ORIGINAL ARTICLE

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A modeling study of the interaction and electron transfer between cytochrome b_5 and some oxidized haemoglobins

Abstract Using Brownian motion simulations we have studied the formation of docked complexes of reduced cytochrome b_5 and oxidized haemoglobin. Our results indicate that the presence of molecular electrostatic fields has a significant role to play in the formation of these complexes. In contrast to previous modeling studies on this system, we clearly identify electron transfer within an ensemble of similarly docked complexes rather than the formation of a single complex. Docking involves a number of acidic residues surrounding the exposed haem edge of cytochrome b_5 and a set of basic residues surrounding the exposed haem edge of the globins. Although amino acids from the partner globin proteins are involved to a small extent in the binding of some of the complexes, the reactivity of any particular globin is essentially independent of the nature of its partner globin chain within the haemoglobin molecule. Comparison of results from adult and embryonic haemoglobins indicates a significant difference in complex formation. Application of electron tunneling analysis to the complexes allows us to predict the rates of electron transfer within each ensemble of complexes. These data provide a theoretical insight into the important process of re-reduction of oxidized haemoglobins as well as explaining the general inability to produce crystalline forms of many docked electron transfer complexes.

Keywords Cytochrome b_5 · Haemoglobin · Brownian simulation · Electron transfer

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Introduction

In many biological processes, from the cytochrome chain of the mitochondrion to photosynthesis, a reaction of major importance is the deceptively simple process of electron transfer. This process often involves the prior formation of protein-protein complexes before the electron transfer event. Few such complexes have been isolated and structurally characterized [1, 2] and questions have been raised as to whether the few isolated complexes are indeed of functional significance [2, 3]. In the absence of well-characterized electron transfer protein complexes, theoretical approaches to the problem of inter-protein electron transfer have been developed. In many cases, analyses of the formation of the initial protein-protein complex have employed numerical simulation based on Brownian dynamic encounters modified to take into account the effects of the electrostatic fields associated with the protein partners [4, 5, 6]; this approach appears to yield realistic models for the initial encounter for a number of systems [4, 5, 6, 7, 8]. However, more detailed investigations clearly require additional analysis of such contributions as relaxation of the initial complex structures [9]. The analysis of the subsequent transfer of electrons from the donor protein to the acceptor protein has often been based on the pathways model developed by Onuchic and Beratan [10, 11], which analyses the process of electron tunneling between the partners in terms of the contributions of covalent bond, H-bond and through-space contributions, whilst explicitly taking into account the detailed structures of the redox partners. The original pathways model may in some circumstances also require modification to take into account vibrational effects and the possible interference between multiple different pathways [12, 13, 14, 15, 16]. In other electron transfer systems, gating is also recognized as a major factor modifying the overall electron transfer process between two proteins [17, 18, 19, 20]. Thus in some systems the combination of Brownian dynamics docking and pathways analysis

gives no more than a "first-order approximation" of the total process of inter-protein electron transfer. However, in the absence of any firm experimental data with which to make comparisons for the reaction of reduced cytochrome b_5 with met-haemoglobin, we have chosen, as a first step, to use this combination of approaches in our analysis of this system to make predictions concerning this important reaction.

One particular electron transfer process is indirectly used to maintain the functional properties of haemoglobin in its role as an oxygen carrying protein. In order to reversibly bind and release oxygen, the iron atom at the center of the haem groups of haemoglobin must remain in its ferrous form. However, under physiological conditions the oxy form of haemoglobin is intrinsically unstable and can be oxidized to the non-functional ferric form by two distinct reactions. Mono-molecular dissociation of the oxy-haemoglobin leads to the formation of superoxide and ferric haemoglobin [21]. Nucleophilic attack on the oxy form of haemoglobin also leads to the formation of ferric haemoglobin [22]. The sum of these two reactions is responsible for approximately 2% oxidation of adult human haemoglobin per day [23]. As the circulatory lifetime of a red blood cell is approximately 120 days [24], it is necessary to constantly re-reduce the ferric haemoglobin to the ferrous form. The reduction is achieved by a small electron transfer chain within the red blood cell, which leads from NADH to cytochrome b_5 reductase to cytochrome b_5 and finally to ferric haemoglobin [25, 26]. The final step involves the transfer of an electron from cytochrome b_5 to haemoglobin within a transient protein-protein complex [27, 28]. By visual inspection of the structures of cytochrome b_5 and isolated globin chains alone, Poulos and Mauk [29] proposed a structure for a unique cytochrome b_5-α globin and cytochrome b_5-β globin complex. It was proposed that the binding within these complexes involved predominantly ionic interactions between surface Lys residues surrounding the haem group on cytochrome b_5 and Glu residues surrounding the haem site on the globin chains. Some support for this suggestion was gained from a small number of studies on the reactions of mutant and natural proteins [30, 31, 32].

