Graham W. Pettigrew · Geoffrey R. Moore

Cytochromes c

Biological Aspects

With 68 Figures

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Chapter 1 Resolution, Characterisation and Classification of c-Type Cytochromes

Functional studies of cytochromes fall into one of two groups. In the first, the intact respiratory system is examined using methods which allow the resolution of individual components. In the second, components are isolated and characterised and their role studied in reconstituted partial systems. Relatively few studies incorporate both approaches and we suggest that this has led to problems in the interpretation of results.

A good example is the investigation of the role of cytochrome c_2 in bacterial photosynthesis (Chap. 3). Models of electron transport were proposed based on the light-induced absorbance changes in the intact photosynthetic system assuming that cytochrome c_2 was the single c-type cytochrome present. Only with the demonstration by Wood (1980) of the presence of a cytochrome c_1 could the kinetic results of the whole system be correctly re-interpreted. Conversely, studies on isolated components without reference to the intact system can be misleading. Thus the study of electron transfer between purified cytochrome c_3 and ferredoxin of the sulfidogenic bacteria has no physiological relevance because the two proteins are separated by the cell membrane and cannot interact in vivo (Chap. 3).

In the following we discuss methods for resolution of the whole system and methods for characterisation of individual purified components. In the final section we describe a classfication scheme for the cytochromes c.

1.1 Resolution

1.1.1 The Location of Cytochrome c

Gram-negative bacteria are surrounded not only by the cell membrane but also by a peptidoglycan cell wall and an outer membrane. Proteins which are secreted into the space between the cell membrane and the outer membrane are termed periplasmic. They can be selectively released by treatment with lysozyme and EDTA in a sucrose medium which provides osmotic support for the spheroplasts as they are formed. The fragile spheroplasts can then be osmotically shocked to release the cytoplasmic contents. Thus periplasmic, cytoplasmic and membrane fractions can be obtained (Fig. 1.1) and their composition investigated.

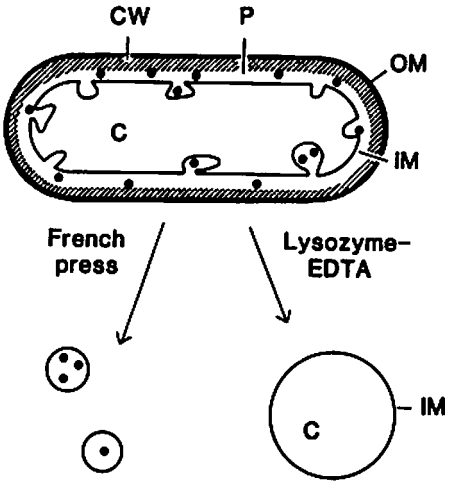


Fig. 1.1. Spheroplast formation in gram-negative bacteria. Gram-negative bacteria contain a periplasmic compartment (*P*) situated between the outer membrane (*OM*) and inner (plasma) membrane (*IM*). The cell wall (*CW*) is denoted by *hatching*. Passage of bacteria through a French pressure cell yields membrane vesicles which may contain periplasmic proteins (●) depending on the way the inner membrane fragments and reseals. In some bacteria, the inner membrane is highly invaginated, increasing the likelihood of trapping the periplasmic proteins in vesicles. Treatment with lysozyme and EDTA removes the outer membrane and cell wall and the spheroplasts that are formed contain an intact cytoplasmic compartment (*C*) if osmotically supported

Table 1.1. The cellular location of cytochrome c in *Pseudomonas stutzeri*

	Cyt.c (%)	ICDH (%)
Periplasmic	52	2
Cytoplasmic	8	98
Membrane	40	0

The amounts of total cytochrome c and isocitrate dehydrogenase (ICDH) present in each fraction were expressed as a percentage of the total present in the three fractions. (G. W. Pettigrew, unpublished results)

Wood (1983) has proposed that all c-type cytochromes are either periplasmic or are bound to the periplasmic side of the cell membrane. This model, which receives increasing experimental support, has important implications for the energy conserving mechanisms of bacterial oxidative phosphorylation (Chap. 3). The criterion for periplasmic location is the release of a protein without release of cytoplasmic contents and this is shown in Table 1.1 for *Pseudomonas stutzeri* where the cytochrome c is measured spectrophotometrically and isocitrate dehydrogenase is used as a cytoplasmic marker.

