glu. We report here the effect of these structural modifications on the cytochrome b_5-mediated reduction of methemoglobin, and the stability of the protein–protein interaction. The results clearly indicate that the lysyl residues β66 (E10) and β95 (FG2) are involved in the formation of a functional cytochrome b_5–hemoglobin complex.

MATERIALS AND METHODS

Trypsin-solubilized cytochrome b_5 was purified from rabbit liver as described (17).* Soluble NADH:cytochrome b_5 reductase was prepared from human erythrocytes as described by Leroux *et al.* (18). The enzyme activity was measured with the methemoglobin–ferrocyanide complex as a substrate according to Hegesh and Avron (19). One unit catalyzes the reduction of 1 μmol of oxidized hemoglobin tetramer per min. Normal and abnormal hemoglobins were prepared in pure form by DEAE-Sephadex chromatography. The three variants used in this study were checked by structural analysis (amino acid analysis of the abnormal tryptic peptides) to be Hb N Baltimore, Hb I Toulouse, and Hb I Philadelphia as defined in refs. 20–22. Methemoglobin species were obtained by oxidation of pure components according to the nitrite procedure described by Kilmartin (23). Taking advantage of their slight difference in isoelectric point (24–26), we purified the valency hybrids ($\alpha_2^{2+}\beta_2^{3+}$ and $\alpha_2^{3+}\beta_2^{2+}$) by isoelectrofocusing (IEF), according to the following procedure. An oxyhemoglobin sample was oxidized to 50% by addition of small amounts of potassium ferricyanide, dialyzed against distilled water, and applied to a preparative Ultrodex flat bed containing pH 6–8 Ampholines (LKB). Once focused, the various species were eluted from the gel and stripped of Ampholines by mixed-bed resin chromatography (27). We assessed the purity of the valency hybrids by oxidation with $CuSO_4$, utilizing the property of cupric ions to oxidize the β subunit selectively (28). The enzymatic reduction of methemoglobin was performed by using a reconstituted physiological system containing 0.15 μmol of NADH, 0.4 nmol of cytochrome b_5, 3.8 milliunits of enzyme, and variable amounts of methemoglobin in 0.5 ml of 0.1 M [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane (Bis-tris), pH 7.0, at 37°C. The reduction of methemoglobin was measured by the increase in absorbance at 576 nm. The change of absorbance was converted into μmol of subunit reduced per min, using a $\Delta\epsilon$ (oxyhemoglobin − methemoglobin) of 10.5 mM^{-1} cm^{-1}. In the range of methemoglobin concentration studied, the velocity of reduction was found to be constant during the first minute of the reaction. In the conditions used in this study, this velocity was directly proportional to the concentration of cytochrome b_5 (in the 0.1–1 μM range), in-

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Abbreviations: IEF, isoelectric focusing; Bis-tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; PMS, phenazine methosulfate.

* The heme peptide core of cytochrome b_5 obtained after trypsin digestion of rabbit liver microsomes that we used in this study can be reasonably assumed to be similar to the human soluble erythrocyte cytochrome b_5 (5).