In common with many other protein-protein redox reactions [33, 34], no complex has been identified in the reaction of reduced cytochrome b_5 and met-haemoglobin Thus, in the absence of any direct structural characterization of a cytochrome b_5-haemoglobin complex, we have undertaken, as an initial step in our study of the reduction of ferric haemoglobin, a theoretical investigation of the structural requirements for efficient electron transfer between cytochrome b_5 and adult ferric haemoglobin, based on Brownian dynamics and electron transfer theory originating in the work of Marcus and Sutin [35]. We have extended this study to an investigation of the likely role of cytochrome b_5 in the reduction of the ferric forms of human embryonic haemoglobins.

Materials and methods

The crystallographic coordinates of cytochrome b_5, adult haemoglobin and embryonic haemoglobin Gower II were obtained from the Protein Data Bank (1CYO, 1BBB and 1A9W, respectively). Disordered amino acids in the crystal structure of haemoglobin Gower II ($\alpha_2\epsilon_2$) were inserted using the graphics program O [36]. For haemoglobin Portland ($\zeta_2\gamma_2$), the globin ζ chain was prepared by computer mutation of the α chain using O. The γ chain structure was obtained from the three-dimensional structure of haemoglobin Barts (γ_4) (R.D. Kidd et al., in preparation). In all cases where protein structures were modified, the resulting protein structure was energy minimized using CNS [37]. All haemoglobin structures used were in the met (ferric) or isostructural ferrous liganded form.

Simulations of electron transfer complex formation were performed using the software developed by Northrup and his colleagues [4, 5, 6, 8, 38, 39]. The simulation process occurs in a number of related steps. The charge on the partner proteins (reduced cytochrome b_5 and ferric haemoglobin) are first calculated using Tanford-Kirkwood principles extended to include amino acid solution accessibility [40, 41]. The resulting charge distribution is then used to determine the electrostatic field surrounding the proteins with the Poisson-Boltzman equation using the Warwicker-Watson method [42]. Brownian motion of the partner proteins within the electrostatic fields is then simulated by employing the Ermak and McCammon algorithm [43]. In order to obtain statistically meaningful outcomes, 10,000 Brownian trajectories were calculated for each simulation. Each simulation was performed on a dual processor SGI Octane and required approximately 9 h c.p.u. time. All simulations were performed at an ionic strength of 0.1, a temperature of 25 °C and a pH of 6.2. Such simulations typically produced 400–700 electrostatically docked complexes. From the results of the docking simulations it is not only possible to identify the structures of the docked complexes but also the frequencies with which any particular amino acid participates in complex formation. Furthermore, it is possible to predict a second-order rate constant for the electron transfer process. We have chosen to use an exponential distance-dependent rate equation as a good approximation of the electron transfer process between two redox centers contained within the partner proteins [44]. This model requires only the distance information generated from the Brownian motion simulation and an appropriate distance decay factor [45]. In order to evaluate an appropriate distance decay factor for this system, we randomly chose 50 individual complexes from the cytochrome b_5-$\alpha_2\beta_2$ trajectories and evaluated the efficiency of electron transfer in each complex using the structure-specific analysis of electron tunneling in proteins developed by Onuchic and Beratan [10, 46, 47, 48]. This set of data was then fitted to an exponential decay model to yield a value of the distance-dependent decay factor, appropriate for this ensemble of complexes, that was then incorporated with the Brownian motion simulation data to provide predicted second-order rate constants for the electron transfer process.

Results

Cytochrome b$_5$-α$_2$β$_2$ reactions

Brownian motion simulation of 10,000 reaction trajectories for the reaction of cytochrome b_5 with oxidized adult human haemoglobin in the absence of molecular electrostatic fields yielded 120 docked complexes exhibiting only moderate rates of intramolecular electron transfer for the ensemble. In the presence of the molecular electrostatic fields, Brownian motion simulations for the reaction of reduced cytochrome b_5