Less is known of gram-positive bacteria which lack the outer membrane. It may be that, in these organisms, cytochromes c are more tightly bound to the surface of the cell membrane (Jacobs et al. 1979) so that they are not lost through the relatively porous cell wall.

1.1.2 SDS Gel Electrophoresis and Heme Detection

The simple spectrophotometric method used in Table 1.1 cannot distinguish the individual cytochromes c that are present. This however is possible using SDS gel electrophoresis followed by heme detection.

One method of heme detection is based on the peroxidase activity of heme using 3 3′ 5 5′ tetramethylbenzidine as an oxidisable substrate (Thomas et al. 1976). The method was developed, and has been widely used, for proteins such as cytochrome P450 and cytochrome b which contain protoheme IX but, because this heme is not covalently bound, only a small fraction is retained after denaturation and this varies depending on the precise conditions employed.

On the other hand, c-type cytochromes contain covalently bound heme and are therefore ideally suited to the application of the peroxidase activity method after SDS gel electrophoresis (Goodhew et al. 1986). In Fig. 1.2, the method is applied to the fractions obtained during spheroplast preparation of aerobic and denitrifying *Pseudomonas stutzeri*. This experiment allows several important conclusions to be made.

First, individual cytochromes can be identified using purified markers. Second, the study of the general location of cytochrome c summarised in Table 1.1 is extended to define the location of the individual cytochromes. The figure of 8% total cytochrome appearing in the cytoplasmic fraction of Table 1.1 can be seen from Fig. 1.2 to be due mainly to membrane material which is difficult to fully sediment from the viscous spheroplast lysate. Third, membrane-bound c-type cytochromes are resolved and shown to be distinct from the soluble cytochromes. With few exceptions such cytochromes are rarely purified and are poorly characterised. Fourth, the effect of growth conditions on the complement of c-type cytochromes can be defined; cytochrome cd_1 (nitrite reductase) and a band of M_r 30 K are induced in denitrifying conditions, while a membrane-bound cytochrome c of M_r 32 K is characteristic of aerobic growth.

Sample preparation for electrophoresis usually involves the addition of a sulfhydryl reducing agent but this must be avoided if heme staining is to be performed because the ferrous iron is readily lost from the heme to give the porphyrin. However, although porphyrins have no peroxidase activity, they are fluorescent and this property is used in an alternative and equally sensitive method for the detection of c-type cytochromes in the presence of reducing agents (Wood 1981).

The heme-staining method is also usefully applied during purification of c-type cytochromes. Chromatographic peaks may contain single cytochromes

