Biochimica et Biophysica Acta, 725 (1983) 409–416
Elsevier

BBA 41413

THE EFFECT OF IRON-HEXACYANIDE BINDING ON THE DETERMINATION OF REDOX POTENTIALS OF CYTOCHROMES AND COPPER PROTEINS

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(Received June 28th, 1983)

Key words: Cytochrome; Copper protein; Redox potential; Azurin; Ligand binding; Iron hexacyanide

The midpoint redox potentials of *Pseudomonas aeruginosa* cytochrome *c*-551 and *Rhodopseudomonas viridis* cytochrome c_2 were measured as a function of pH in the presence of *Euglena* cytochrome *c*-558 and the results compared with those obtained in the presence of ferro-ferricyanide. The pattern of pH dependence observed for the two bacterial cytochromes was the same whether it was measured by equilibrium with another redox protein or with the inorganic redox couple. Thus, the pH dependence of redox potential is not a consequence of pH-dependent ligand binding. The midpoint potential of *Ps. aeruginosa* azurin was measured as a function of pH using both ferro-ferricyanide mixtures and redox equilibrium with horse cytochrome *c* or *Rhodopseudomonas capsulata* cytochrome c_2. In this case also the pattern of pH dependence obtained did not vary with the redox system used and it closely resembled that of *Ps. aeruginosa* cytochrome *c*-551. This is consistent with the observation that the equilibrium between cytochrome *c*-551 and azurin is relatively independent of pH. An equation was derived which described pH-dependent ligand binding and which can produce theoretical curves to fit the experimental pH dependence of redox potential for both cytochrome and azurin. However, the pronounced effect on such curves produced by varying the ligand association constants, and the insensitivity of the experimental data to changes in ionic strength, suggest that ligand binding effects do not account for the pH dependence of redox potential.

Introduction

The ferro-ferricyanide couple has been widely used in the determination of redox potentials of high-potential electron-transfer proteins [1–4] and ferricyanide is commonly used as a mediator in the anaerobic titration of unfractionated systems and oxidases [5–8]. Yet it has become clear that ferricyanide binds to horse cytochrome *c* [9–12] and if differential binding occurred for the two redox states then a perturbation of redox potential would be observed according to the equation derived by Dutton [13]:

$$E_m = E_{m(NL)} + \frac{0.06}{n} \log \frac{1 + K_R[L]}{1 + K_o[L]} \quad (1)$$

where $E_{m(NL)}$ is the midpoint potential in the absence of ligand and K_R and K_o are association constants for the binding of ligand (L) to the protein.

In our studies of bacterial cytochrome *c* a principal interest has been the pH dependence of redox potential and we have interpreted this in terms of ionisations in the protein which are influenced by the redox state of the haem. We have recently distinguished two groups of cytochromes in this respect, one of which is characterised by the redox-linked ionisation of a haem propionic acid and is exemplified here by *Pseudomonas* cytochrome *c*-551 [14,15], while the second group is characterised by a redox-linked deprotonation of a

histidine and is represented here by *Rhodopseudomonas viridis* cytochrome c_2 [16,17]. However, the possibility exists that the apparent pH dependence is due simply to differential binding of ferricyanide to protonated and unprotonated forms of the proteins and that this pH dependence would disappear if the redox potential could be determined by another method.

Such an explanation has been proposed by Lappin et al. [18] for *Ps. aeruginosa* azurin on the basis of the variations in the pH dependence of redox kinetics using different redox agents. These authors propose that with appropriate redox agents the midpoint potential of azurin would be independent of pH. If this is true for azurin it may also hold for *Ps. aeruginosa* cytochrome *c*-551, since the redox equilibrium between the two is known to be relatively pH independent [19,20].

In this paper we present evidence that the pH dependence of redox potential observed for some *c*-type cytochromes and azurin is not due to pH-dependent ligand binding.

Methods

Preparation of redox proteins

Azurin ($A_{625}/A_{280} = 0.5$) and cytochrome c-551 ($A_{550(red)}/A_{280} = 1.15$) were prepared from cells of *Ps. aeruginosa* and cytochrome *c*-551 ($A_{551(red)}/A_{280} = 1.35$) was prepared from *Ps. stutzeri* strain 221 essentially as described by Ambler [21,22]. *Euglena gracilis* cytochrome *c*-558 was purified as described by Pettigrew et al. [23] and cytochrome c_2 from *Rhodopseudomonas capsulata* strain *St. Louis* was prepared essentially as described by Bartsch [24]. Horse cytochrome *c* (type VI) was from Sigma.

