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pH VARIATION OF MIDPOINT POTENTIAL FOR THREE PHOTOSYNTHETIC BACTERIAL CYTOCHROMES *c'*

A LINK BETWEEN PHYSICAL AND FUNCTIONAL PROPERTIES

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Midpoint redox potential (E_M) versus pH curves are reported over the pH range 5 to 10 for the cytochromes *c'* from three species of purple photosynthetic bacteria: *Rhodospirillum rubrum*, *Rhodopseudomonas palustris* and *Chromatium vinosum*. In each case, theoretical curves are fitted to the data and p*K* values for the reduced (pH 5–5.5) and oxidized (pH 8–8.5) forms of the protein are found to influence the midpoint redox potentials. The oxidized form p*K* values in each case are found to correlate with previously determined p*K* values for variation in physical and/or spectroscopic properties. This correlation of functional and physical observables is discussed in terms of a possible mechanism of control of midpoint redox potential through heme iron-ligand bonding as moderated by the protein conformation in response to solution conditions. The reduced form p*K* values are discussed in terms of a mechanism which would alter the polarity of the heme environment, thereby influencing redox potentials.

Introduction

The cytochromes *c'* are an anomalous class of *c*-type cytochromes found [1,2,40] principally in several species of photosynthetic bacteria. Since the first reports [3,4] of their isolation and purification, the atypical spectroscopic and physical properties of this protein have been a focus of study. The initial reports detailed [4] the pH variation of the ultra-violet-visible spectrum of the oxidized and reduced forms, the latter showing a spectral transition only at the high extreme of pH, near pH 12.0. The oxidized form showed band shifts and intensity variations as a continuous function of pH from 6 to 13. The oxidized form of cytochrome *c'* has since been the subject of numerous physical studies [5–16] with the aim of elucidating the nature of the unusual, reversible pH dependence of the heme iron state and its relationship to protein structure and function. Among the techniques employed to monitor this phenomonon are proton NMR [5], ESR [6–8], Mössbauer spectroscopy [10,11], magnetic susceptibility [12, 13] and resonance Raman spectroscopy [14–16], each focussing on a different aspect of the same phenomonon.

One of the more novel findings of these studies is the conclusion [6–8,17] that under certain conditions the heme iron of cytochrome *c'* exists in a ligand environment which maintains the ferric ion in a ground state with significant contribution from the $S = 3/2$ (or intermediate spin) state. This situation is rarely found for iron, but studies [16,18–20] of model heme compounds have provided further support for the protein studies which have proposed the $S = 3/2$ state. Furthermore, the work to date shows that this heme iron electronic state is subject to con-

tinuous control and variation as the protein conformation responds to bulk solution conditions such as pH.

Any proposed purpose for this unique pH sensitivity must certainly await the discovery of the precise function of the cytochromes c'. In any event, the relationship of the observed pH induced spectral changes to functional properties is of interest in this connection. Reported midpoint redox potentials (E_M) for isolated cytochromes c' at pH 7 are near zero in most cases, with the E_M for the cytochrome c' from *Rhodopseudomonas palustris* near +100 mV. The pH variation of midpoint potential of soluble cytochrome c' and chromatophore bound cytochrome c' from *Rhodospirillum rubrum* have been reported [21] in the pH range 5 to 9.5 and a variation of about 90 mV over this range was observed in each case.

In this study we report the variation of the midpoint redox potentials for the cytochromes c' from the three species of purple photosynthetic bacterium for which extensive phsyical and spectroscopic data are available: *Chromatium vinosum* ($E_M = -5$ mV), *Rhodospirillum rubrum* ($E_M = -8$ mV) and *Rhodopseudomonas palustris* ($E_M = +102$ mV). The results are used to establish pK values for protein ionizations which affect the midpoint potentials, following the method of Clark [22] in the same manner as previously reported [23,24,41] for other classes of c-type cytochromes. These results are discussed with reference to heme-iron axial ligation and pH-induced variations of this ligation as it affects the heme-iron redox potential. Also discussed are pH-induced alteration of the polarity of the heme environment and possible alteration of redox potential via this mechanism.

Experimental

Rhodospirillum rubrum (ATCC No. 11170) was cultured on a modified Hutner's medium [25] with malate as a primary carbon source. *Rhodopseudomonas palustris* (ATCC No. 17007) was cultured on a modified Hutner's medium [26] with malate and succinate as primary carbon sources. *Chromatium vinosum* (ATCC No. 17899) was cultured on a malate medium [27]. Cells were grown in 5-l flat-walled bottles from a 1 liter innoculum at 30°C under illumination by General Electric showcase bulbs and harvested after 3 days growth.