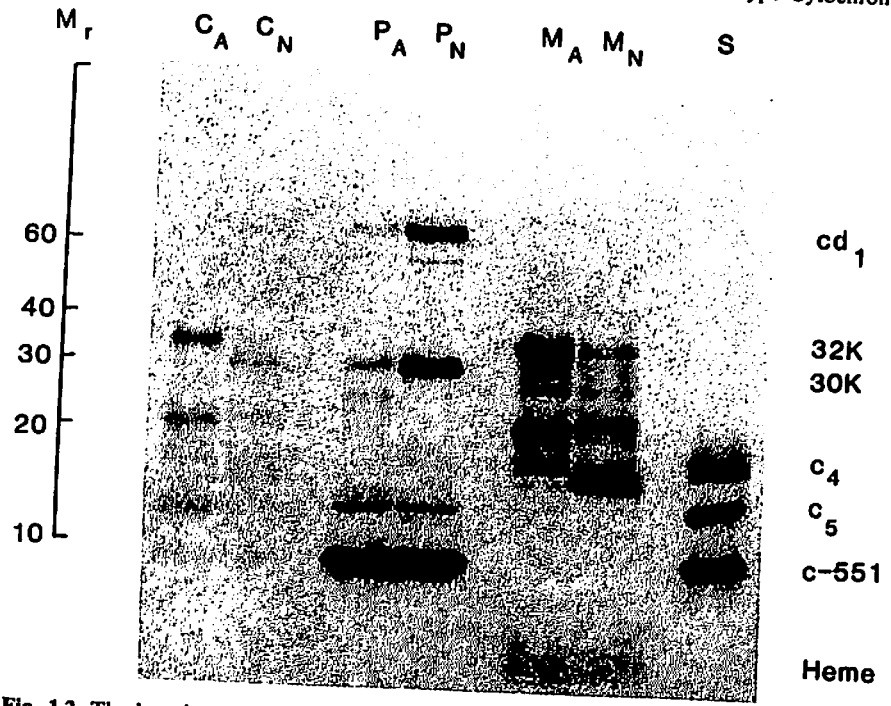
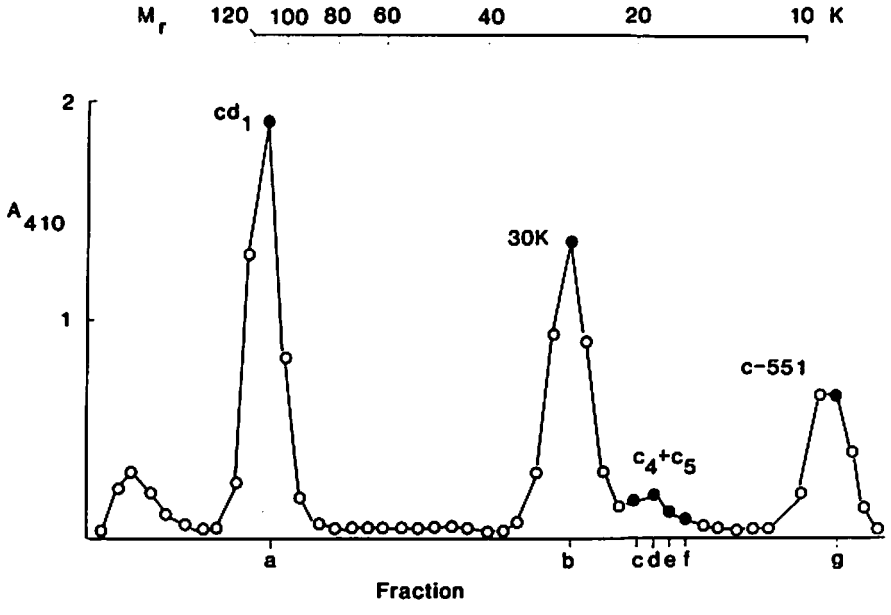


Fig. 1.2. The location and induction of the c-type cytochromes of *Pseudomonas stutzeri*. Cytoplasmic (*C*), periplasmic (*P*) and membrane (*M*) fractions obtained from aerobic (*A*) and nitrate-grown cells (*N*) cells of *Ps. stutzeri* stain 224 were subjected to SDS gel electrophoresis and then heme peroxidase staining for the detection of c-type heme. The positions of bands were compared with the purified standards (*S*) containing cytochromes c_4, c_5 and c-551 of *Ps. stutzeri* and to a set of molecular weight marker proteins (not shown) which were used to construct the scale of M_r (in kilodaltons). The channels were scanned at 560 nm and the following areas (in arbitrary units) were obtained for individual bands

	P_N	P_A
c-551	251	243
30 K	197	40
cd_1	184	12

or unresolved mixtures as shown in Fig. 1.3. In this molecular exclusion chromatography of the periplasmic fraction of denitrifying *Ps. stutzeri* 224, the cytochrome cd_1, the band of M_r 30 K and cytochrome c-551 are each separated from other cytochromes c but cytochromes c_4 and c_5 are unresolved. This mixture would be difficult to observe by visible spectroscopy but is readily detected by SDS gel electrophoresis of selected fractions followed by heme staining.



