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REDOX POTENTIALS OF THE PHOTOSYNTHETIC BACTERIAL CYTOCHROMES c_2 AND THE STRUCTURAL BASES FOR VARIABILITY

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Summary

The cytochromes c_2 of the Rhodospirillaceae show a much greater variation in redox potential and its pH dependence than the mitochondrial cytochromes *c* that have been studied. It is proposed that the range of redox potential for cytochromes c_2 functioning as the immediate electron donor to photo-oxidised bacteriochlorophyll may be 345—395 mV at pH 5.

Closely related cytochromes c_2 with different redox potentials show patterns of amino acid substitution which are consistent with changes in hydrophobicity near the haem being at least a partial determinant of redox potential. More distantly related cytochromes are difficult to compare because of the large number of amino acid substitutions and the probability that there are subtle changes in overall peptide chain folding.

The redox potential versus pH curves can be analysed in terms of either one ionisation in the oxidised form or two in the oxidised form and one in the reduced. The pK in the oxidised form at higher pH values can be correlated with the pK for the disappearance or shift of the near infrared absorption band located near 695 nm. The structural bases of these ionisations are not known but the possible involvement of the haem propionate residues is discussed.

Introduction

The cytochromes c_2 are a class of electron transport proteins found in the non-sulphur purple bacteria (family Rhodospirillaceae). They are distinguished from other natural groups of cytochrome *c* by functional role, by distinctive reactivity with bovine mitochondrial cytochrome *c* oxidase and reductase,

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by amino acid sequence and by redox potential. Some cytochromes c_2 have been shown to act as the immediate electron donor to the photooxidised bacteriochlorophyll molecule [1—4]. Cytochromes c_2 react slowly with purified bovine cytochrome oxidase (complex IV) but react similarly to mitochondrial cytochromes *c* with NADH : cytochrome *c* reductase (complex I + III) [5], whereas nearly all other bacterial cytochromes are unreactive. The amino acid sequence work of Ambler and co-workers [6,7] has shown that the cytochromes c_2 are closely related to the mitochondrial cytochromes *c*; in fact the sequence of some cytochromes c_2 more closely resembles the mitochondrial cytochromes *c* than they do other cytochromes c_2 [6]. X-ray crystallographic studies of members of the two cytochrome *c* families have shown that the haem-binding sites are very similar [8]. Despite near continuity

TABLE I

IONISATIONS AFFECTING THE REDOX POTENTIAL AND THE NEAR INFRARED BAND OF THE CYTOCHROMES c_2

Abbreviations for genera are: *R.*, *Rhodospirillum*; *Rm.*, *Rhodomicrobium* and *Rps.*, *Rhodopseudomonas*. pK_{01}, pK_{02} and pK_r define the three ionisation equilibria of the best-fit theoretical curves for the pH dependence of the redox potential (Figs. 1A—G). The p*K* values for single ionization cases are shown under pK_{02}. The $pK_{NIR\ band}$ column is for the titration of the oxidised cytochrome at the wavelength maximum for the near infrared band which varies slightly from one example to the next. Complex cases including that of *R. photometricum* cytochrome c_2 are discussed in the text. Figure numbers refer to this paper, whereas the data marked by * are published in Ref. 12.

Cytochrome c_2	pK_{01}	pK_r	pK_{02}	$pK_{NIR\ band}$	Fig.
R. rubrum 1.1.1 (ATCC 11170)	6.2	7.0	8.4	1/2 loss at pH 9.1	1A, 2G
Rm. vannielii 3.1.1 (ATCC 17100)	6.3	7.4	9.4	9.1	*, 2E
Rps. sphaeroides 2.4.1 (ATCC 17023)	6.1	6.4	8.0	7.9	*
Rps. palustris 2.1.6 (ATCC 17001)	6.9	7.4	8.6	8.4	1B
Rps. palustris 2.1.37 (ATCC 17007)	6.8	7.4	8.6	8.3	1B
Rps. palustris x (La Jolla isolate)	6.8	7.4	8.6	8.2	1B
Rps. viridis (NTHC 133)	6.7	7.1	9.2	1/2 loss at pH9.7	1C, 2B
Rps. capsulata 2.3.1 (ATCC 11166)	—	—	9.3	8.6	1D, 2D
Rps. capsulata SL (ATCC 23782)	—	—	9.4	9.0	1D, 2C
R. molischianum iso-1 (Stanier, R., Berkeley)	—	—	7.4	7.3	1E, 2A
R. molischianum iso-2 (Stanier, R., Berkeley)	—	—	8.3	8.5	1E
R. fulvum iso-1 (ATCC 15798)	—	—	7.2	6.9	1E
R. fulvum iso-2 (ATCC 15798)	—	—	8.5	8.4	1E
Rps. acidophila 7050 (ATCC 25092)	—	—	8.5	8.7	1F
R. photometricum SP113 (Dept. of Microbiology, Bloomington, Ind.)	—	—	7.5	7.4 and 9.4	1G, 2F

in primary structure between the two groups the redox potentials of cytochromes c_2 (compiled in ref. 9) are higher than those of the few mitochondrial cytochromes *c* measured [10,11].

We have analysed species variations of the redox potential and its pH dependence for various cytochromes c_2 as well as the pH dependence of the near infrared absorption band in terms of relatively subtle changes in the amino acids that contribute to the haem environment. We have already published such analyses for cytochrome c_2 from three species [12].

Materials and Methods

Cytochromes c_2 from the organisms listed in Table I were purified by the procedures of Bartsch [13]. Organisms were maintained in agar stab cultures made up in a previously described medium [14]. The same medium was used for large scale anaerobic growth of liquid cultures. The strains used are listed in Table I.