Measurement of redox potentials using ferri-ferrocyanide mixtures

Midpoint redox potentials were determined by spectrophotometric examination of the redox state of the protein on a Cary 219 spectrophotometer in ferri-ferrocyanide solutions of known potential [25,4]. Buffers used were 3.3 mM in acetate, phosphate Tris or borate and the pH was determined at the redox equilibrium before addition of dithionite. Under these conditions, with an ionic strength contribution of 5 mM from the 0.5 mM ferrocyanide and 2–10 mM from the buffers, the ferri-ferrocyanide midpoint potential can be taken from Fig. 1 in Ref. 25. Some experiments were carried out in the presence of 0.1 M NaCl and under these conditions the midpoint potential of ferri-ferrocyanide was taken as 430 mV [3,26]. This should be regarded as an approximate figure as our conditions are not those described in these references, but the absolute value of E_m does not affect the relative pH-dependent patterns that we wish to emphasise.

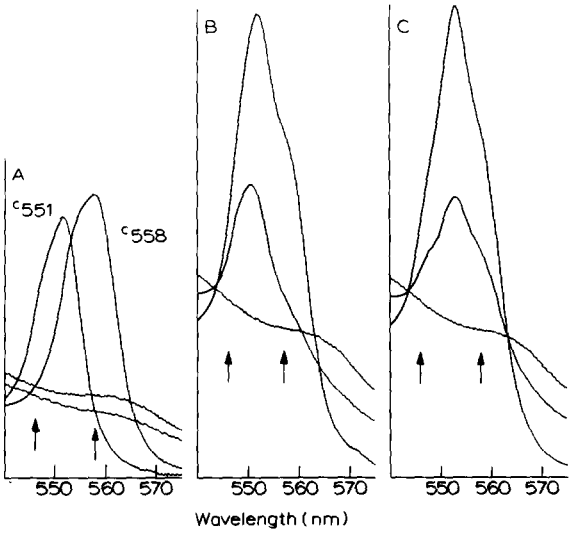

Fig. 1. Determination of the redox potential of *Ps. aeruginosa cytochrome c*-551 by mixture with *Euglena* cytochrome *c*-558. (A) Visible spectra of the oxidised and reduced forms of cytochromes *c*-551 and *c*-558. Arrows indicate isosbestic points at 546 nm (for cytochrome *c*-558) and 558 nm (for cytochrome *c*-551) at pH 8. (B, C) Spectra at redox equilibria after the addition of ferricyanide and after the addition of dithionite to a mixture of ferrocytochrome *c*-551 and ferricytochrome *c*-558 in $3.3 \cdot 10^{-3}$ M acetate, pH 5.17 (B), and $3.3 \cdot 10^{-3}$ phosphate, pH 7.91 (C). Arrows indicate isosbestic points for the individual cytochromes.

Measurement of redox potentials using mixtures with Euglena cytochrome c-558

Ps. aeruginosa cytochrome *c*-551, or cytochrome c_2 from *Rps. viridis* was reduced by millimolar ascorbate and excess reductant removed by passage through Sephadex G-25 (fine) equilibrated in 10 mM NaCl, 0.5 mM EDTA, 0.5 mM sodium phosphate, pH 7. A redox equilibrium was estab-

lished and spectrophotometrically recorded after mixing ferrocytochrome *c*-551 or c_2 with ferricytochrome *c*-558 in 3.3 mM acetate, phosphate, Tris or borate buffer. Two examples of such equilibria are shown in Fig. 1. After measurement of pH, a crystal of ferricyanide was added to achieve complete oxidation followed by a few grains of sodium dithionite for complete reduction. In the case of cytochrome *c*-551 and *c*-558 shown in Fig. 1, measurements at 546 nm (an isosbestic point for the cytochrome *c*-558) allow calculation of the redox state of cytochrome *c*-551 and, conversely, measurements at 557–558 nm (an isosbestic point for cytochrome *c*-551, the exact position of which was dependent on the pH) allow calculation of the redox state of cytochrome *c*-558.