Cytochrome c' was purified from sonicated cells by standard procedures [28]. The heme/protein absorbance ratio or purity index [28] was used as a criterion of purity.

Redox titrations were performed in an anaerobic cell under a continuous flush of oxygen-free nitrogen. Rubber septa were punctured and gas-tight syringes were used to make additions. The potential was monitored by a Metrohm No. EA237 platinum combination microelectrode (Ag|AgCl reference) which was calibrated with the hydroquinone/quinhydrone couple at several pH levels. The potential was varied at stable intervals by additions of ferrous iron to the titration system containing EDTA, ferric iron, pH buffer and cytochrome c'. The iron-EDTA complex concentration was at least 20-times the cytochrome c' iron concentration at all times. This provided a redox buffer, since the E_M of ferrous-ferric/EDTA is in the range of +100 to +10 mV in the pH range 5 to 10.

The reduced/oxidized cytochrome c' ratio was determined at the reduced maximum wavelength (423–426 nm) as a function of measured potential. A Cary 14 recording spectrophotometer and an Orion 601A pH/mV meter were employed. For reported values, the potential varied by 3 mV or less during an absorbance measurement, as recorded before and after each measurement, a time span of several minutes.

A typical sample for titration contained, in a volume of 0.5 ml: 2.5 μM cytochrome c'; mixed buffer with (80 mM each) Tris-acetate, potassium phosphate and glycine, 10 mM EDTA and 0.1 mM ferric iron (complexed by EDTA). Ferrous iron was added as an anaerobic solution of ferrous ammonium sulfate (0.5 M). A typical titration saw the addition of 4–7 μl of ferrous iron for a final iron concentration of 4–7 mM. Sodium dithionite was added to record the 100% reduced cytochrome c' spectrum. The initial spectrum, prior to addition of ferrous iron, was used to determine the 100% oxidized absorbance, since the potential was sufficiently positive. The pH was measured before and after the titration, prior to dithionite addition. During several titrations for each cytochrome, the potential was varied in an oxidative sense (i.e., additional ferric iron was added) in order to test for reversibility. No significant deviation from the Nernst relation for a one-electron change was found for data points obtained in this way.

Results

Representative results for titrations of *Chromatium* cytochrome c' are presented in Fig. 1. The plots followed the Nernst relationship for the pH range investigated in these studies, approx. 5 to 10, for all three cytochromes c'. Slopes for the plots shown in Fig. 1, and for all data reported, were 59 + 3 mV/log concn. unit, as expected for a one-electron process.

In Fig. 2–4, the experimental values (open circles) for E_M are plotted versus pH for the three cytochromes c' studied. The solid curves represent the calculated values using the appropriate equation for pH-dependent redox potential according to the methods of Clark:

$$E_M = E_0 + 59 \log \frac{[H^+]^2 + K_R[H^+]}{[H^+] + K_O} \quad (1)$$

where E_0 is the midpoint potential at unit proton concentration (pH = 0), K_R and K_O represent ionization constants for the reduced and oxidized forms of the protein. Analysis of the three sets of data indicate that the slope of the E_M vs. pH curves is –59 at high pH and that a reduced form ionization occurs at lower pH than an oxidized form ionization. The latter is indicated by a more positive fluctuation of slope, followed by a more negative fluctuation as pH increases.

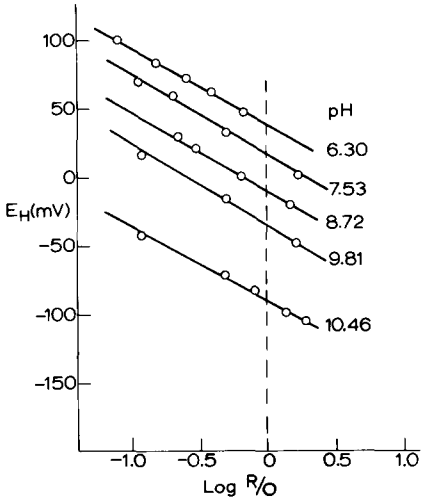

Fig. 1. Redox titration data for *Chromatium* cytochrome c' at pH values as indicated. The dashed line indicates log $R/O=O$, or midpoint potential.