Mid-point oxidation-reduction potentials were measured using potassium ferrocyanide/ferricyanide mixtures [15,16,12] with optical spectrophotometric determination of the percentage reduction of the cytochrome. Experimental details are shown in the legend to Figs. 1A—1G. The difference extinction coefficient at 550 nm (reduced minus oxidised) was assumed to be 20 $mM^{-1} \cdot cm^{-1}$ in all cases. This assumption is justified on the grounds that cytochrome c_2 spectra are very similar and the small deviations from this value that might be expected, e.g. $\pm 2\ mM^{-1} \cdot cm^{-1}$ will affect only the calculation of the amount of ferricyanide produced on reduction of the cytochrome by ferrocyanide. For most of the pH range examined, ferricyanide is present in considerable excess over cytochrome and therefore the ratio ferro- to ferricyanide will be insensitive to such small differences.

Titration of the near infrared band centered around 695 nm in the ferricytochrome spectra was carried out as described in ref. 12. The wavelength maximum of this band differs in different cytochromes c_2 and it will be referred to as the near infrared band.

Results

The pH dependence of the mid-point potential (E_m) for different cytochromes is shown in Figs. 1A—1G. In all cases the experimental data could be fitted to theoretical curves defined by one proton ionisation equilibrium (K) in the oxidised form

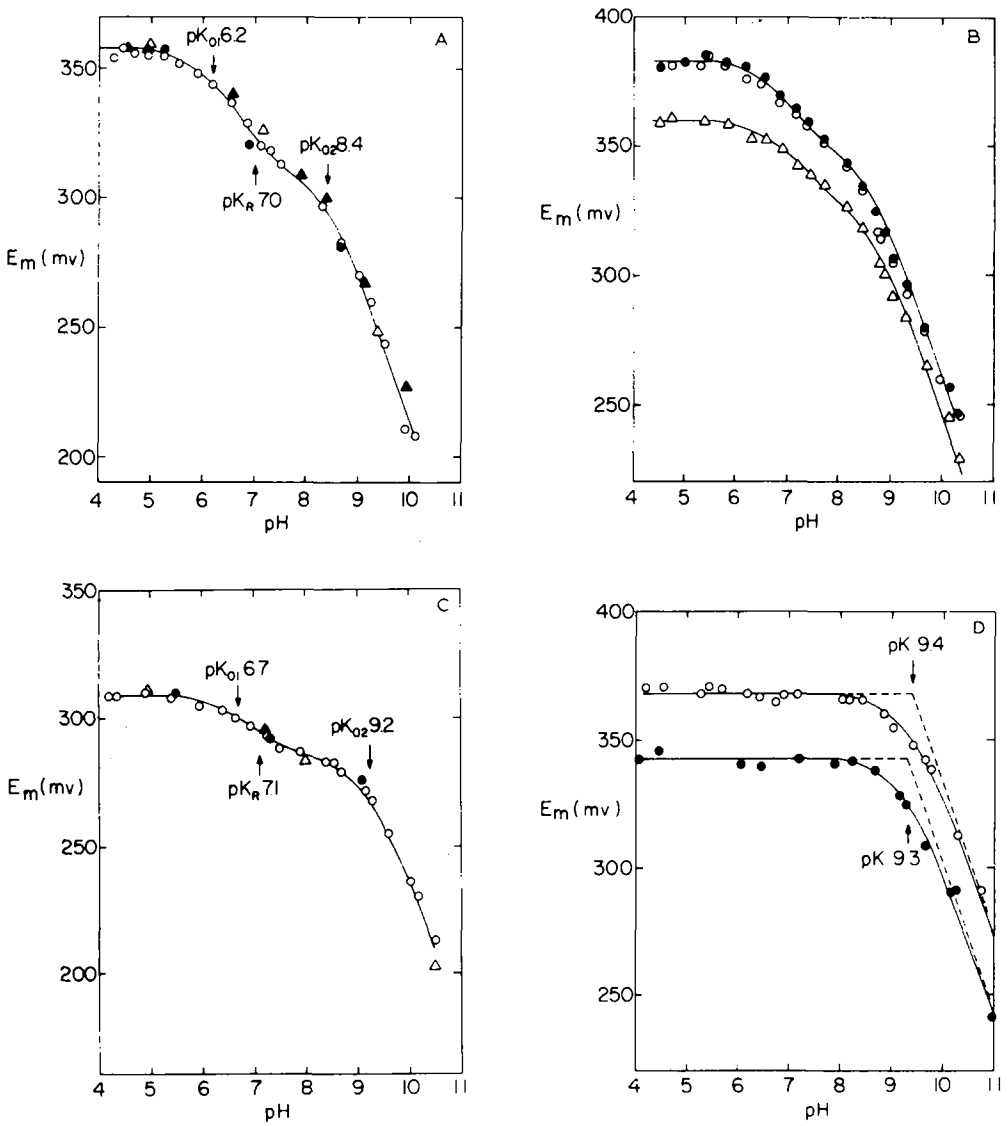
$$E_m = \tilde{E} + \frac{RT}{nF} \ln \frac{[H^+]}{[H^+] + K} \tag{1}$$

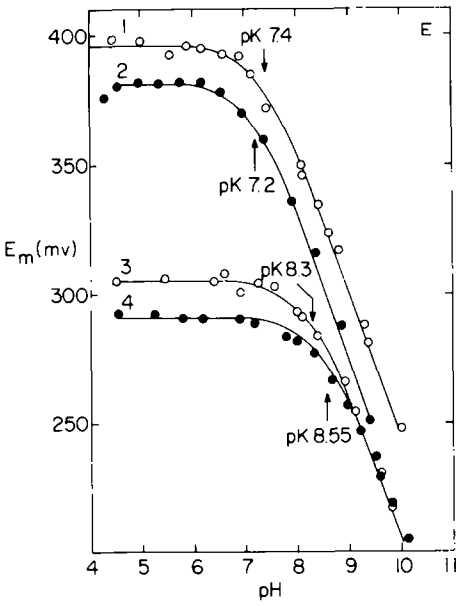
or by two proton ionisation equilibria in the oxidised form (K_{o1},K_{o2}) and one in the reduced form (K_r).