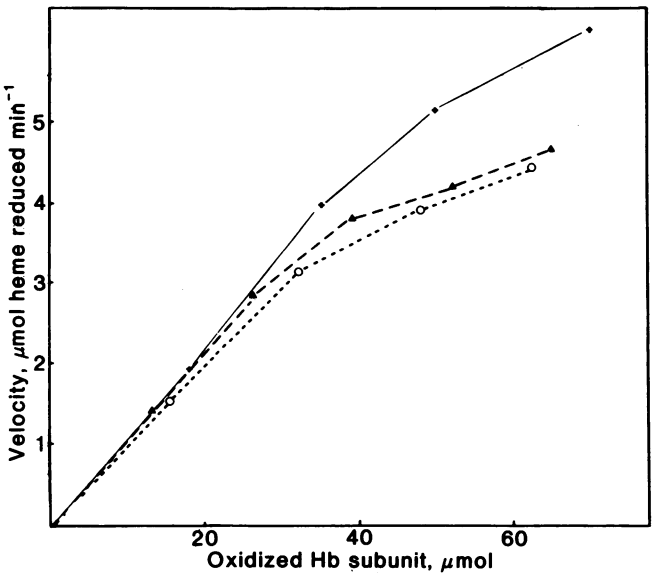

FIG. 1. Kinetics of the reduction of Hb A oxidized species. + — +, metHb A; ▲ -- ▲, α-oxidized valency hybrid $\alpha_2^{3+}\beta_2^{2+}$ A; and O --- O, β-oxidized valency hybrid $\alpha_2^{2+}\beta_2^{3+}$ A. Conditions: 0.3 mM NADH, 0.8 μM cytochrome b_5, 7.6 milliunits of cytochrome b_5 reductase per ml, 0.1 M Bis-tris, pH 7.0, 37°C.

dicating that, as stated by Hultquist and Passon (4), the first step of the reaction (i.e., the reduction of cytochrome b_5) was not rate limiting. Control experiments showed that, in these conditions, there was no detectable reoxidation of any of the investigated species, normal or abnormal.

The chemical reduction of methemoglobin was performed according to Kajita *et al.* (29) at 25°C, using the following reaction mixture: 0.15 μmol of NADH and 0.055 μmol of oxidized hemoglobin in 0.5 ml of 0.1 M Bis-tris·HCl, pH 7.0. The reaction was initiated by the addition of 0.77 nmol of phenazine methosulfate (PMS). The conversion of methemoglobin to oxyhemoglobin was measured by the increase of absorbance at 576 nm. Combined IEF/electrophoresis on thin-layer polyacrylamide gels was performed as described (30), with minor modifications (for technical details, see legend to Fig. 6).

RESULTS

The dependence of the reduction velocity on metHb A concentration is illustrated in Fig. 1. Similar curves are obtained for metHb A, α-oxidized, and β-oxidized valency hybrids. It is to be noted, however, that above 30 μM, the velocity of reduction of the valency hybrids is significantly slower than that of metHb A. This could be accounted for by the presence within the hybrid species of reduced subunits behaving like inhibitors. Of special interest is the fact that, within the valency hybrids, the α_A- and β_A-oxidized subunits appear to be reduced with the same velocity. All of these observations suggest that the respective reactivities of the two valency hybrids towards reduced cytochrome b_5 are quite similar.†

In an attempt to define the role of individual basic residues of methemoglobin in the interaction with cytochrome b_5, the reducibility of several available lysine variants of hemoglobin was investigated. As shown in Fig. 2, metHb I Philadelphia is reduced at the same rate as metHb A. Conversely, the velocity of the reduction is markedly decreased by Lys → Glu substitutions of the residues β66 and β95, corresponding respectively to Hb I Toulouse and Hb N Baltimore. The defect is even more apparent when the β-oxidized valency hybrids are used as substrate of the reduction. As shown in Fig. 3, the velocity of the reduction of the β-oxidized hybrids of Hb I Toulouse and N Baltimore is dramatically decreased in comparison with that of the β_A subunit. This suggests that the poor reducibility of the two variants is mainly due to the β chain abnormality. An additional indication was provided by the following experiment. A lysate containing both Hb A and Hb N Baltimore was first completely oxidized and subsequently half-reduced by the cytochrome b_5–enzyme system. The products of this partial reduction were then analyzed by IEF. As shown in Fig. 4, the two Hb A valency hybrids are present in equivalent amounts, whereas in the case of Hb N Baltimore only one valency hybrid band is visible. This species, which cannot be reoxidized by

† After completion of this work, a paper by Tomoda *et al.* (31) appeared in which the authors stated that the β subunit is more rapidly reduced than the α subunit in the cytochrome b_5-dependent system. The main reason for this apparent contradiction is that in our case we measured the reduction of the hybrids during the first minute, whereas Tomoda *et al.* carried out their measurements between 2 and 52 min. In their case some concomitant reoxidation of the more autoxidable α chain could have taken place.