In Figs. 2–4, the pK values for the reduced and oxidized forms which gave the best fit to the data, as represented by the solid curves, are included in each figure. The E_0 values were first approximated by extrapolating the experimental points to pH 0. They were then allowed to vary to provide a better fit to the experimental values, but the final values are within 20 mV of the values determined by extrapolation. E_0 values are not physically significant, but have been chosen by this procedure to facilitate the curve fitting in the pH range of interest. All three sets of data reported here do suggest a continued slope of about –59 mV/pH 'unit' below pH 5. A previous study [21] of *R. rubrum* cytochrome c' reported a variation from the Nernst relationship for a one-electron process below pH 5.5. Their redox titration system was quite different from the one used here, but did show results similar to ours in the pH range above 5.5. We did not attempt to obtain data below pH 5.

Reference to Figs. 2–4 will perhaps suggest that more than two ionizations could be determined by a closer fit to the data. This is most evident for the *Chromatium* cytochrome c' curve in Fig. 2 and is indeed found to be the case upon calculation with the equation including more pK values. We believe that a reasonable fit is achieved by using only the two ionizations for all three proteins reported here.

The E_M values which we observe are consistent with previously reported [1] values. Our values at pH 7 are: *Chromatium*, +20 mV; *R. rubrum*, +10 mV and *R. palustris*, +100 mV. Our values for the midpoint potentials of the *R. rubrum* cytochrome c' are also quite close to those previously reported [21] as a function of pH, employing a different system.

Discussion

The values of the redox potential influencing pK values for the reduced and oxidized forms of cytochromes c' which we have determined are presented in Table I. The uncertainties associated with our 'redox titrations' values in the table are based on calculated curves using various pK values, and the judgement that the curves are similar fits to the data within the indicated range. We found that a pK value of 0.1 higher or lower made only a small difference in terms of the appearance of the fit. Previously reported pK values which have been correlated with changes in

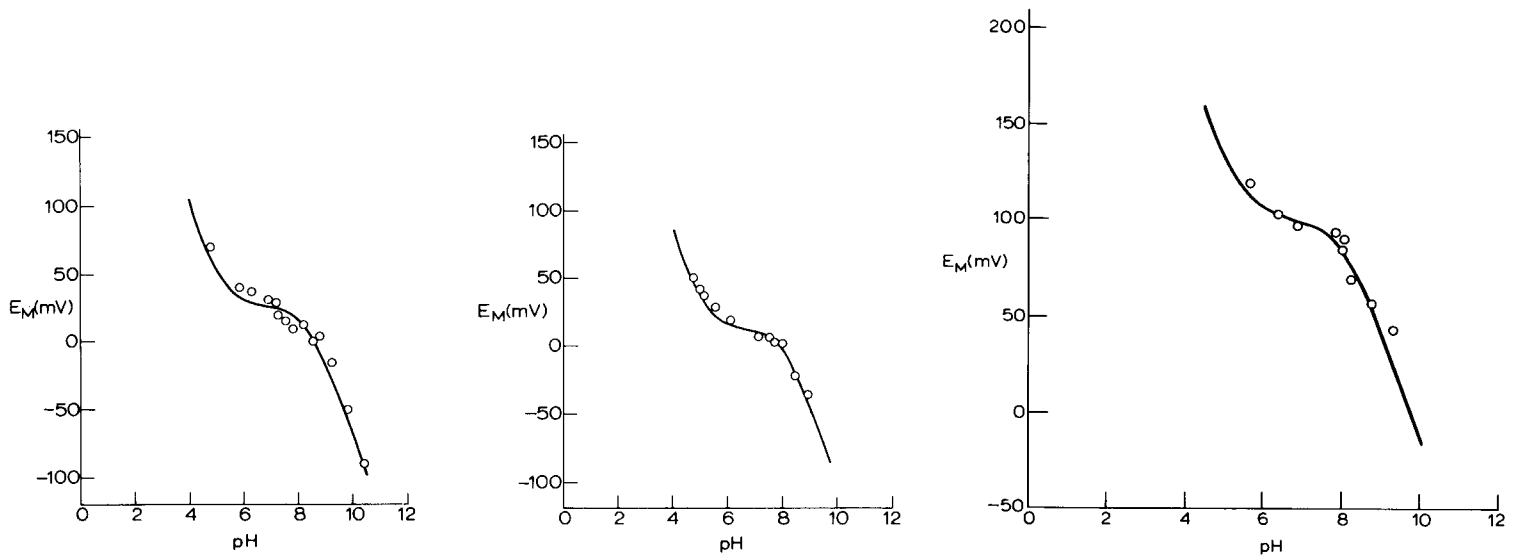

Fig. 2. Experimental points (open circles) and calculated curves (solid lines) using Eqn. 1 for cytochrome *c'* from *Chromatium*. Values used in calculation are: E_0 = 340 mV; pK_R = 5.3; pK_O = 8.4.