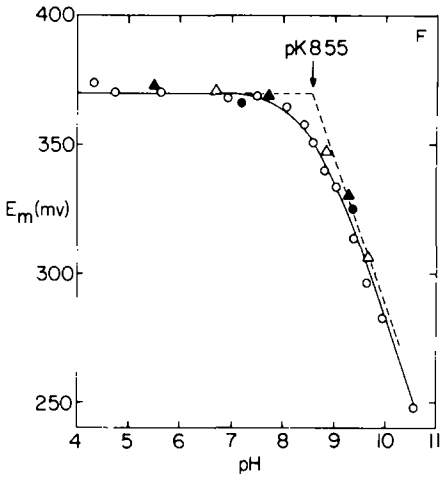
$$E_m = \tilde{E} + \frac{RT}{nF} \ln \frac{[H^+]^2 + K_r[H^+]}{[H^+]^2 + K_{o1}[H^+] + K_{o1}K_{o2}} \tag{2}$$

These equations are more fully explained in ref. 12. E_m is the mid-point oxidation-reduction potential at a defined pH. $\tilde{E}$ is derived by extrapolating E_m to pH 0 along a line of constant slope [17]. Thus the solid lines in Fig. 1 are the theoretical curves for the ionisations shown. Table I compares the pK values observed in the redox potential studies with those observed in the near infrared band titrations. Particular problems encountered in individual cases are described below.

A slow approach to the redox equilibrium for the iso-cytochromes c_2 of *Rhodospirillum molischianum* and *Rhodospirillum fulvum* was observed above a certain pH which correlates approximately with the pK observed for the titration of the near infrared band. This slow approach to equilibrium did not







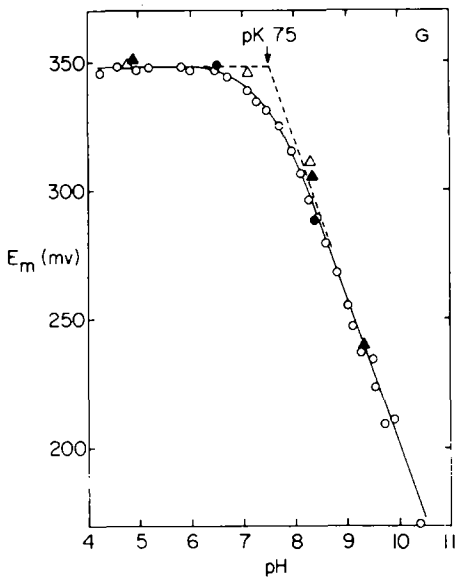


Fig. 1. (A—G) pH dependence of the oxidation reduction potentials of the cytochromes c_2. (A) *R. rubrum* cytochrome c_2. (B) ○, *Rps. palustris* strain 37 cytochrome c_2; ●, *Rps. palustris* strain x cytochrome c_2; △, *Rps. palustris* strain 6 cytochrome c_2. (C) *Rps. viridis* cytochrome c_2. (D) ○, *Rps. capsulata* strain SL cytochrome c_2; ●, *Rps. capsulata* strain 2.3.1. cytochrome c_2; (E) 1, *R. molischianum* iso-1-cytochrome c_2 (○); 2, *R. fulvum* iso-1-cytochrome c_2 (●); 3, *R. molischianum* iso-2-cytochrome c_2 (○); 4, *R. fulvum* iso-2-cytochrome c_2 (●). (F) *Rps. acidophila* cytochrome c_2. (G) *R. photometricum* cytochrome c_2. For Figs. A, C, F, and G the data points are: single equilibrium measurements in air (○); titration results (●); potentials measured directly with a platinum-calomel electrode (▲) and anaerobic single equilibrium results (△). In the figures B, D and E where the data for more than one cytochrome is included in a single figure, a single designation is used for all the data for one cytochrome. Ionic strengths were 0.006—0.008 mol/l, temperature was 25°C, cytochrome concentration was $5 \cdot 10^{-1}$ M. Solution redox potentials were set using known concentrations of potassium ferrocyanide (0.17—0.67 mM) and potassium ferricyanide (0—0.33 mM). Buffers were 1 mM acetic acid/sodium acetate (pH 4—5.8), 1 mM NaH_2PO_4/Na_2HPO_4 (pH 5.8—7.5), 1 mM Tris · HCl (pH 7.5—8.8), 1 mM glycine · NaOH (pH 8.8—10.5). The pH was measured before the addition of dithionite at the end of each experiment. Solid lines are theoretical curves according to Eqn. 1 or Eqn. 2.