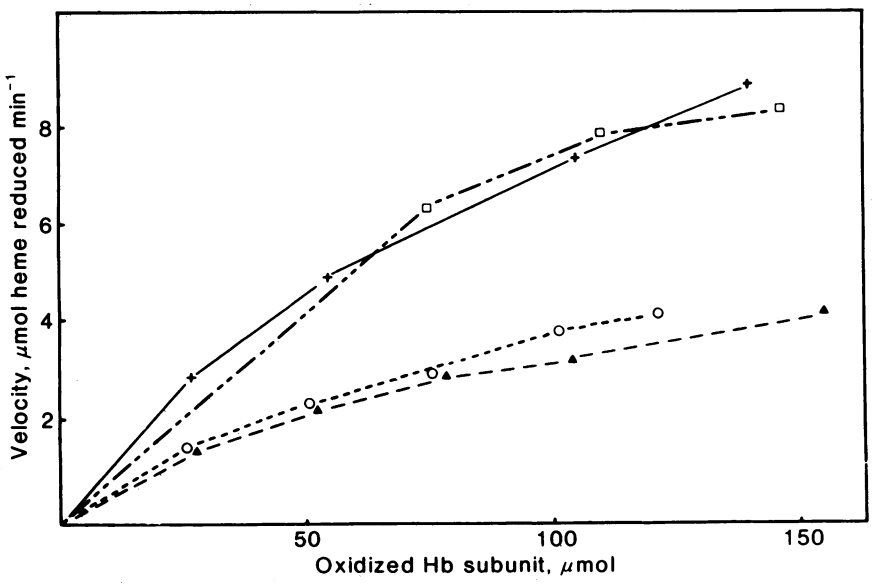

FIG. 2. Kinetics of the reduction of fully oxidized abnormal hemoglobins. □— - -—□, MetHb I Philadelphia; O --- O, metHb I Toulouse; ▲ -- ▲, metHb N Baltimore; and + — +, control metHb A. Conditions were the same as for Fig. 1.

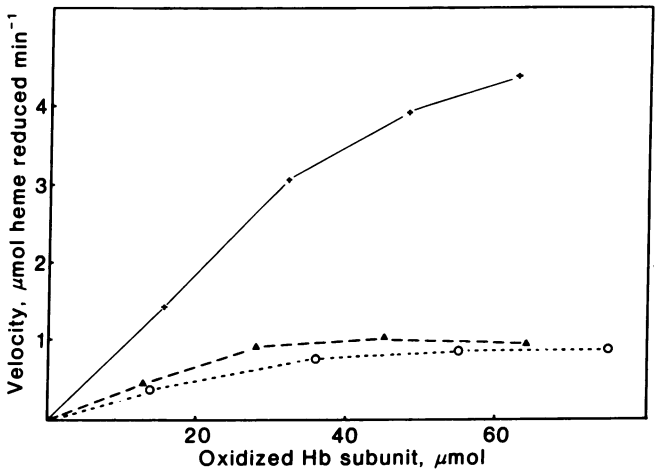

FIG. 3. Kinetics of the reduction of the β-oxidized valency hybrids. O --- O, $\alpha_2^{2+}\beta_2^{3+}$ I Toulouse; ▲ -- ▲, $\alpha_2^{2+}\beta_2^{3+}$ N Baltimore; and + -- +, $\alpha_2^{2+}\beta_2^{3+}$ A. Conditions were the same as for Fig. 1.

cupric ions under the conditions described by Winterbourn and Carrell (28), is therefore a β-oxidized hybrid. This further demonstrates that the abnormal β subunit of Hb N Baltimore is reduced much more slowly than the normal β subunit.