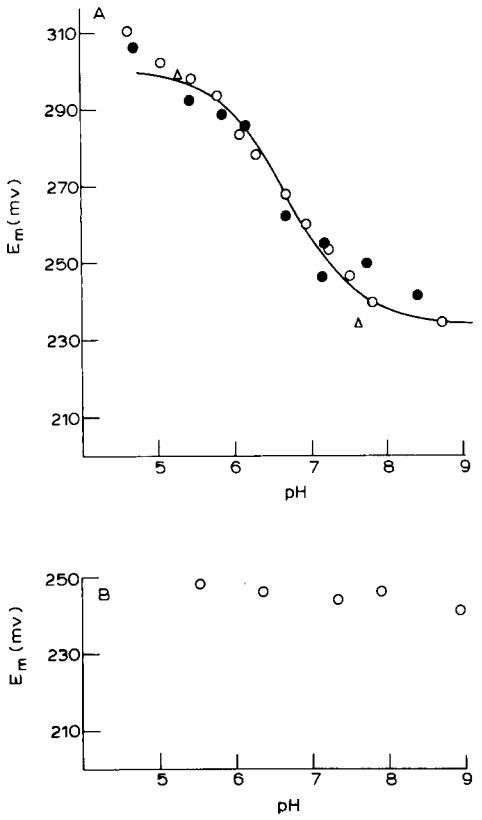

Fig. 2(A). The pH dependence of redox potential of *Ps. aeruginosa* cytochrome *c*-551. Cuvettes contained $2.5 \cdot 10^{-6}$ M cytochrome *c*-551 in $3.3 \cdot 10^{-3}$ M acetate, phosphate, tris or borate buffer. (○) Midpoint potentials measured in $5 \cdot 10^{-4}$ M ferrocyanide and $1.7 \cdot 10^{-5}$ M ferricyanide. (●) Midpoint potentials measured by mixture with $2 \cdot 10^{-6}$ M *Euglena* cytochrome *c*-558. (△) Midpoint potentials measured anaerobically in the presence of 10^{-5} M diaminodurol and a combination Pt/Ag/AgCl electrode. The solid line is a theoretical curve defined by Eqn. 2 with $pK_{ox} = 6.3$ and $pK_{red} = 7.2$ [15]. (B). The pH-independent redox potential of *Euglena* cytochrome *c*-558. At redox equilibrium the cuvettes contained $2.5 \cdot 10^{-6}$ M cytochrome *c*-558, $5 \cdot 10^{-4}$ M ferrocyanide, $8.3 \cdot 10^{-6}$ M ferricyanide and $3.3 \cdot 10^{-3}$ M acetate, phosphate, Tris or borate buffers.

After mixing, spectra were always recorded twice, immediately and after 1 min, to ensure stable redox equilibria had been achieved. From determination of the redox equilibrium constant the difference between the midpoint potentials of cytochrome *c*-558 and its partner in the mixture could be calculated. The midpoint potential of cytochrome *c*-558 was independently determined in ferri-ferrocyanide solutions to be 245 mV with very little variation with pH (Fig. 2B).

Measurement of redox potentials in mixtures of cytochromes and azurin

It is not possible to determine accurately the redox state of the proteins in a cytochrome/azurin mixture because neither protein has an isosbestic point near the peak maximum of the other. However, the contribution of azurin to absorbance at 550 nm is small, as is the contribution of cytochrome to absorbance at 625 nm. The redox state of each protein could therefore be approximately calculated and any pH dependence of equilibria studied.

Ferrocytochrome *c* from horse, *Rps. capsulata* or *Ps. aeruginosa* was mixed with Cu(II)-azurin in 3.3 mM acetate, phosphate, Tris or borate buffer and redox equilibrium, ferricyanide-oxidised and dithionite-reduced spectra were recorded on the Cary 219 spectrophotometer.

Results

The pH dependence of redox potential of Ps. aeruginosa cytochrome c-551

The midpoint potential of *Euglena gracilis* cytochrome *c*-558 measured using mixtures of ferri- and ferrocyanide was essentially independent of pH between pH 5 and 9 (Fig. 2B). A stable redox equilibrium between this cytochrome and *Ps. aeruginosa* cytochrome *c*-551 was rapidly established in the absence of hexacyanide by mixing ferrocytochrome *c*-551 with ferricytochrome *c*-558 and the midpoint potential of the cytochrome *c*-551

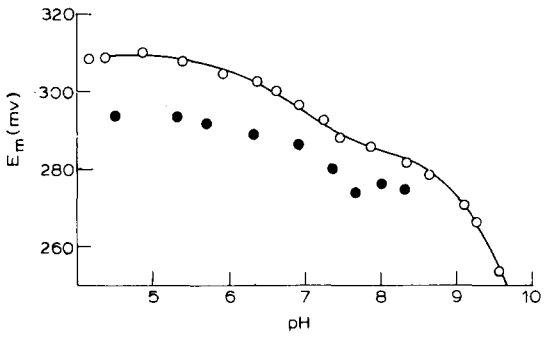

Fig. 3. The pH dependence of redox potential of *Rps. viridis* cytochrome c_2. (○) Results obtained in the presence of $5 \cdot 10^{-4}$ M ferrocyanide and $1.7 \cdot 10^{-5}$ M ferricyanide, 10^{-3} M acetate, phosphate, Tris or glycine buffers [16]. The solid line is a theoretical curve defined by the equation $E_m = E + (0.06/n)\log\{([H^+]^2 + K_R[H^+])/([H^+]^2 + K_{01}[H^+] + K_{01}K_{02})\}$, derived using the procedures of Clark [26] with $pK_{01} = 6.7$, $pK_R = 7.1$ and $pK_{02} = 9.2$. (●) Midpoint potentials measured by mixture with *Euglena* cytochrome *c*-558. The cuvette contained $2 \cdot 10^{-6}$ M cytochrome *c*-558, $2.5 \cdot 10^{-6}$ M cytochrome *c*-551, 0.1 M NaCl and $3.3 \cdot 10^{-3}$ M acetate, phosphate or Tris buffer.

was calculated from the mixed spectra (Fig. 1) assuming $E_m(c\text{-}558) = 245$ mV [23]. The derived pH dependence of E_m for cytochrome *c*-551 is shown in Fig. 2A and compared with published results obtained in the presence of ferri-ferrocyanide.