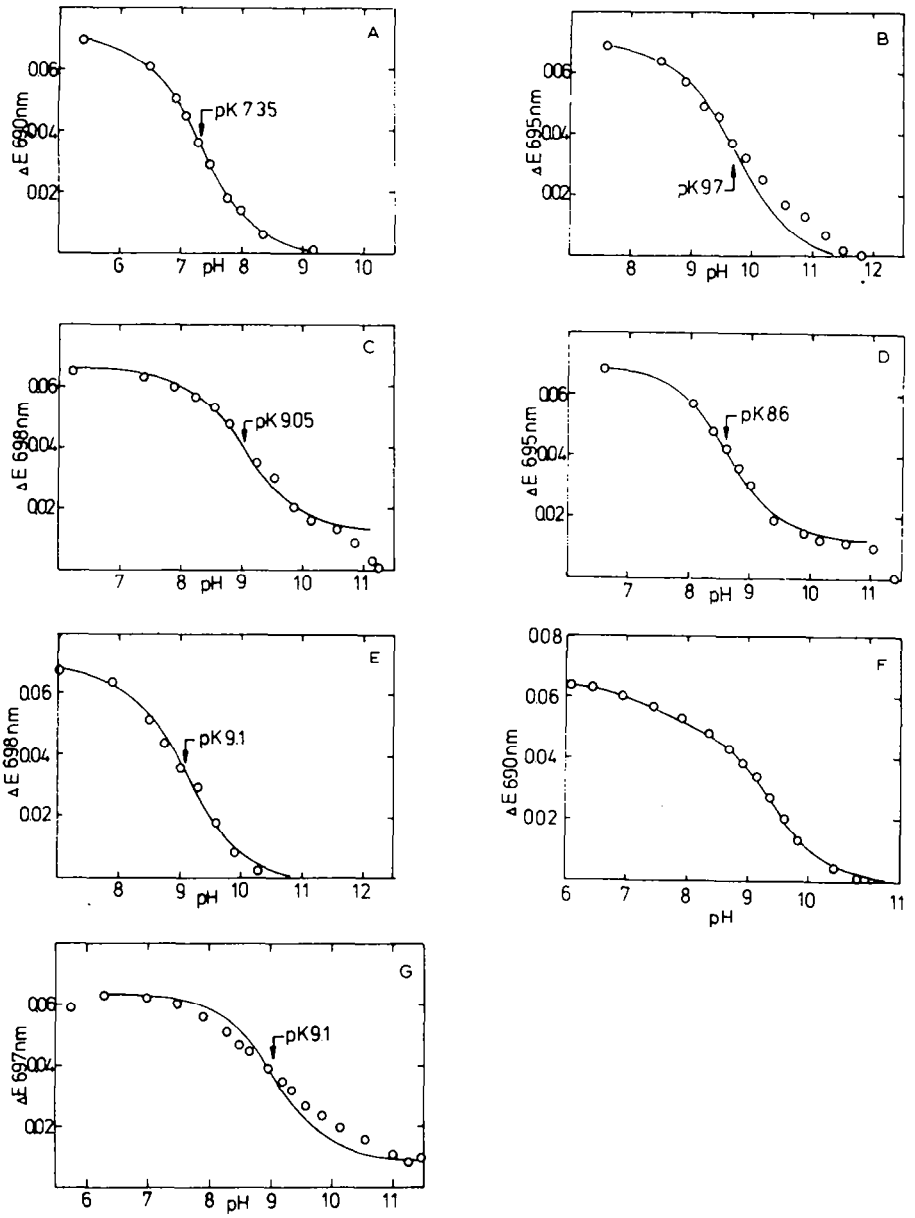


Fig. 2. (A—G) pH titration of the near infrared (NIR) absorption band for selected cytochromes c_2. Titration curves are for cytochromes c_2 from (A) *R. molischianum* (the iso-1-cytochrome), (B) *Rps. viridis*, (C) *Rps. capsulata* strain SL, (D) *Rps. capsulata* strain 2.3.1., (E) *Rm. vannielii*, (F) *R. photometricum* and (G) *R. rubrum*. The pH of an unbuffered solution of ferricytochrome c_2 (approx. 10^{-4} M in 5 mM NaCl and $3 \cdot 10^{-4}$ M potassium ferricyanide) was adjusted with 0.1 M NaOH and the spectrum of the near infrared absorption band was recorded after each addition. $\Delta E_{\text{NIR band}}$ is the extinction of the near infrared band maximum at a particular pH minus the extinction after complete loss of the near infrared band. The open circles are the experimental points while the solid lines are calculated from the Hill equation for a single ionisation. $pH = pK + \log[(\text{alkaline species})/(\text{neutral species})]$. The p$K$ values shown are for these theoretical curves. In the case of the data for *R. photometricum* cytochrome c_2 a theoretical curve for two ionisations was calculated and is shown in F. The pK values for this curve are 7.4 and 9.4 and the contribution of each ionisation to the loss in absorption was 22 and 78%, respectively.

occur with other cytochromes c_2 nor with mitochondrial cytochromes *c* and its cause is not known. A similar complexity was observed in the binding of CO to the reduced forms of *R. molischianum* and *R. fulvum* iso-2-cytochromes c_2. A fraction of both (20 and 2%, respectively) bound CO rapidly (60 s) but a further slow reaction with CO occurred which resulted in 30 and 7% binding, respectively, after 1 h. This may be due to the presence of denatured cytochrome but there was no indication of this during re-purification. Alternatively, Ambler (personal communication) has found that there is micro-heterogeneity at several positions in the sequence of *R. molischianum* iso-2-cytochromes c_2 and it is possible that a naturally occurring component of the presumed mixture may react with CO.

The pH titration curves for the loss of the near infrared band for many cytochromes c_2 could be fitted to a single ionisation curve. An example of such a case is that of *R. molischianum* iso-1-cytochrome c_2 shown in Fig. 2A. The slopes of the Hill plots varied between 0.9 and 1.1 and the pK values obtained were close to the pK values observed in the E_m versus pH curves (pK_{O2} in the three ionisation cases). These pK values are shown in Table I.