Whereas substitutions of the β95 (FG2) lysyl residue (Hb N Baltimore Lys → Glu and Hb Detroit Lys → Asn) do not give rise to any detectable disorders in the variants (unpublished data and ref. 32), Hb I Toulouse β66 (E10) Lys → Glu exhibits abnormal redox properties, namely an increased rate of autoxidation (21), which could lead to a nonspecific impairment of the reducibility. Therefore, the reduction of Hb I Toulouse by chemicals was investigated. Conversely to what is observed in the cytochrome b_5-dependent reaction, Hb I Toulouse appears to be quite reducible in the NADH/PMS system. Under the conditions used, the reaction follows pseudo-first-order kinetics. As shown in Fig. 5, the rate of reduction of the β I subunit is not significantly different from that of the β A subunit.

In order to explain this defect of the cytochrome b_5-mediated reduction, the interaction between hemoglobin and cytochrome b_5 was studied by using the IEF/electrophoresis technique recently developed by Righetti and coworkers (30, 33). This technique, which had allowed us to visualize the mutual interaction of cytochrome b_5 and methemoglobin (14), was used

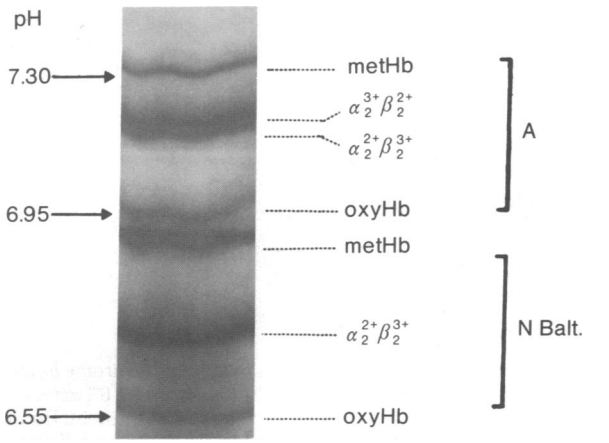

FIG. 4. IEF pattern of the products of partial reduction of metHb A and metHb N Baltimore. The analysis was performed on an Ultrodex flat bed in a pH 6–8 gradient as described for preparation of valency hybrids.

to detect the possible impairment of this protein–protein binding in the case of the lysyl-substituted hemoglobins. As illustrated in Fig. 6*a–c*, when this procedure was applied to an equimolar mixture (on a heme basis) of cytochrome b_5 and metHb A, a markedly delayed migration of both components was observed, particularly in the pH 6–8 range. The same perturbation was obtained with metHb I Philadelphia (not shown). In contrast, Hb N Baltimore and Hb I Toulouse displayed a clearly different pattern (Fig. 6 *d* and *e*). In these cases the migration of cytochrome b_5 was much faster than in the presence of Hb A. After a 7-min migration, the titration curves of cytochrome b_5 and metHb N Baltimore or metHb I Toulouse appeared to be widely separated (Fig. 6 *d* and *e*), whereas in the case of the cytochrome b_5–metHb A mixture, they were still close to each other around pH 7 (Fig. 6*c*). These titration patterns suggest that the two abnormal hemoglobins interact only weakly with cytochrome b_5.

Taken together, the poor reducibility of Hb N Baltimore and Hb I Toulouse and the impairment of their interaction with cytochrome b_5 strongly favor the idea that lysyl residues β66 and β95 play a critical role in the formation of a functional hemoglobin–cytochrome b_5 complex. Conversely, the lysyl residue α16 is not required for the interaction to take place, as indicated by the normal reducibility of metHb I Philadelphia and its normal ability to bind cytochrome b_5.