The midpoint potential of cytochrome *c*-551 was also determined using diaminodurol and a Pt/Ag/AgCl combination electrode with the results shown in Fig. 2A. $Ru(CN)_6^{4-}$ or $Co(CN)_6^{3-}$ (final conc. 0.5 mM) had no effect on the redox equilibrium in these experiments.

The pH dependence of redox potential of Rps. viridis cytochrome c_2

Stable redox equilibria were established at different pH values by mixing ferrocytochrome c_2 from *Rps. viridis* and ferricytochrome *c*-558 from *Euglena gracilis*. Midpoint potentials were calculated for the cytochrome c_2 assuming $E_m(c\text{-}558) = 245$ mV and these are shown in Fig. 3 in comparison with published results obtained in the presence of ferri-ferrocyanide. It was necessary to conduct the experiments in the presence of 100 mM NaCl because in the absence of added salt the variations in ionic strength for phosphate buffer at different pH values led to perturbations of redox potential superimposed on those caused by pH differences alone. This effect was much less pronounced for the original measurements with ferri-ferrocyanide [16], where the dominant components of ionic strength are the hexacyanides themselves.

The pH dependence of redox potential of Ps. aeruginosa azurin

The midpoint potential of azurin was measured by single point mixtures with known ferri-ferrocyanide concentrations at different pH values and under two conditions of total ionic strength. An extinction coefficient of 3.5 $mM^{-1} \cdot cm^{-1}$ at 625 nm for azurin [19] was used in calculations. This figure is controversial but application of the highest literature value of 5.9 $mM^{-1} \cdot cm^{-1}$ [18] made very little difference to the calculated potentials. Theoretical curves were constructed using the equation:

$$E_m = \tilde{E} + \frac{0.06}{n}\log\frac{K_{red} + [H^+]}{K_{ox} + [H^+]} \qquad (2)$$

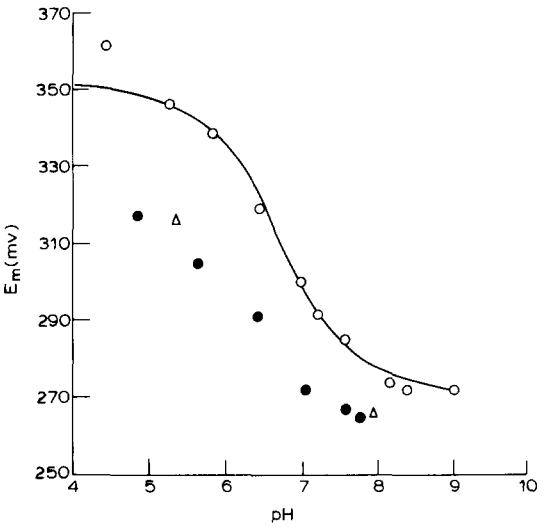

Fig. 4. The pH dependence of redox potential of azurin. The cuvette contained $5 \cdot 10^{-6}$ M azurin. (○) Midpoint potentials measured in $5 \cdot 10^{-4}$ M ferrocyanide and $3.3 \cdot 10^{-5}$ M ferricyanide, $3.3 \cdot 10^{-3}$ M acetate, phosphate, Tris or borate buffer. The solid line is a theoretical curve defined by Eqn. 2 with $pK_0 = 6.1$ and $pK_R = 7.4$. (●, △) Midpoint potentials measured by mixture with horse cytochrome *c* (●) and *Rps. capsulata* cytochrome c_2 (△) (each $2.5 \cdot 10^{-6}$ M) in $2 \cdot 10^{-3}$ M acetate, phosphate or Tris buffers.

in which K_{ox} and K_{red} are proton dissociation constants for a group in the two redox states and $\tilde{E}$ is the midpoint potential at acid pH [15]. Midpoint potentials were also calculated from equilibrium mixtures of horse cytochrome *c* and azurin, and *Rps. capsulata* cytochrome c_2 and azurin (Fig. 4). These cytochromes have pH-independent redox potentials [4] and thus pH-dependent redox equilibria with azurin must be due to pH dependence of the azurin itself. The rate of reaction of ferrocytochrome *c* with Cu(II)-azurin was very dependent on the nature of the cytochrome. That with horse cytochrome *c* was very slow ($t_{1/2} = 45$ s at pH 5 and 29 s at pH 7.4), while those with *Rps. capsulata* cytochrome c_2 and *Ps. aeruginosa* cytochrome *c*-551 were too rapid to measure without rapid reaction techniques.

The redox equilibrium of *Ps. aeruginosa* cytochrome *c*-551 and azurin was measured at several pH values (Fig. 5) and expressed as a difference in midpoint potentials in Fig. 6A. Comparison with such differences calculated from ferri-ferrocyanide experiments on the separate proteins (Fig. 6A) indicates that the equilibrium is essentially independent of pH and that ferri-ferrocyanide does not perturb this equilibrium.