Fig. 3. Experimental points (open circles) and calculared curve (solid line) using Eqn. 1 for cytochrome *c'* from *R. Rubrum*. Values used in calculation are: E_0 = 320 mV; pK_R = 5.2; pK_O = 8.1.

Fig. 4. Experimental points (open circles) and calculated curve (solid line) using Eqn. 1 for cytochrome *c'* from *Rsp. palustris*. Values used in calculation are: E_0 = 420 mV; pK_R = 5.4; pK_O = 8.0.

TABLE I

OBSERVED pK VALUES: OXIDIZED CYTOCHROMES c'

Method	*R. rubrum*	*Chromatium*	*Rsp. palustris*	Ref.
Optical	–	9.1	7.4	5
	8.2	9.0	~7.8	33
Raman	9.0	–	–	15
NMR	8.7	–	–	5
EPR	–	9.8	–	8
Mössbauer	8.5	–	–	10
Near infrared MCD	8.5	9–10	7.8	9
Redox titrations	8.1 ± 0.1	8.4 ± 0.1	8.0 ± 0.1	this work
	5.2 ± 0.1	5.3 ± 0.1	5.4 ± 0.1	(reduced form)

various physical and spectroscopic properties are included for comparison. These values are in fact all for the oxidized form of the protein, with the exception of the pK_R values we have determined.

In comparing these numbers one must keep in mind that conditions under which they have been obtained vary from 77 K and below (EPR, Mössbauer) to room temperature (NMR, optical). Also, the phenomona employed, yielding results of varying precision, have then been used to obtain a pK value for some unspecified protein ionization which affects the observable. Any statement that the same ionization is responsible for the different phenomona must be based on a reasonable basis for correlation among the phenomona, in addition to actual correspondence of the numerical values.

Comparison of the oxidized form pK values shows that the pH-dependent transition in the pH range 8–10 which results in the observed alteration of many physical and spectroscopic properties of the heme may be closely linked to the variation in a functional property – the midpoint redox potential of the heme iron. If related, our pK values should most closely parallel the room temperature values. This is the case for *R. palustris* (range, 7.4 to 7.8; our value, 8.0) and *R. rubrum* (range, 8.2–8.7; our value, 8.1) cytochromes c', but the *Chromatium* agreement is not as close (range: 9–10; our value: 8.4). There are no previous reports of observed pK values for the reduced form of cytochromes c'. We submit that our values for the oxidized-form pK values are in fact sufficiently similar, with the largest deviation of 0.6 pH units, to be considered a manifestation of the same phenomonon as all the reported pK values in Table I. This alkaline ionization, in the pH range 8–9.5 for various cytochromes c', is responsible for both the variation in spectroscopic and functional (i.e., redox) properties. The pK we have observed for the reduced form is, of course, quite independent of the oxidized form pK values.

The combined effect of the two ionizations is to moderate the pH variation of an essentially pH dependent redox process – as evidenced by the low and high pH slopes of the E_M vs. pH curves. The net result is that in the pH range of about 6 to 8, the change in midpoint potential is small – about –20 mV in the three cases reported – as compared to the overall variation in the pH range 5–10, which is more than –100 mV. The more positive slope in the pH range 5–5.5 is due to the reduced-form ionization. The trend back to a more negative slope in the pH range 8–9 is then due to the oxidized-form ionization. Since this ionization is correlated with that determined in other studies, one can better comment further on the basis of the observed functional property varaition, in the pH range 8–10. We will then return to a discussion of the reduced-form ionization.

Magnetic studies have shown [5,12,13] that for various cytochromes c' the ferric heme iron undergoes a change in the magnitude of unpaired spin in the pH range 7–10. The pH 7 form is closer to ferric ion in an $S = 3/2$ spin state than is the pH 10 form, which is

better described by ferric iron in the $S = 5/2$, or high-spin, state. Recent X-ray crystallographic and magnetic results [18] for ferric porphyrins suggest that the lower pH form, with $S = 3/2$ contribution, is stabilized by unusual axial ligation which results in the splitting of the iron sigma antibonding $d(x^2-y^2)$ and $d(z^2)$ orbitals so as to favor the $S = 3/2$ d orbital occupancy (see Fig. 1 of Ref. 18). This orbital splitting situation is delicately balanced and a slight increase in the strength of axial ligand bonding (i.e., ligand field strength) shifts the orbital splitting pattern to the high-spin state.