DISCUSSION

In 1971, Hultquist and Passon proposed a two-step mechanism for the physiological reduction of methemoglobin in erythrocytes (4). In *step 1*, soluble cytochrome b_5 (5) is reduced by NADH in the presence of a soluble cytochrome b_5 reductase (6). This enzyme has been shown to be identical with the so-called NADH:methemoglobin reductase (or NADH-diaphorase) and probably derived from the ubiquitous microsomal NADH:cytochrome b_5 reductase (EC 1.6.2.2) (35–37). In *step 2*, ferriheme groups of methemoglobin are reduced nonenzymatically by reduced cytochrome b_5. To our knowledge, the mechanism of this direct reduction of methemoglobin by reduced cytochrome b_5 has not yet been investigated. In our first approach to this problem we demonstrated a direct interaction between the two hemoproteins (14). The cytochrome b_5–methemoglobin complex was shown to be stabilized in the pH range 6–8. This suggested the involvement of electrostatic interactions between basic residues of hemoglobin and acidic residues of cytochrome b_5. According to this model the binding of cytochrome b_5 to α and β chains should be equivalent because the number and distribution of basic surface residues are similar in the two chains. Furthermore, the interaction between cytochrome b_5 and hemoglobin should be hindered by substitutions of basic residues of hemoglobin located in the binding domain.

The results obtained here support these predictions. Within the valency hybrids, the α- and β-oxidized subunits of Hb A appear to be reduced with identical velocities. In addition, two Lys → Glu variants—Hb I Toulouse and Hb N Baltimore—exhibit low rates of reduction of their abnormal β subunit in the cytochrome b_5-dependent reducing system. The possible involvement of the abnormal redox properties of Hb I Toulouse in the poor reducibility of this variant can be ruled out because (*i*) as indicated under *Materials and Methods*, no concomitant oxidation could be detected during the measurement of the initial velocities of the cytochrome b_5-mediated reaction; (*ii*) Hb I Toulouse is easily reducible by chemicals such as reduced PMS. Therefore it is likely that the dramatic defect observed in the cytochrome b_5-dependent reduction of the variants is related to a specific impairment of the cytochrome b_5–hemo-

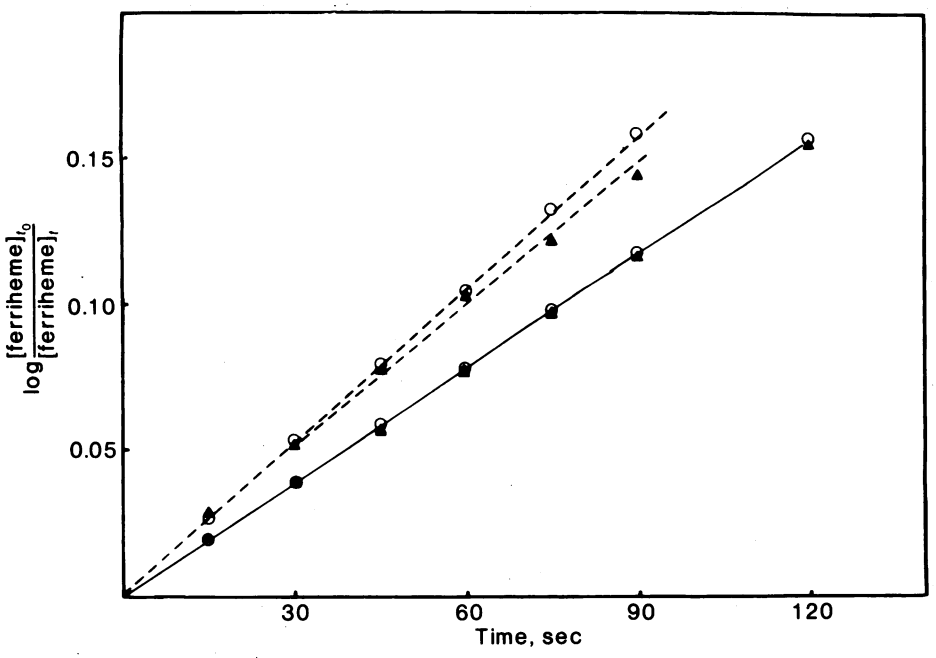

FIG. 5. Kinetics of the reduction by PMS of Hb A and Hb I Toulouse oxidized species. O — O, MetHb A; ▲ — ▲, metHb I Toulouse; O --- O, β-oxidized valency hybrid $\alpha_2^{2+}\beta_2^{3+}$ A; and ▲ --- ▲, β-oxidized valency hybrid $\alpha_2^{2+}\beta_2^{3+}$ I Toulouse. $[\text{Ferriheme}]_{t_0}$ is the initial concentration of oxidized subunits; $[\text{ferriheme}]_t$ is the concentration of oxidized subunits at a given time of the reaction. Conditions: 0.3 mM NADH, 3.08 μM PMS, 0.11 mM ferriheme (at t_0), in 0.1 M Bis-tris, pH 7.0, 25°C.