A similar analysis for *Ps. stutzeri* cytochrome *c*-551 and *Ps. aeruginosa* azurin is shown in Fig. 6B. As we report elsewhere [14] pK_0 and pK_R in *Ps. stutzeri* cytochrome *c*-551 are shifted to pH 7.6 and 8.3, respectively. This leads to a mismatch in

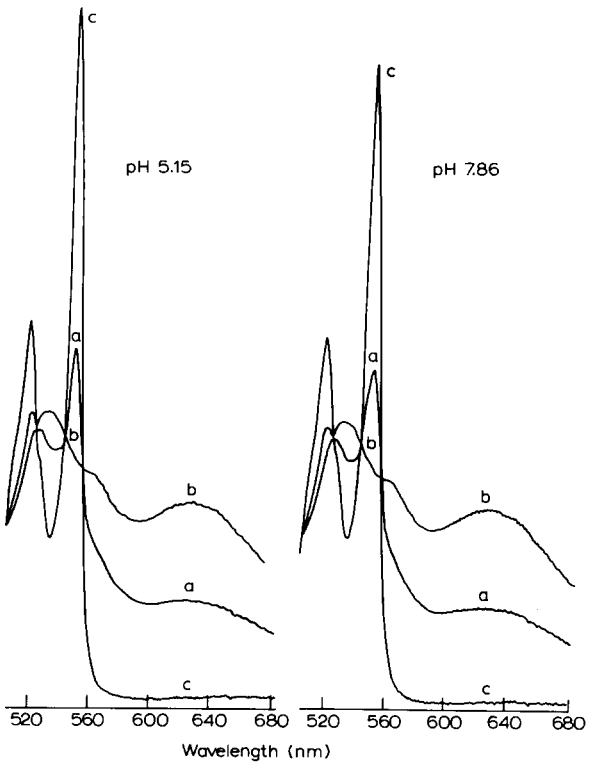

Fig. 5. The redox equilibrium of *Ps. aeruginosa* cytochrome *c*-551 and azurin. The cuvette contained $3 \cdot 10^{-3}$ M cytochrome *c*-551 and $5 \cdot 10^{-3}$ M azurin in $3.3 \cdot 10^{-3}$ M acetate, pH 5.15, or phosphate, pH 7.86. (a) Redox equilibrium achieved by adding ferrocytochrome *c*-551 to Cu II azurin. (b) Ferricyanide oxidised. (c) Dithionite reduced.

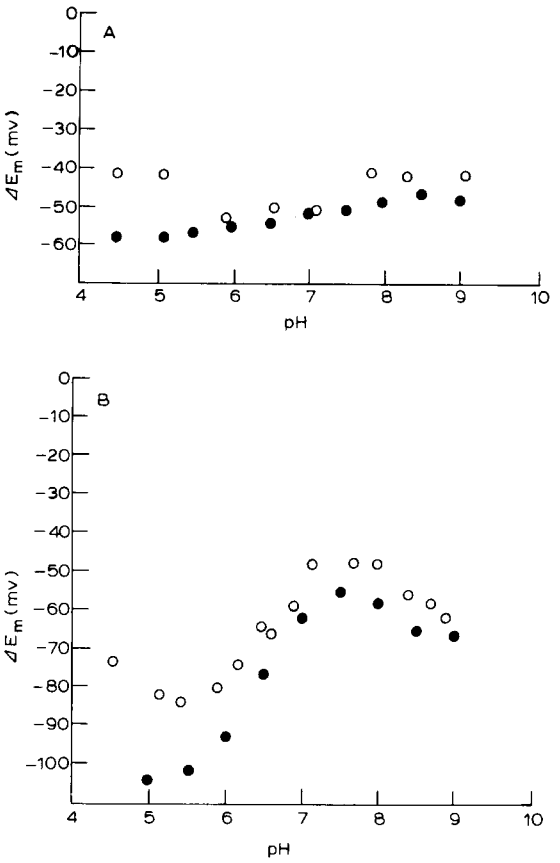

Fig. 6. pH dependence of the equilibria between *Ps. aeruginosa* cytochrome *c*-551 and *Ps. aeruginosa* azurin (A) and *Ps. stutzeri* cytochrome *c*-551 and *Ps. aeruginosa* azurin (B). (●) E_m (*c*-551) $- E_m$(azurin) calculated from separate determinations of midpoint potential in the presence of ferri-ferrocyanide. Data for (A) are from Figs. 2A and 4. Data for (B) appear elsewhere [14] and from Fig. 4. (○) E_m (*c*-551)$- E_m$ (azurin) calculated from redox equilibria of the type shown in Fig. 5.

the pH-dependent patterns of the cytochrome and the azurin and a pH-dependent equilibrium is observed which reflects the pH dependence of the azurin below pH 7.5 and the pH dependence of the cytochrome above this pH.