Fig. 1 The results of Brownian simulation of the docking of cytochrome b_5 and adult haemoglobin. The center of mass of each member of the docked ensemble of cytochrome b_5 molecules is shown as a *red sphere*. The haemoglobin molecule is oriented with the α chain at the *bottom left* and β chain at the *top right* of the figure
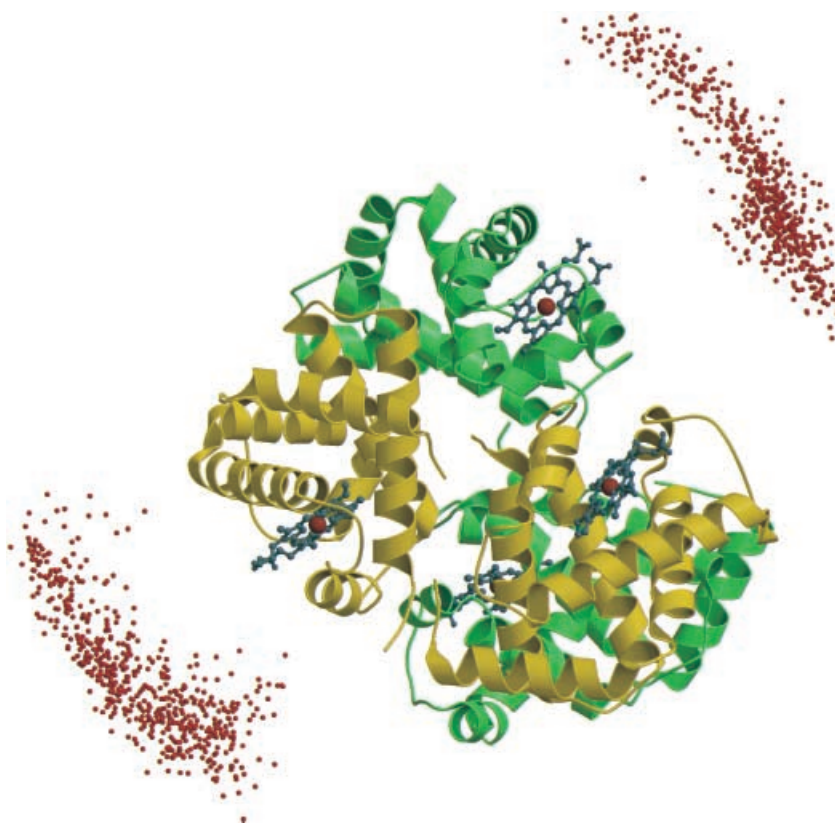
with each of the globin chains in adult human ferric haemoglobin yielded 500–700 docked complexes for each chain (Fig. 1). Inspection of the structures of this set of complexes clearly shows that rather than predicting the formation of a single complex, or even an overwhelmingly common complex, the calculations predict the formation of an ensemble of complexes. Examination of the constituent complexes indicates that the proteins have a common relative orientation, interacting at common surfaces, whilst exhibiting a range of detailed structural differences. Further analysis of the ensemble of complexes allows us to identify those amino acids which have a significant role in complex formation, together with the frequency with which they appear in the binding surface of the ensemble. Such an analysis (Table 1) clearly indicates that the general pattern of interaction involves a set of acidic surface residues surrounding the exposed haem edge of cytochrome b_5 and a set of basic residues surrounding the exposed haem edges of the haemoglobin molecule (Fig. 2). Repeating the simulations using both the adult human haemoglobin ($\alpha_2\beta_2$) and the human embryonic protein Gower II ($\alpha_2\epsilon_2$) suggests that the nature of the partner globin present in the haemoglobin molecule has very little effect on the formation of cytochrome b_5-α globin complexes, with the average energy of interaction being essentially identical in the two cases (Table 1).

Energy of interaction and rates of electron transfer within cytochrome b_5-$\alpha_2\beta_2$ complexes

Using the analysis of electron transfer pathways developed by Beratan and Onuchic [10], we observe that cytochrome b_5 shows a "hot spot" for electron transfer identified with the exposed haem edge. Likewise, the globin chains of haemoglobin exhibit a "hot spot" for electron transfer surrounding the exposed haem edge. Closer inspection of a sample of 50 individual complexes identified a wide range of binding energies for the docked complexes of –8.7 to +0.8 kcal mol^{-1}. For each of these complexes we have determined the electron transfer efficiency and compared the predicted best pathway for electron transfer between the two proteins (see, for example, Fig. 3A and B). Interestingly, no correlation is found between the predicted binding energy and the efficiency of the electron transfer process (Fig. 4). By comparison, a reasonable correlation is found between the efficiency of the electron transfer process and the distance of approach between the haem groups on the reacting proteins (Fig. 5). The data of Fig. 4 yields a decay factor of 1.4 Å for the distance-dependent rate of electron transfer, from which we calculate that the rate of electron transfer from reduced cytochrome b_5 to either α globin or β globin is essentially identical (1.15×10^5 M^{-1} s^{-1}), irrespective of its partner chain