globin reaction. This defect could be accounted for by the perturbation of this protein–protein interaction, by the impairment of the electron transport reaction itself, or by both. Due to the complexity of the reaction and to the heterogeneity of the substrates (coexistence of normal and abnormal subunits in the abnormal methemoglobin tetramers, coexistence of the β-oxidized and α-reduced subunits in the valency hybrids), the analysis of the kinetic data did not permit us to discriminate between the different possibilities. In contrast, the IEF/electrophoresis technique, which had already proved useful to visualize the interaction between cytochrome b_5 and methemoglobin (14), provided a direct and unambiguous demonstration that the binding of cytochrome b_5 to methemoglobins I Toulouse and N Baltimore is indeed impaired. Thus we conclude that the poor reducibility of the abnormal β subunits of these two hemoglobin variants is due, at least in part, to a defect in the binding of cytochrome b_5. Because of the normal oxygen-binding properties of these variants, their overall tertiary structure is thought to be normal (unpublished results; ref. 21). Therefore the impairment of the cytochrome binding must arise from the substitutions themselves. This implies that the E10 and FG2 residues are located in the cytochrome b_5-binding area. If these lysines are directly involved in electrostatic interactions with carboxyl groups of cytochrome b_5 (14), their replacement by glutamic acid residues should introduce repulsive forces, preventing the formation of the cytochrome