A Nernst equation incorporating pH-dependent ligand binding

The treatment of Dutton [13] describing the effect of ligand binding on redox potential can be extended to include pH-dependent ligand binding. If the simplifying assumption is made that a negative ligand such as ferri- or ferrocyanide binds only to the protonated state of the protein the following equilibria have to be taken into account:

$$\text{ox} + H^+ \rightleftharpoons \text{ox H},\ K_{OH} = \frac{[\text{ox H}]}{[\text{ox}][H^+]}$$

$$\text{red} + H^+ \rightleftharpoons \text{red H},\ K_{RH} = \frac{[\text{red H}]}{[\text{red}][H^+]}$$

$$\text{ox H} + L \rightleftharpoons \text{ox HL},\ K_{OL} = \frac{[\text{ox HL}]}{[\text{ox H}][L]}$$

$$\text{red H} + L \rightleftharpoons \text{red HL},\ K_{RL} = \frac{[\text{red HL}]}{[\text{red H}][L]}$$

Considering these, Eqn. 3 can be derived:

$$E_m = E_{m(NL)} + \frac{0.06}{n} \log \frac{1 + K_{RH}[H^+] + K_{RL}K_{RH}[H^+][L]}{1 + K_{OH}[H^+] + K_{OL}K_{OH}[H^+][L]} \qquad (3)$$

Using Eqn. 3 a theoretical curve was produced to fit the experimental data for cytochrome *c*-551 (the data for azurin can be fitted to the same equation using the same parameters – not shown). K_{OH} was set equal to K_{RH} so that the redox potential was not affected by differential proton binding in the two redox states, and the curve is therefore due to ligand binding effects only. With the parameters $K_{OH} = K_{RH} = 1.8 \cdot 10^6\ M^{-1}$, $[L] = 5 \cdot 10^{-4}$ M, $K_{RL} = 2.3 \cdot 10^4\ M^{-1}$ and $K_{OL} = 0$ the theoretical curve is that shown in Fig. 7. This of course is not a unique solution but it serves to demonstrate three important points. Firstly, in the absence of other evidence the experimental data can equally well be fitted by a pH-dependent ferri-ferrocyanide binding treatment as by a differential proton binding treatment (cf. Eqn. 2 and Fig. 2A). Secondly, the pK_R value (6.25) required to fit the experimental data to a pH-dependent ligand binding model is very different from the pK_R value (7.2) required for the H^+ binding model of Eqn. 2. Thirdly, a factor of 10 increase or decrease in K_{RL} or [L] produces dramatic changes in the theoretical curve (Fig. 7). We would expect that K_{RL} would be sensitive to ionic strength and therefore the pH dependence of redox potential of azurin and cytochrome *c*-551 was also measured in 0.1 M NaCl. Although the level of redox potential at a particular pH was altered, the pH-dependent pattern remained the same. In particular, the fall in mid-

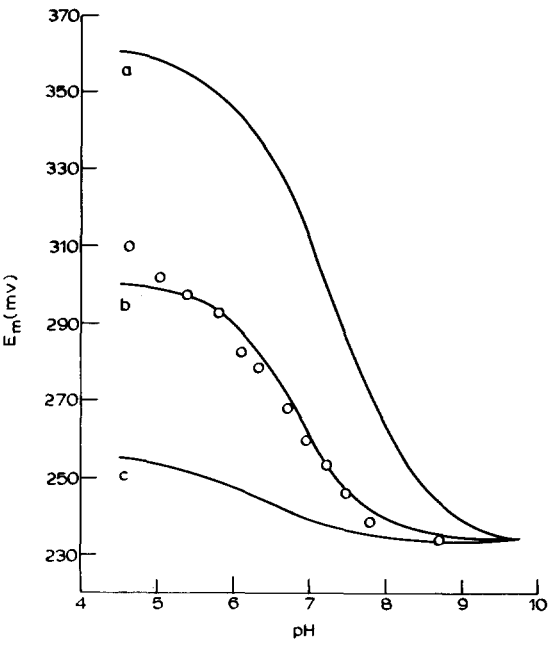

Fig. 7. Theoretical curve for pH dependent ligand binding. Solid lines were constructed from the equation:

$$E_m = E_{m(NL)} + \frac{0.06}{n} \log \frac{1 + K_{RH}[H^+] + K_{RL}K_{RH}[H^+][L]}{1 + K_{OH}[H^+] + K_{OL}K_{OH}[H^+][L]}$$

(Eqn. 3)

with the parameters defined in the text. All curves have $K_{OH} = K_{RH} = 1.8 \cdot 10^6\ M^{-1}$ and $[L] = 5 \cdot 10^{-4}$ M (the ferrocyanide concentration used in redox potential measurements). K_{OL} is set at 0 and K_{RL} is varied between $2.3 \cdot 10^5\ M^{-1}$ (a), $2.3 \cdot 10^4\ M^{-1}$ (b) and $2.3 \cdot 10^3\ M^{-1}$ (c). (○) Experimental points for *Ps. aeruginosa* cytochrome *c*-551 obtained in the presence of ferri-ferrocyanide (from Fig. 4).

point potential between pH 5 and pH 9 was not altered in the way predicted for a decline in K_{RL} at high ionic strength.