Recent X-ray crystallographic results [29] for the cytochrome c' from *Rhodospirillum molischianum* indicate that a single histidine is axially coordinated to heme iron with the sixth coordination site vacant. Control of bonding of such a histidine to heme-iron bond through response of the protein conformation to solution pH has been proposed [6,17] as a possible mechanism for alteration of the heme iron magnetic state. The control of E_M change has been proposed [34] to proceed via a similar mechanism. The E_M variation in the pH range 8–10 is then suggested to be under the control of protein modulated axial ligand binding. Such control need not involve large changes in conformation, and could reasonably be maintained by some ionizable protein group in the vicinity of the heme. In cytochromes c', the conserved heme-binding sequence Cys-X-X-Cys-His is followed by a carboxylic acid, an aromatic residue and a basic residue (lysine for *Chromatium* and *R. rubrum*). In addition, a lysine is found in the first X position for these two cytochromes c'. The X-ray structure for the *R. molischianum* protein shows these residues in a non-helical region of the structure and relatively accessible to solvent. Although the pK for the free lysine amino group is above 10, an apparent pK of about 9 is observed for oxidized eucaryotic cytochrome c, for a process which involves a deprotonated lysine displacing axially coordinated methionine. In the cytochromes c', a slight localized structural alteration moderated by such an ionization – but not involved in bonding to heme iron – could modify the binding of histidine to heme iron and therefore affect the E_M value.

Previous results [24,30–32] concerning the dependence of heme redox potential on axial ligands have shown that the potential becomes more negative (heme is more easily oxidized) as the donor strength of the ligands increases. This is attributed [31] to the greater charge stabilization of the ferric porphyrin complex relative to the ferrous porphyrin complex. Based on this reasoning, the trend toward more negative potentials which is induced by the protein ionization in the pH range 8–10 must allow better donation to heme iron by the axially coordinated histidine ligand (in combination with the pyrrole nitrogens of the porphyrin). This is consistent with the magnetic studies since the transition from $S = 3/2$ to $S = 5/2$ could involve increased bonding interaction for histidine with the $d(z^2)$ orbital, resulting in a smaller separation from the $d(x^2-y^2)$ orbital. This could be coincident with some movement of iron out of the porphyrin plane, as has been suggested [6,18] which would lower the $d(x^2-y^2)$ orbital energy, further favoring the $S = 5/2$ state.

Another variable which has been demonstrated [35–37] to affect the heme redox potentials is the polarity of the heme environment, including especially the degree of exposure of the heme to solvent. If this degree of exposure is affected by ionizations of the protein another mechanism may be operable in varying redox potential with pH.

For the pH 8.7 transition monitored [5] by NMR for the *R. rubrum* cytochrome c', the relaxation data indicate no significant change in overall protein conformation for the oxidized form of the protein. This is consistent with the earlier reports [1] that anionic ligands such as cyanide and azide do not bind to oxidized cytochrome c' despite the 5-coordinate nature of the heme iron [29] in the pH range 5–12. The heme iron must be then relatively shielded from solvent on the side which is uncoordinated. This is in accord with the *R. molischianum* structural data. The reduced form, however, is known to bind CO, indicating a somewhat more open access to the heme iron.

The observed pK_R near pH 5.3 for the reduced forms of the cytochromes c' may be associated with an ionization which alters the polarity of the heme environment, thereby affecting the redox potential, without altering the observed physical properties such as the optical spectrum or magnetic susceptibility. One possibility is the ionization of an exposed propionate side chain [29] of the heme, the additional negative charge thereby altering the polarity of the heme pocket so as to stabilize the reduced form

(more positive going slope) in the pH region above 5.3. Such an ionization in itself would not be expected to affect the heme optical spectrum or magnetic properties since the carboxylate groups are isolated from the heme π system.

In summary, the pH 8–10 transition in functional property, namely midpoint redox potential, which we observe can be correlated with pH induced variation in a number of physical properties. The observed decrease in E_M is consistent with interpretations of the alteration of heme-iron electronic state and provides a basis for linking the pH variation of functional and physical properties. The pK_R for the reduced form in the region near pH 5.3 can likewise be explained in terms of a mechanism in which the polarity of the heme environment is altered, inducing a change in the observed redox potentials.

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