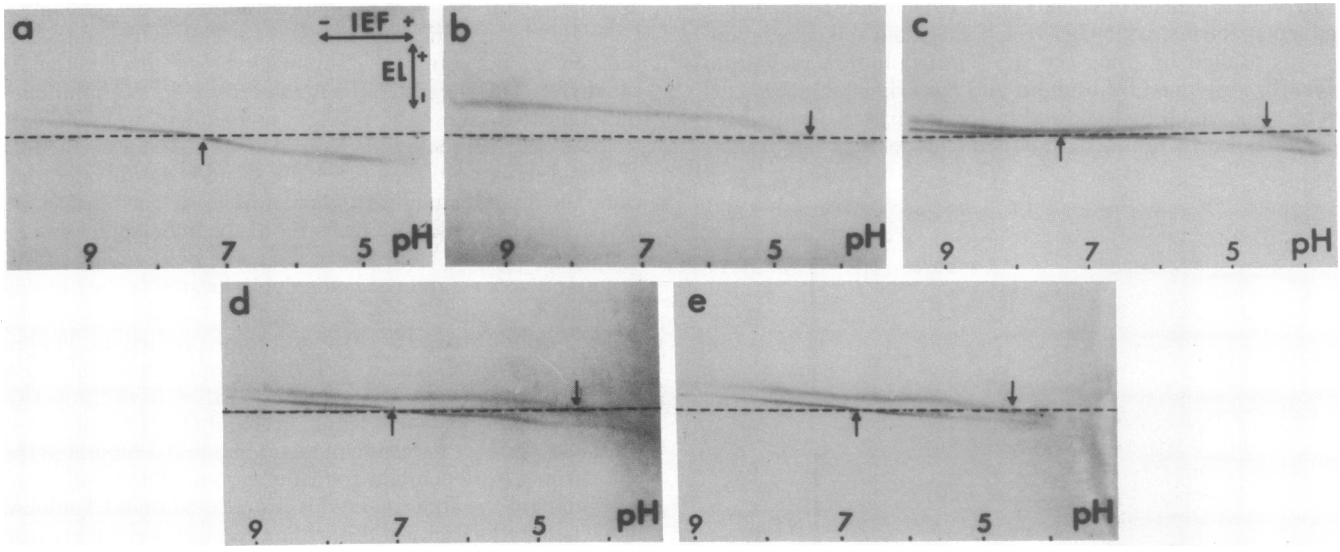

FIG. 6. Titration curves of interacting cytochrome b_5 and normal and abnormal hemoglobins. (*a*) MetHb A alone; (*b*) cytochrome b_5 alone; (*c*) metHb A + cytochrome b_5; (*d*) metHb I Toulouse + cytochrome b_5; (*e*) metHb N Baltimore + cytochrome b_5. The gel contained 6% acrylamide (ratio acrylamide to bisacrylamide = 25:1 wt/wt) and 2% Ampholine pH 3.5–10 (LKB). Each gel was loaded with 150 μg of each protein. Running conditions: LKB Multiphor 2117 chamber run at 13 W (600 V at equilibrium) for 90 min at 4°C in the first dimension (IEF). Second dimension (EL): 7-min run at 600 V (constant voltage), immediately followed by Coomassie brilliant blue staining as in ref. 34. The linearity of the gradient was checked after each run on a strip of unstained gel and found quite reproducible. The bidirectional arrows and + and − symbols represent the direction and polarity of IEF and electrophoresis. The broken lines indicate the sample application trough and the intersection point of each curve indicated by vertical arrows represents the pI of each protein.

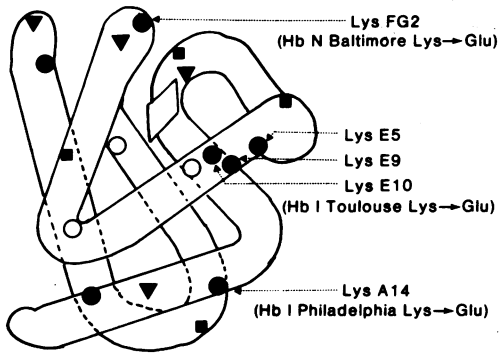

FIG. 7. Diagram of a hemoglobin subunit, with the heme indicated by a rectangle drawn in perspective [after Dickerson (38)]. The basic residues of both α and β chains are indicated by the following: O, ●, Lys and Arg residues shared by both subunits; the residues directly visible on this view are indicated by ●, those behind the plane are indicated by O. ▼, Lys and Arg residues specific for α subunit. ■, Lys and Arg residues specific for β subunit.

b_5–methemoglobin complex. Noteworthy is the fact that both E10 and FG2 residues are located in the vicinity of the heme crevice. Conversely, the Lys → Glu substitution of Hb I Philadelphia, which lies in a region remote from the heme pocket, did not produce any abnormal behavior towards cytochrome b_5.

These results suggest that the positively charged groups of the cytochrome b_5-binding domain of hemoglobin are located in the vicinity of the heme crevice. From the distribution of basic residues in α and β subunits, it is apparent that, in the heme-surrounding region, only four surface lysyl residues are shared by both chains, namely Lys E5, E9, E10, and FG2 (Fig. 7). Because the α and β subunits exhibit identical reducibility by cytochrome b_5, it seems reasonable to assume that these residues constitute the main part of the cytochrome b_5 binding domain.

Similar conclusions have been drawn from studies of the cytochrome b_5-cytochrome *c* complex, in which four lysyl residues of cytochrome *c* have been proposed to interact with carboxylic groups surrounding the heme of cytochrome b_5 (15, 16). Similar interactions have also been demonstrated to take place between cytochrome *c* and various redox enzymes (39–43).

It is then tempting to assume that the same general mechanism is involved in all these cases: namely, that the interaction between complementary charged domains around heme brings the heme groups close to each other, allowing the electron transport reaction to take place. However, the confirmation of such a mechanism will probably have to await the solution of the structure of the hemoprotein complexes by x-ray crystallography.

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