It might be argued that a more reasonable model would assign a value to K_{OL} greater than zero. When this was done with $K_{OL} = 10^4$, $K_{RL} = 1.5 \cdot 10^5$ a fall in E_m with pH of the correct magnitude was obtained from Eqn. 3. However, a pK_R of 5.5 was required in order to fit the experimental data, which is even further removed from the pK of 7.2 required for the H^+ binding model. If K_{OL} and K_{RL} were then raised or lowered to the same degree the theoretical curves shifted markedly above or below the experimental results.

Discussion

The patterns of pH dependence of redox potential for *Ps. aeruginosa* cytochrome *c*-551 are virtually identical whether the potential is measured by the ferri-ferrocyanide method at two different ionic strengths, the cytochrome *c*-558 mixture method or the diaminodurol method. To produce such effects by pH-dependent ligand binding would require that these very different redox agents have identical binding affinities for the cytochrome *c*-551. This is so unlikely that we therefore favour an explanation based on a single ionisable group, the pK of which differs in the two redox states, that difference being imposed by the electrostatic influence of the haem [4]. This interpretation is strongly supported by the pH dependence of the NMR spectrum and visible spectrum of cytochrome *c*-551 in the two redox states. From these studies, values of pK_{ox} and pK_{red} were independently derived which agree with those deduced from the redox potential analysis [15].

In the case of cytochrome c_2 from *Rps. viridis*, the pH dependence of redox potential [16] was consistent with a much smaller imposed separation of pK values (p$K_{ox} = 6.7$; p$K_{red} = 7.1$) and the only spectroscopic indicators of pH dependence were the proton resonances of a histidine residue (p$K_{ox} \simeq 6.8$, p$K_{red} \simeq 7.1$) [17]. Eley et al. [12] have shown that a histidine can form a pH-dependent binding site for ferricyanide in mitochondrial cytochrome *c* and therefore it was important to examine the pH dependence of redox potential of *Rps. viridis* cytochrome c_2 in the absence of ferri-ferrocyanide. The results of Fig. 3 indicate that under such conditions the pH dependence of redox potential is still observed. We therefore conclude that the deprotonation of histidine in *Rps. viridis* cytochrome c_2 results directly in the pH dependence of redox potential without pH-dependent ligand binding.

In their work on the pH dependence of kinetics of oxidation and reduction of azurin, Lappin et al. [18] found that ferricyanide oxidation of azurin was influenced by a group on the protein with a pK of 7.1 and ferrocyanide reduction by a group with a pK of 6.1. However, the kinetics of oxidation by tris[4,7-di(phenyl-4-sulphonate)-1,10-phenanthroline] Co(III) were pH independent and Lappin et al. predicted that the redox potential of azurin measured in the presence of this reagent would be independent of pH.

We have confirmed their observation that the redox potential of azurin measured in the presence of ferri-ferrocyanide is markedly pH dependent, but we observed a similar pH dependence in the presence of either horse cytochrome *c* or *Rps. capsulata* cytochrome c_2 as the redox partner in the absence of ferri-ferrocyanide (Fig. 4). Also we found that the equilibrium constant for *Ps. aeruginosa* cytochrome *c*-551/azurin mixtures was essentially independent of pH in agreement with previous work [19,20]. In view of the established pH dependence of cytochrome *c*-551 this implies a matching pH dependence for azurin. Because of the alkaline shift in pK_0 and pK_R in the case of *Ps. stutzeri* cytochrome *c*-551 such a matching is not obtained and the equilibrium constant in this case exhibits a pH dependence predictable from the separate patterns of pH dependence of the cytochrome and the azurin. Finally, the pattern of pH dependence of the azurin iron hexacyanide equilibrium was not affected by raising the ionic strength. These results appear to preclude pH-dependent ligand binding as the factor defining pH dependence of redox potential of azurin.

We therefore conclude that the pH dependence of redox potential for *Ps. aeruginosa* azurin may be interpreted in a similar way to that for cytochrome *c*-551. The difference in the electrostatic environment in the two redox states imposes a separation of pK values for a group on the protein in the vicinity of the redox centre. Our results suggest a group with a pK_{ox} of 6.1 and pK_{red} of

7.4, values in reasonable agreement with the kinetically derived pK_{ox} of 6.1 and pK_{red} of 7.1 [18]. Such an electrostatic explanation may be an oversimplification in the case of azurin because the deprotonation event (at least in the reduced protein) appears to be coupled to a conformational change. It has been shown that deprotonation of His-35 with a $pK_{red} \approx 7$ causes local structural rearrangements in the vicinity of the Cu and it may be this structural change rather than electrostatic interactions that lead to stabilisation of the oxidised protein with respect to the reduced form [27–29].

The pH independence of the kinetics of oxidation of azurin by tris[4,7-di(phenyl-4-sulphonate)-1,10-phenanthroline] Co(III) [18] might be a result of complications introduced by the pH-dependent conformational change of azurin. A minimal reaction scheme is:

$$\begin{array}{ccc} \mathrm{Az(I)} & \overset{k_f}{\rightleftharpoons} & \mathrm{Az^*(I)} + \mathrm{H^+} \\ k_o \downarrow & & \\ \mathrm{Az(II)} & & \end{array} \qquad (k_b)$$

where k_f and k_b are the rate constants for the conformational change in the reduced azurin (Az(I) and Az*(I), resp.) and k_o the oxidation rate constant.