For some cytochromes c_2 the titration of the near infrared band could not be described by a single ionisation. In the case of *Rhodospirillum rubrum* cytochrome c_2 a Hill slope of 0.7 was obtained (Fig. 2G). In the case of *Rhodopseudomonas viridis*, *Rhodopseudomonas capsulata* strains SL and 2.3.1. and

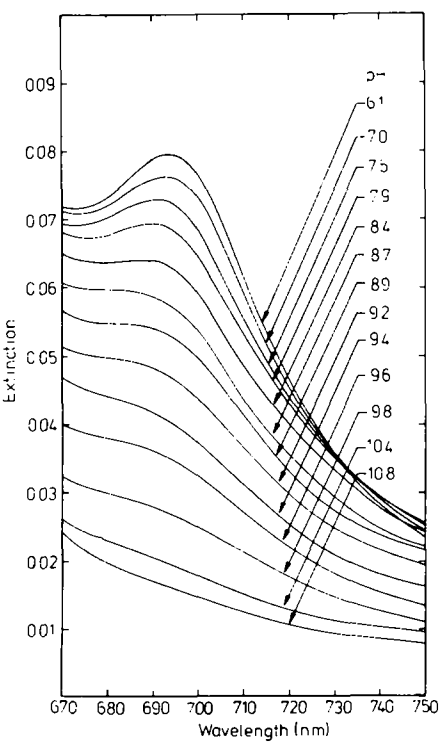


Fig. 3. Titration of the near infrared absorption band of *R. photometricum* ferricytochrome c_2. The conditions of titration were as described in the legend to Fig. 2. The spectroscopic changes were slow and several minutes were required after each addition of NaOH for equilibrium to be achieved.

Rhodomicrobium vannielii cytochromes c_2 titration curves were complex although for all but *Rps. viridis* cytochrome c_2 single ionisation curves could be fitted to data at lower pH values (Figs. 2B—2E). In the case of *Rm. vannielii* cytochrome c_2 a high spin species with a band at 630 nm was present between pH 10 and 11 but disappeared at higher pH. This type of pattern was previously observed in the titration of *Euglena* cytochrome *c*-552 [18].

The most interesting anomaly in the near infrared band titration data is that of *Rhodospirillum photometricum* cytochrome c_2. In this case the near infrared band located at 693 nm appears to shift to 688 nm with an approximate pK of 7.4 (Fig. 2F and Fig. 3). This pK is the same as that observed in the E_m versus pH curve (Table I, Fig. 1G). However, the bulk of the absorption in this region is lost with a pK of 9.4, an ionisation not detectable in the E_m versus pH curve.

Discussion

The range of redox potentials in the cytochromes c_2

This survey includes all the Rhodospirillaceae species known to contain cytochrome c_2; those species which lack cytochrome c_2 are *Rhodopseudomonas tenue* and *Rhodopseudomonas gelatinosa*. Species which have not yet been examined for cytochrome content include *Rhodopseudomonas sulphidophila*, *Rhodopseudomonas globiformis* and *Rhodopseudomonas sulphoviridis*. The redox potentials of the cytochromes c_2 have been compared at pH 5 because the E_m versus pH curves were found to have zero slope in this region (see Fig. 1A—1G). In Table II the cytochromes are arranged into two groups. Most have a redox potential at pH 5 of between 345 and 395 mV while the iso-2-cytochromes of *R. molischianum* and *R. fulvum* and *Rps. viridis* cytochrome c_2 have a redox potential at pH 5 of near 300 mV. However, both the iso-2-cytochromes occur in organisms which also have an iso-1-cytochrome of higher redox potential. Rapid light-induced oxidation of cytochrome c_2 has been observed in several of the Rhodospirillaceae, eg. *Rps. capsulata* [1] and *Rhodopseudomonas sphaeroides* [1], but not in *Rps. viridis* where a complex membrane-bound cytochrome *c*-558 · *c*-553 has been implicated as the immediate electron donor to photooxidised bacteriochlorophyll [19,20]. Therefore it is conceivable that the redox potential for these cytochromes c_2 that function in cyclic electron transport may be restricted to the range 345—395 mV at pH 5.

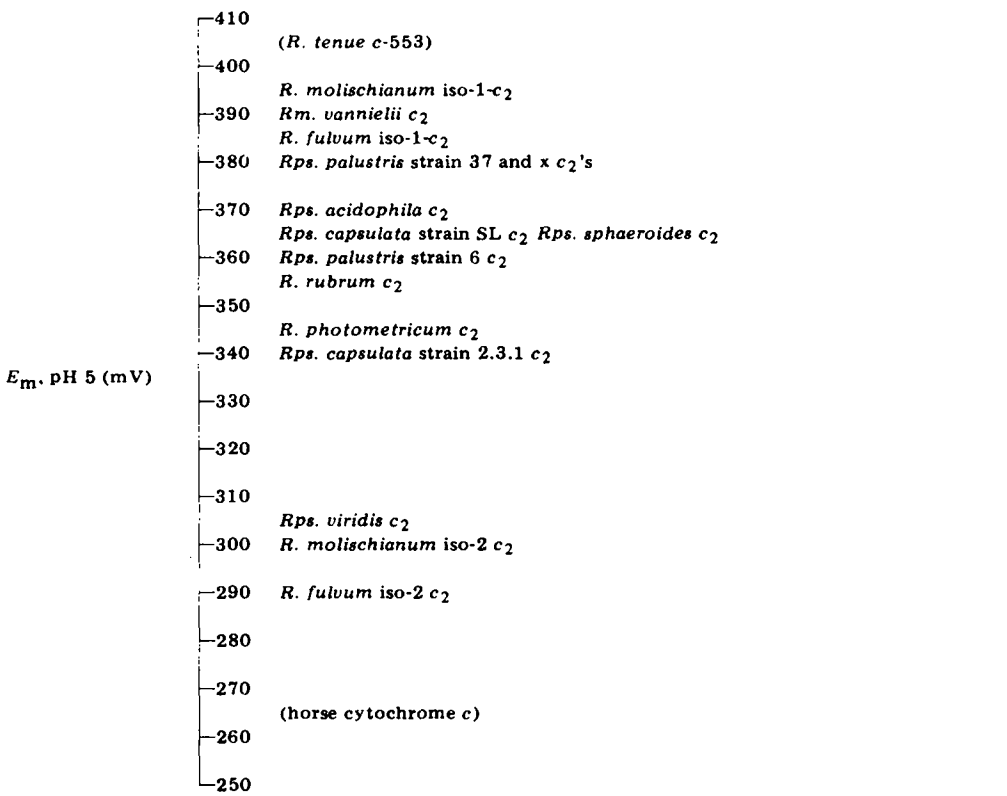
Two species of Rhodospirillaceae, *R. tenue* and *Rps. gelatinosa*, lack cytochrome c_2 but do have a cytochrome related in structure to the *Pseudomonas* cytochromes *c*-551 [21]. This cytochrome in *R. tenue* has a redox potential of 405 mV and may perform the function of a cytochrome c_2. In *Rps. gelatinosa* on the other hand, the *c*-551-like cytochrome has a redox potential of below 150 mV [22] and, as in *Rps. viridis* there is a membrane-bound cytochrome which may perform the role of electron donor to photooxidised reaction centre bacteriochlorophyll [23].