Table 1 Amino acids and their frequencies at the interface of docked complexes

b5-α2ε2 docking, α active		b5-α2β2 docking, α active		b5-ζ2γ2 docking, ζ active	
Residue	Freq.	Residue	Freq.	Residue	Freq.
Haem	1[a]	Haem	1	Haem	1
Asp85	0.5	Asp85	0.16	Lys82	0.3
Lys90[b]	0.3	Lys90[b]	0.3	Glu85	0.3
ε Asp43[c]	0.15	β Glu43	0.2	Lys61	0.3
Lys6[b]	0.2	Lys61[b]	0.25	γ Asp43	0.2
His45	0.15	His45	0.2	His45	0.2
Asp64	0.1	Asp64	0.2	Asp22	0.1
Arg92	0.07	Arg92	0.1	Asp68	0.09
Lys60[b]	0.06	Lys60[b]	0.2	Arg92	0.09
Asp47	0.05	Asp47	0.1	Asp47	0.08
Asp75	0.02	Asp75	–	γ Lys59	0.06
Av. energy[d]	−2.88		−2.79		−0.71
Rate const.[e]	1.1×10^5		1.1×10^5		7.9×10^3

b5-α2ε2 docking, ε active		b5-α2β2 docking, β active		b5-ζ2γ2 docking, γ active		Amino acids of b5 used in docking	
Residue	Freq.	Residue	Freq.	Residue	Freq.	Residue	Freq.
Haem	1	Haem	1	Haem	1	Haem	1
Lys59	0.29	Lys59[b]	0.4	Lys95	0.30	Glu44[b]	0.23
Lys66	0.28	Lys95[b]	0.35	Lys59	0.30	Glu48[b]	0.17
Glu90	0.27	Glu90	0.32	Lys66	0.30	Glu38	0.17
Lys95	0.27	Glu43	0.29	Asp43	0.17	Asp66	0.17
Asp43	0.18	Arg40	0.17	Lys65	0.15	Asp60[b]	0.16
Lys87	0.17	Lys66[b]	0.14	Glu90	0.12	Glu56	0.07
Lys65	0.08	Asp94	0.09	Glu21	0.11	Glu43[b]	0.07
Gly73	0.06	α His45	0.08	Asp73	0.10	Glu37	0.04
Arg40	0.06	Lys65[b]	0.08	Asp94	0.07	Glu69	0.03
Glu21	0.06	Asp47	0.07	Lys76	0.03	Glu59	0.02
Asp94	0.05	Asp21	0.07	ζ His45	0.02		
Av energy[d]	−4.9		−2.86		−2.4		
Rate const.[e]	1.7×10^7		1.2×10^5		8.4×10^4		

[a]Frequencies are normalized to haem = 1
[b]Refers to residues identified by Poulos and Mauk [29]
[c]Prefix Greek symbols refer to residues on partner chains
[d]Average energies are kcal mol^{-1}
[e]Rate constants are M^{-1} s^{-1}

(Table 1). It is interesting to note that this rate is almost exactly one order of magnitude greater than that calculated for the complexes in the absence of the molecular electrostatic fields.

Comparison of α and ζ globin-cytochrome b5 complex formation

When reduced cytochrome b_5 reacts with the ζ chain of haemoglobin Portland ($\zeta_2\gamma_2$), significant differences from the reactivity with the adult haemoglobin ($\alpha_2\beta_2$) are predicted. The average binding energy is reduced from −2.8 kcal mol^{-1} to only −0.7 kcal mol^{-1} and the predicted second-order rate constant for intermolecular electron transfer is reduced from 1.5×10^5 to 7.9×10^3 M^{-1} s^{-1} (Table 1). These drastic changes are correlated with a significant change in the pattern of amino acids involved in complex formation. In particular, the significant roles of Lys90 and Lys60 in the interactions involving cytochrome b_5 and the α globin protein are lost and replaced by an interaction in the ζ globin involving Lys82. It is also interesting to note that in the case of complex formation between cytochrome b_5 and embryonic haemoglobin Portland ($\zeta_2\gamma_2$), significant interactions involve participation of amino acids from the partner γ chain not apparent when β is the partner chain in the adult protein (Table 1).