If $k_f \gg k_b$ the pH-jump kinetic experiment used by Lappin et al. [18] does not detect the conformational change when $k_o \gg k_f$, conditions which apply to the oxidation of Az(I) by ferricyanide. However the oxidation of Az(I) by the substituted cobalt reagent is very slow and in this case k_o may be less than k_f with the consequence that the rate equation would include a term for the conformational change.

Acknowledgements

We are indebted to Drs. R.G. Bartsch and T.E. Meyer for providing the facilities and expertise for the growth of *Rps. viridis* and *Rps. capsulata.* We are grateful to Professors M. Brunori, A.G. Sykes and R.J.P. Williams and to Drs. G.W. Canters, H.A.O. Hill, N.A. Kitchen and M.T. Wilson for many helpful discussions. G.W.P. would like to dedicate this paper to the memory of the late Professor G.S. Boyd, who was a constant example and encouragement to him. G.R.M. thanks the SERC for an Advanced Fellowship and G.W.P. and F.A.L. thank the SERC and the Wellcome Trust for financial support.

References

1 Velick, S.F. and Strittmatter, P. (1956) J. Biol. Chem. 221, 265–275
2 Margalit, R. and Schejter, A. (1973) Eur. J. Biochem. 32, 492–499
3 O'Reilly, J.E. (1973) Biochim. Biophys. Acta 292, 509–515
4 Pettigrew, G.W., Meyer, T.E., Bartsch, R.G. and Kamen, M.D. (1975) Biochim. Biophys. Acta 430, 197–208
5 Dutton, P.L., Wilson, D.F. and Lee, C.P. (1970) Biochemistry 9, 5077–5082
6 Wilson, D.F., Erecinska, M., Leigh, J.S. and Koppelman, M. (1972) Arch. Biochem. Biophys. 151, 112–121
7 Dutton, P.L. and Jackson, J.B. (1972) Eur. J. Biochem. 30, 495–510
8 Reid, G.A. and Ingledew, W.J. (1979) Biochem. J. 182, 465–472
9 Butler, J., Davies, D.M. and Sykes, A.G. (1981) J. Inorg. Biochem. 15, 41–53
10 Miller, W.G. and Cusanovich, M.A. (1975) Biophys. Struct. Mech. 1, 97–111
11 Stellwagen, E. and Cass, R.D. (1975) J. Biol. Chem. 250, 2095–2098
12 Eley, C.G.S., Moore, G.R., Williams, G. and Williams, R.J.P. (1982) Eur. J. Biochem. 124, 295–303
13 Dutton, P.L. (1974) Biochim. Biophys. Acta 346, 165–212
14 Leitch, F.A., Moore, G.R. and Pettigrew, G.W. (1983) Biochemistry, in press
15 Moore, G.R., Pettigrew, G.W., Pitt, R.C. and Williams, R.J.P. (1980) Biochim. Biophys. Acta 590, 261–271
16 Pettigrew, G.W., Bartsch, R.G., Meyer, T.E. and Kamen, M.D. (1978) Biochim. Biophys. Acta 503, 509–523
17 Moore, G.R., Leitch, F.A. and Pettigrew, G.W. (1983) Biochim. Biophys. Acta, submitted for publication
18 Lappin, A.G., Segal, M.G., Weatherburn, D.C., Henderson, R.A. and Sykes, A.G. (1979) J. Am. Chem. Soc. 101, 2302–2306
19 Silvestrini, M.C., Brunori, M., Wilson, M.T. and Darley-Usmar, V.M. (1981) J. Inorg. Biochem. 14, 327–338
20 Rosen, P. and Pecht, I. (1976) Biochemistry 15, 775–786
21 Ambler, R.P. (1963) Biochem. J. 89, 341–349
22 Ambler, R.P. and Brown, L.H. (1967) Biochem. J. 104, 784–825
23 Pettigrew, G.W., Leaver, J.L., Meyer, T.E. and Ryle, A.P. (1975) Biochem. J. 147, 291–302
24 Bartsch, R.G. (1971) Methods Enzymol. 23, 344–363
25 Hanania, G.I.H., Irvine, D.H., Eaton, W.A. and George, P. (1967) J. Phys. Chem. 71, 2022–2030
26 Clark, W.M. (1960) in Oxidation Reduction Potentials of Organic Systems, Williams and Wilkins, Baltimore
27 Hill, H.A.O., Leer, J.C., Smith, B.E. and Storm, C.B. (1976) Biochem. Biophys. Res. Commun. 70, 331–338
28 Ugurbil, K. and Bersohn, R. (1977) Biochemistry 16, 3016–3023
29 Adman, E.T., Canters, G.W., Hill, H.A.O. and Kitchen, N.A. (1982) FEBS Lett. 143, 287–292