The non-sulphur bacteria, therefore, appear to employ at least three different cytochromes in performing this electron transfer to reaction centre bacteriochlorophyll. Most organisms utilise soluble cytochromes, either c_2 or

TABLE II

MID-POINT OXIDATION-REDUCTION POTENTIALS OF THE CYTOCHROMES c_2

Species names are positioned to indicate the redox potential determined at pH 5. Strain designations are given where appropriate. The redox potentials of two *c*-type cytochromes which are not members of the cytochrome c_2 family are shown for comparison. These are horse cytochrome *c* [11], and *R. tenue* cytochrome *c*-553 which resembles the *Pseudomonas* cytochrome *c*-551 family in amino acid sequence [21].



Pseudomonas c-551-like, having redox potentials between 345 and 405 mV, while a few have a complex membrane-bound cytochrome *c*, one haem of which operates near this range of redox potential.

Changes in hydrophobic residues as a structural basis for the differences in the redox potential at pH 5

The redox potential of *meso*-haem dimethyl ester · pyridine complex in benzene solution compared to that in water suggested that the very high redox potential of the cytochromes *c*, having histidine and methionine ligands, was probably due to the local non-polar environment provided by the protein [24]. Theoretical calculations were made by Kassner [25] to show that changes in the size of the hydrophobic shell surrounding the haem in cytochromes or a change in the ratio of polar to non-polar residues and therefore a change in the dielectric constant of the protein would alter the redox potential in a predictable way. Furthermore it was proposed that the higher redox poten-

tials of the cytochromes c_2 relative to the mitochondrial cytochromes *c* were due to the approx. 11 amino acid larger size of the cytochromes c_2 [25]. It is now known that there are cytochromes c_2 which are the same size as mitochondrial cytochromes *c* but are indistinguishable from the larger cytochromes c_2 in having similarly high redox potentials and in having the same relative proportion of non-polar residues. Two related classes of cytochrome, approx. 16 amino acid residues smaller than the mitochondrial cytochromes *c*, also appear to provide an exception to the Kassner theory. *Chlorobium* cytochromes *c*-555 have a lower redox potential as predicted for a smaller protein structurally related to the mitochondrial cytochromes *c* [26] but the equally small algal cytochromes *f* have high redox potentials in the same range as the cytochromes c_2 [27].

Because of the extreme sensitivity of Van der Waals forces to distance small perturbations in the positions or nature of hydrophobic side chains close to the haem may be a more important determinant of redox potential than the overall dielectric constant of the protein interior. This would be consistent with the relatively constant ratio of polar to non-polar residues found in cytochromes of different redox potentials. Hydrogen bonding between amino acids must also be considered as a factor influencing the relative stability of reduced and oxidised species since hydrogen bond networks within cytochromes can help dissipate and stabilise internal charge [28].

Theoretically, cytochromes which are similar in amino acid sequence but which show differences in redox potential should allow conclusions as to the effect of amino acid substitutions on redox potential. *Rps. capsulata* strains SL and 2.3.1. cytochromes c_2 differ from one another at only two positions in the amino acid sequence [7] but the redox potentials are 30 mV apart (Fig. 1D). The two differences are leucine 87 in strain SL for threonine in strain 2.3.1. and threonine 94 for serine (The numbering used here and throughout is the actual residue number in a sequence without the use of gaps. The cytochromes c_2 are clearly homologous in sequence but require different patterns of gaps in order to align them so that structural homologues may have different residue numbers in different cytochromes.)

The leucine-threonine substitution involves a decrease in hydrophobicity in the case of strain 2.3.1. and this may be correlated with the 30 mV lower redox potential. That is, the more hydrophilic threonine may contribute to a haem environment which tends to favour the oxidised species. However, this residue is situated in a surface loop quite remote from the haem (residues 80—87 in *R. rubrum* cytochrome c_2 [28]). On the other hand threonine-serine 94 (serine 89 in *R. rubrum* cytochrome c_2) is only 6.5 Å from the plane of the haem and is hydrogen bonded to tyrosine 52 in the case of *R. rubrum* cytochrome c_2 [28]. This change therefore seems the likelier cause for the change in redox potential although the exact nature of the effect whether in terms of residue bulk, polarity or hydrogen bonding ability cannot be assessed.

In the comparison of three *Rps. palustris* strains, two of the cytochromes c_2 (those from strains x and 37) have essentially identical redox potentials throughout the pH range studied while that from strain 6 is 20 mV lower (Fig. 1B). The amino acid sequences (ref. 7 and Ambler, R.P., personal communication) have the differences listed in Table III. Of these differences only two

TABLE III

AMINO ACID SUBSTITUTIONS IN CYTOCHROMES c_2 FROM THREE STRAINS OF *RPS. PALUSTRIS* USING THE HOMOLOGOUS *R. RUBRUM* CYTOCHROME c_2 AS REFERENCE STRUCTURE

The differences in the amino acid sequences of cytochromes c_2 from three *Rps. palustris* strains are compared with corresponding positions in *R. rubrum* cytochrome c_2. The distances of Tyr 52 and Phe 77 from the haem group in *R. rubrum* cytochrome c_2 are taken from Table II of Salemme et al. [28] which lists those residues bounding the haem crevice.