Comparison of γ, ε and β globin-cytochrome b5 complex formation

The ensemble of complexes formed between γ globin and reduced cytochrome b_5 exhibits slightly lower binding energies and second-order rate constants of electron transfer than the β complexes (Table 1). The ε chain, on the other hand, shows a marked increase in average binding energy for the ensemble of complexes, together with a predicted second-order rate of electron transfer for the reaction with cytochrome b_5 which is nearly two orders of magnitude greater than that for the β chain. In the case of the γ globin, the major difference between the frequency with which particular amino acids appear
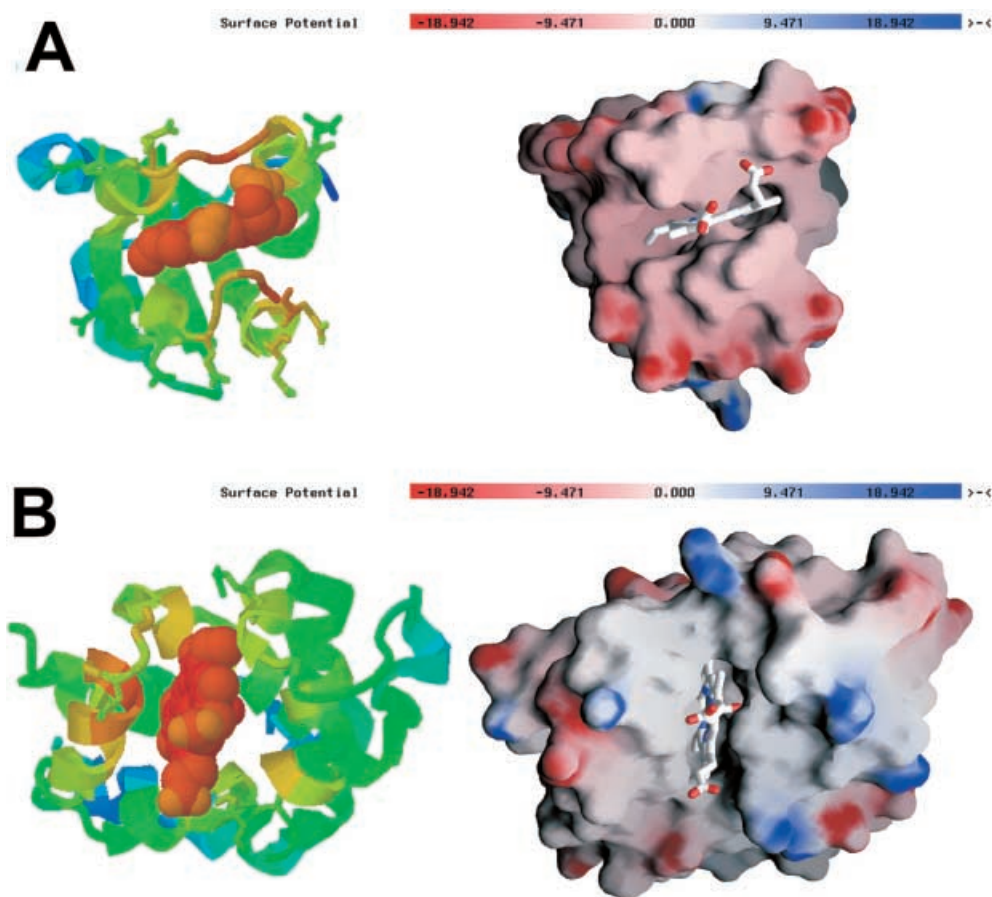
Fig. 2 Representations of the molecular characteristics of **A** cytochrome b_5 and **B** β globin. The figure on the *left*, in each case, shows the positions of the amino acids most commonly involved in docking (shown in "stick" representation) overlayed on the molecule showing electron transfer decay coupling (coupling is identified by colour with *red* indicating a coupling of $> 10^{-1}$ graded through to *blue* which indicates a coupling of $> 10^{-10}$) calculated using HARLEM [10]. The figure on the *right*, in each case, indicates electrostatic potential mapping of the surface of each molecule obtained using GRASP [51], with the potential scale indicated above each figure

within the γ complexes and the β complexes appears to be the relatively high frequency of appearance of the very basic Arg40 in the β complexes, whereas Lys65 appears to play the comparable role in the case of the γ complexes. In the case of the ϵ chain, the higher binding energy and rate of electron transfer appears to correlate with the involvement of an additional basic group provided by Lys87 in the ϵ chain, which appears in the cytochrome b_5-ϵ globin complexes with high frequency (Table 1).

Discussion

The outcome of our calculations using Brownian motion simulation of the interaction of cytochrome b_5 with human adult haemoglobin predicts that the binding of the two proteins leads to the formation of an ensemble of complexes rather than the production of a single or

common reaction intermediate as proposed by Poulos and Mauk [29]. Although a range of structures is predicted, all of the structures show an interaction interface consisting of a limited number of amino acids from each partner protein. Furthermore, each of these interfaces shows the common structural feature of a central haem core surrounded by polar residues which interact primarily via charge-charge interaction [49]. As suggested by Poulos and Mauk, the most important residues consist of a set of acid residues on cytochrome b_5 and a set of basic residues on haemoglobin (Table 1). However, in contrast to the predictions made by Poulos and Mauk, our analysis fails to identify the presence of α Lys56 or β Lys61 within any of the complexes. Our simulation of the interaction of cytochrome b_5 with the whole haemoglobin molecule indicates that the formation of any particular complex may also involve some contribution from the partner chain (Table 1) (this type of interaction was not predicted by Poulos and Mauk, who investigated only the reactions of cytochrome b_5 with isolated globin chains). Closer study of the individual complexes, in terms of the correlation between electron transfer efficiency and binding energy or haem-haem separation, shows that binding energy is not a good indicator of efficiency of electron transfer. Indeed, many of the individual tighter binding complexes show the lowest efficiency of electron transfer (Fig. 4). A comparison of our data with that reported by Mauk