Rps. palustris strain 6 c_2	*Rps. palustris* strain 37 c_2	*Rps. palustris* strain x c_2	*R. Rubrum* c_2	Residues bounding the haem crevice in *R. rubrum* cytochrome c_2
Ala 4	Lys 4	Ala 4	Ala 5	
Lys 5	Ala 5	Lys 5	Ala 6	
Asn 48	Asn 48	His 48	Tyr 52	5.5 Å
Gln 60	Ala 60	Ala 60	Glu 64	
Glu 61	Asp 61	Glu 61	Ala 65	
Asp 62	Asn 62	Asx 62	Asn 66	
Ala 65	Asn 65	Ala 65	Ala 69	
Pro 68	Asn 68	Ala 68	Lys 72	
Tyr 73	Phe 73	Phe 73	Phe 77	10 Å
Gln 83	Lys 83	Gln 83	—	
Lys 86	Gln 86	His 86	—	
Thr 88	Val 88	Ala 88	—	
Ser 90	Val 90	Val 90	Lys 88	
Asp 100	Glu 100	Glu 100	Asp 98	
Ala 107	Val 107	Val 107	Ile 105	

involve residues near the haem. These are asparagine-histidine 48 (tyrosine 52 in *R. rubrum* cytochrome c_2) and tyrosine-phenylalanine 73 (phenylalanine 77 in *R. rubrum* cytochrome c_2). The more polar tyrosine 73 of strain 6 cytochrome c_2 may account for the lower redox potential but the changes at residues 88, 90 and 107 are all in the direction of decreased hydrophobicity in strain 6 cytochrome c_2 and may also contribute. The other substitutions involve no pronounced change in hydrophobic character and many are presumed to be on the surface of the molecule. The cytochrome c_2 of strain 37 does however have a different set of potential hydrogen-bond forming residues at positions 65, 68 and 83 which makes it difficult to draw firm conclusions.

These results for the cytochromes of *Rps. capsulata* and *Rps. palustris* do suggest that the Kassner theory (that redox potential is related to hydrophobicity of the haem environment) may be a partial explanation for the differences in redox potential among closely related cytochromes. It is clear that the most suitable subjects for this type of analysis are the very closely related cytochromes which show differences in properties because for more distantly related cytochromes the number of amino acid differences is too great to allow interpretation of the effects on redox potential.

However, even in the favourable cases the assumptions that the basic polypeptide chain folding is the same as that in *R. rubrum* cytochrome c_2 and that conserved residues are in the same relative orientation with respect to the haem may not be valid. The most satisfactory approach may be the use of NMR spectroscopy to investigate the proximity of side chains to the haem group. In comparing *R. rubrum* cytochrome c_2 with horse cytochrome *c* for instance

there are no major differences in the residues near the haem which might explain the 100 mV disparity in redox potential. However, the NMR spectra of the two cytochromes [29] indicate that leucine 32, common to both proteins, is further from the haem in horse cytochrome *c*, an effect which may contribute to the redox potential difference.

The length of the iron-methionine bond has been proposed by Moore and Williams [30] to be an important determinant of redox potential. Any changes in protein structure which alter the length or orientation of this bond may therefore markedly influence redox potential but the causative role of such changes remains to be clarified.

An additional way in which the redox potential of the haem may be influenced is by surface charge. Goldkorn and Scheijter [31] have shown that the redox potentials of *Euglena* cytochrome *c*-552 and horse cytochrome *c* tend to the same value at very low ionic strength while at high ionic strength they differ by 100 mV. This behaviour can be explained by Debye-Hückel theory in terms of total charge on the reduced and oxidised species. *Euglena* cytochrome *c*-552 has an overall negative charge and therefore a negative value for $q^2_{ox} - q^2_{red}$. The redox potential falls with decreasing ionic strength. Horse cytochrome *c* is basic and $q^2_{ox} - q^2_{red}$ is positive so that the redox potential rises with decreasing ionic strength (in a non ion-binding medium). However, this may not be the origin of the differences between the redox potentials of the cytochromes c_2 and cytochrome *c* because some cytochromes c_2 are also positively charged molecules at neutral pH so that $q^2_{ox} - q^2_{red}$ will be positive unless specific ion binding takes place. Therefore extrapolation to zero ionic strength would give even higher values of E_m than those recorded here. The cytochromes c_2 as a family are highly variable in iso-electric point (pI 5.9—9.8) and ionic strength effects would have to be investigated for each protein to determine the possible contribution of overall charge to the redox potential.

pH dependence of the redox potential and the near infrared band absorption

Two patterns of redox potential dependence on pH can be distinguished, the first where a single pK in the oxidised form defines the E_m versus pH curve, and the second where three pK values are required to fit the experimental data (refer to Figs. 1A—1G and Table I). It is possible that the former is a special case of the latter when $pK_{01} = pK_r$. Pettigrew et al. [12] discussed the assignment of the three pK values in general terms but were unable to positively identify a particular pK with a specific protein group. For all of the cytochromes studied, pK_{02} is close to the pK observed for the change in the near infrared absorption band. There also appears to be a correlation between the loss of the near infrared band in electronic absorption spectra and the loss of the "upfield shift" for the sixth ligand methionine protons in NMR spectra [32]. Therefore redox potentials, electronic absorption spectra and NMR spectra simultaneously appear to reflect a change in the orientation of the sixth ligand with respect to the haem.

The nature and cause of this change is not however known. It is widely held opinion that the loss of the near infrared band is due to the replacement of methionine by an adjacent lysine residue at alkaline pH. However, this residue is not found in *Rps. capsulata* cytochrome c_2 and yet the pattern of spectral

changes at alkaline pH is unaffected. Also, in the present studies the pK for the near infrared band titration varies between 6.9 for *R. fulvum* iso-1-cytochrome c_2 and 9.4 for *Rps. capsulata* cytochrome c_2 which would be an unexpectedly large variation in the ionisation of a conserved lysine residue. The *R. molischianum* and *R. fulvum* iso-1-cytochromes do have a distinctively low pK for the near infrared band loss and these are also the two cytochromes which have the substitution of tryptophan 62 (*R. rubrum* cytochrome c_2 numbering) by isoleucine (Ambler, R.P., personal communication). It is possible that the loss of this tryptophan lowers the pK of the rear haem propionate which, on ionisation, perturbs the haem crevice. The ionisation constant for this carboxyl group is not known but the hydrophobic environment of the haem would be expected to result in a raised pK.

The other important feature of the pK for the near infrared band loss is that corresponding spectral changes are not observed in the reduced species. In *R. fulvum* iso-1-cytochrome c_2, for instance, the redox potential versus pH curve was followed for three pH units above pK_{02} and no further ionisation was detected. This absence of a pK in the reduced form may be due to a simultaneous occurrence of ionisations in both the reduced and oxidised forms [33] but it is perhaps more plausibly explained by a surface ionisation which occurs in both oxidised and reduced species but which triggers a conformational change only in the oxidised state [34].

In the case of pK_{01} and pK_r no changes are detectable in the visible spectrum (with the possible exception of *R. photometricum* cytochrome c_2 discussed below). However, Wood et al. [35] observed a pK of 6.2 in the kinetics of the reduction of *R. rubrum* cytochrome c_2 with various small molecules and Smith [36] has shown a small shift in the methionine methyl resonance of *R. rubrum* ferricytochrome c_2 which occurs with a pK of 6.25, identical to the pK_{01} observed in these redox potential studies. Thus pK_{01}, at least in the case of *R. rubrum* cytochrome c_2, appears to affect the methionine-iron bond. It is likely that the ionisations giving rise to pK_{01} and pK_r are due to the same group influenced by the proximity to the haem [12]. Different cytochromes show varying degrees of separation of these two pK values with *R. rubrum* and *Rm. vannielii* cytochromes c_2 having the most pronounced separation (Table I). The amino acid sequences of these two cytochromes differ from all other cytochromes c_2 (and all the mitochondrial cytochromes c) at residue 38 which is asparagine or glutamine in place of the conserved arginine. This residue lies at the "bottom rear" of the molecule, turned in to the surface and quite close to the rear haem propionate [28]. We speculate that it may help stabilise the internal negative charge produced when the rear propionic acid group ionises. In its absence (in *R. rubrum* and *Rm. vannielii* cytochromes c_2) the pK values of the propionate in the oxidised and reduced species may be separated by the influence of the haem group. An attempt is in progress to modify this single arginine residue in *Rps. capsulata* cytochrome c_2 to determine whether the redox potential versus pH curve can be changed from the simple case involving a single pK to the complex form with three pK values. Wood et al. [35] also feel that the pK_{01} of 6.2 and pK_{red} of 7.0 which they observe in their kinetic experiments are due to the ionisation of a propionic acid but they propose that the front haem propionate may be involved because of its proximity to the

proposed electron transfer site at the edge of pyrrole ring 2.

R. photometricum cytochrome c_2 has a distinctive pattern of ionisations affecting the haem group. The apparent shift of the near infrared band to the blue does not occur in any other cytochrome and this change is distinct from the loss of the near infrared absorption because it shows a unique isosbestic point at 733 nm (Fig. 3). One interpretation of this result is that there is a conversion of one cytochrome species with near infrared band at 693 nm to one with a near infrared band at 688 nm with a pK of 7.4. The continued presence of a near infrared band above pH 7.4 suggests that the methionine coordination is still present but the marked effect on redox potential indicates that this coordination has been perturbed. NMR studies will show whether the changes in the methionine proton resonances can be correlated with these pK values.

Comparison with in vivo results

One serious problem in this survey is what relation does the redox potential in solution have to that measured in vivo. This is underlined by a recent study by Prince and Dutton [37] who found that both the redox potential and its pH dependence can change when the cytochrome c_2 is bound to the membrane containing the physiological oxidant. These authors report that at pH 7, the *Rps. sphaeroides* cytochrome c_2 contained in membrane vesicles has a redox potential of 290 mV rather than the 340 mV measured for the cytochrome in solution, and that the redox potential of the bound form is nearly independent of pH between pH 5 and 11. Also they found that the redox potential of *R. rubrum* cytochrome c_2 in chromatophores was independent of pH between pH 5 and 11, whereas in our study and those of Kakuno et al. [38] and Wood and Cusanovich [39] the redox potential of the pure cytochrome showed a pronounced pH dependence. Thus the process of attachment of the cytochrome to the membrane appears to modify the properties in quite profound ways. This may reflect a change in the structure of the cytochromes on interaction with the membrane-bound oxidant, or as Prince and Dutton [37] suggest, a differential binding of the redox species to the membrane may be the explanation. This is of interest in itself, but it does cast serious doubt on the conclusions concerning function made on the basis of the properties of the cytochromes in solution or in the crystal. For example, these authors emphasise that the possibility suggested by our studies that *R.rubrum* cytochrome c_2 might act as a hydrogen carrier in the physiological pH range was not borne out by their studies of the cytochrome in situ.

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