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Fig. 3 The best pathway, calculated using HARLEM [10], for the transfer of an electron from cytochrome b_5 (on the *right*) to the ferric haemoglobin β chain (on the *left*) is shown in **A** for a complex with a binding energy of –8.7 kcal mol^{-1} and an electron transfer efficiency of 6.2×10^{-12} and in **B** for a complex with a binding energy of –0.8 kcal mol^{-1} and an electron transfer efficiency of 2×10^{-5}. The parts of the pathway involving covalent bonds are shown in *thick colour* and the through-space components are shown as *thin red lines*
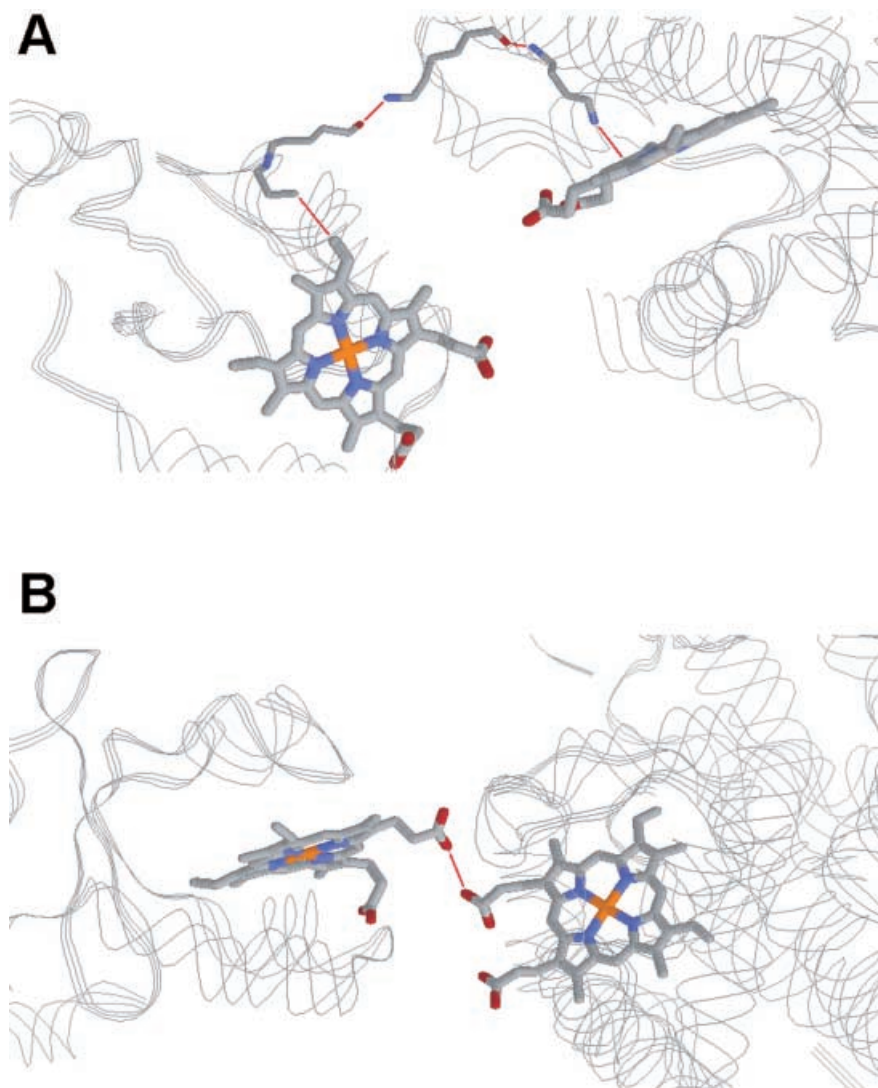
Fig. 4 The relationship between binding energy and electron transfer efficiency in complexes between cytochrome b_5 and haemoglobin β chain
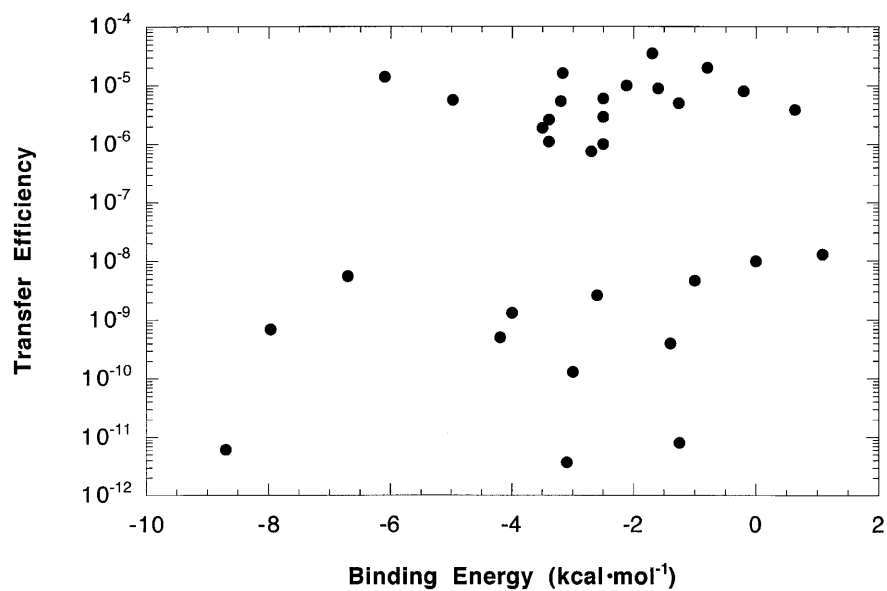
Fig. 5 The relationship between haem-haem distance and electron transfer efficiency in complexes between cytochrome b_5 and haemoglobin β chain. The distance represents the shortest haem edge-haem edge distance achieved in the complex, minus a constant shift factor of 5 Å to take account of the haem edge-iron distance in a perfect edge-to-edge contact (see [5, 8])
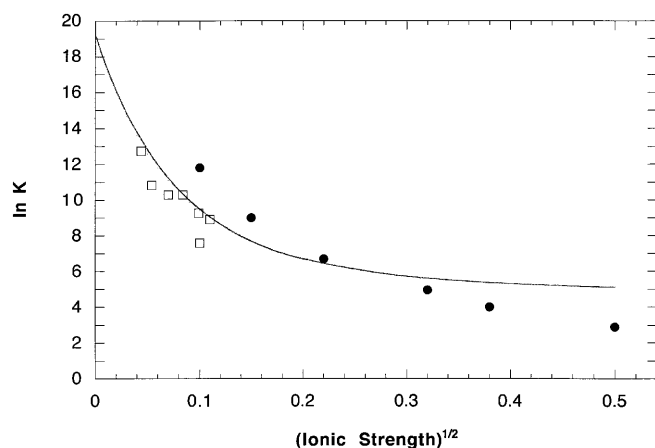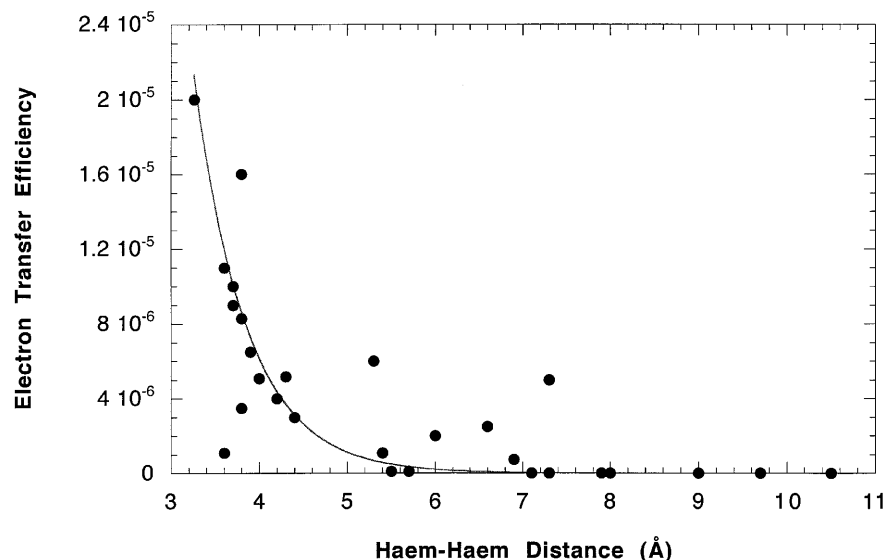
Fig. 6 The effect of ionic strength on the binding of cytochrome b_5 to adult haemoglobin. The calculated binding constant for the interaction of ferrous cytochrome b_5 with ferric adult haemoglobin (●) is shown compared with that measured for the interaction of ferric cytochrome b_5 and ferric haemoglobin (□), taken from [10]. The *line* shows the best fit of the data of [10] to a simple Debye-type function

and Mauk [27] for the interaction of the ferric forms of the proteins (Fig. 6) supports the view that the binding of cytochrome b_5 and haemoglobin in both oxidation states arises from charge-charge interactions and accounts for the marked ionic strength dependence observed for the reaction [28]. The rather moderate binding energies of these complexes, together with the diversity of detailed structure presented in the ensemble, almost certainly accounts for the lack of success in attempts to crystallize such docked complexes. By comparison, the haem-haem distance does show a reasonable correlation with electron transfer efficiency (Fig. 5) and allows us to predict that the reduction of both chains of adult haemoglobin should occur at very similar rates, so avoiding the production of mixed va-

lence intermediate species. Unfortunately, no independent experimental second-order rate constants for the process of electron transfer from cytochrome b_5 to haemoglobin have been reported.

Although the embryonic γ chain shows properties quite similar to the β chain of the adult protein, the embryonic ϵ and ζ chains exhibit markedly different properties to the adult β and α chains, respectively (Table 1). In terms of the important amino acids involved in the interaction of cytochrome b_5 with the embryonic ϵ chain, most of the functional differences appear to arise from the presence of a Lys residue at position 87 in the ϵ chain (β Thr). The extra positive charge, which appears in nearly 20% of the complexes, raises both the average binding energy and the rate of electron transfer. In the case of the ζ chain, three significant alterations in amino acid sequence appear to impact on the formation and properties of the cytochrome b_5-haemoglobin complexes. In the ζ chain, Ile and Ser occupy positions 90 and 60 of the amino acid sequence, respectively, whereas these amino acids are both Lys in the α chain. The potential impact of these differences appears to be somewhat moderated by the replacement of α Ala82 by ζ Lys82. Nevertheless, the ζ chain shows a markedly lower average binding energy and rate of electron transfer.

The embryonic globin chains thus show distinct reactivities towards cytochrome b_5. At present it is unclear whether this difference in reactivity impacts on the biological functioning of these important oxygen-carrying proteins during the first few weeks of human life. The embryonic haemoglobins occur only from week three to twelve of gestation, during which time these three proteins must equip the developing fetus with both oxygen scavenging and transport functions. We have previously shown that the embryonic haemoglobins are more resistant than adult haemoglobin to both auto-oxidation

and nucleophile stimulated oxidation [50]. This fact, taken together with their rather short biological lifetime, may well mean that the reactivity of these proteins with the cytochrome b_5 reducing system is of little biological significance and the amino acid differences seen between these proteins and the adult protein may relate to different oxygen-binding requirements. Nevertheless, our investigations of the reactivities of the embryonic globins have given us good insight into the roles of specific amino acids in the formation of cytochrome b_5-haemoglobin complexes and their impact on the rate of intramolecular electron transfer. At a more general level, our data clearly identify the origins of our inability to isolate complexed proteins in crystalline form as arising from the existence of an essentially iso-energetic ensemble of reaction intermediates.

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