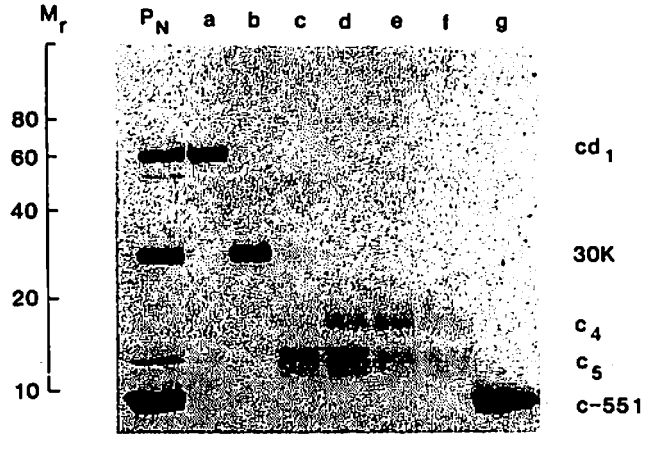


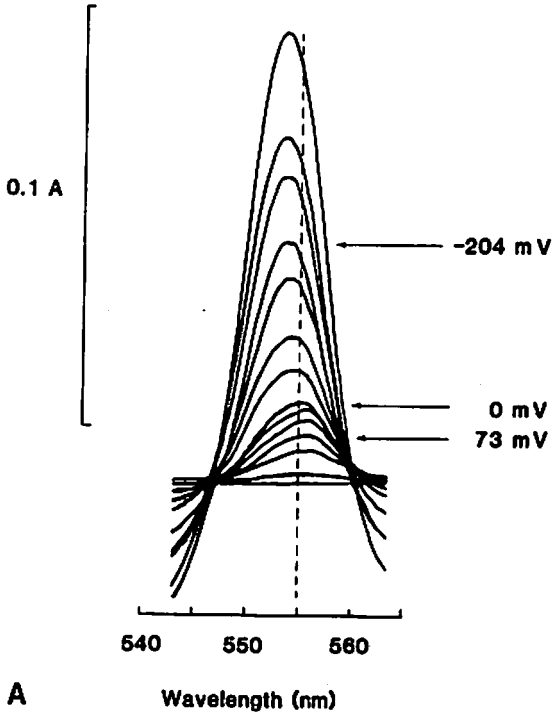
Fig. 1.3. Molecular exclusion chromatography of the periplasmic cytochromes c of denitrifying *Pseudomonas stutzeri*. The periplasmic fraction from a spheroplast preparation of denitrifying *Ps. stutzeri* was concentrated on DEAE cellulose and the concentrated eluate was applied to a Sephadex G-150 superfine column (1.5×90 cm). Portions of selected fractions ($a-g$) were subjected to SDS gel electrophoresis and heme staining in parallel with the periplasmic concentrate before chromatography (P_N). Both the molecular exclusion column and the SDS gel were calibrated using known molecular weight marker proteins and the logarithmic scales of their relative exclusion and relative mobility are shown

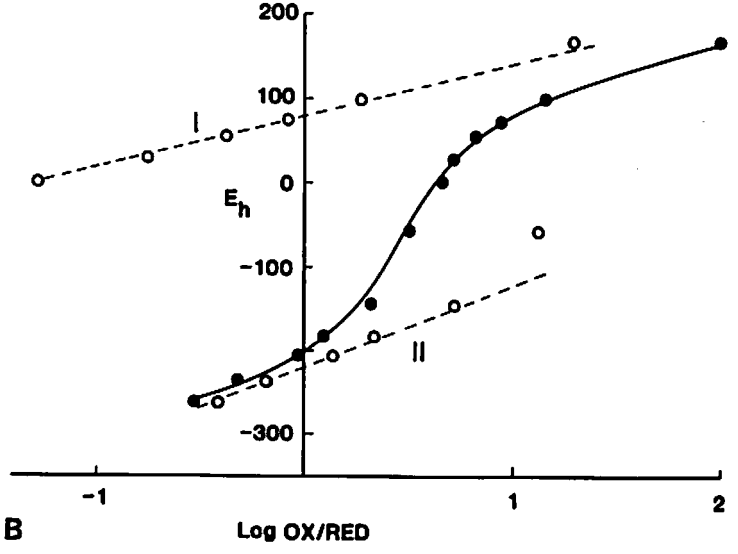
1.1.3 Redox Potentiometry and Spectroscopy

At room temperature the visible spectrum of a mixture of c-type cytochromes will contain featureless, composite α-peak which cannot be directly resolved. Although lower temperatures may sharpen individual α-components and allow their partial resolution, each α-component may also be split leading to an increase in complexity which may be difficult to interpret. However, stepwise reduction of the mixture may reveal spectral components not evident from the fully reduced α-band and, if the redox potential of the solution is measured at each step, even spectroscopically similar components may be potentiometrically resolved.

A relatively simple example is the redox titration of the soluble cytochromes of *"Chloropseudomonas ethylica"* (Fig. 1.4A). This consortium of two organisms synthesises a cytochrome c-555 of $E_{m,7}+103$ mV (from the green sulfur bacterium, Shioi et al. 1972) and a triheme cytochrome c_3 of $E_{m\ \text{average, pH7}}-194$ mV (from the sulfidogenic bacterium, Meyer et al. 1971). It is clear from the spectra of the soluble extract that although the fully reduced

Fig. 1.4. A Redox titration of the soluble extract of *Chloropseudomonas ethylica.* The titration was carried out anaerobically in a stirred cuvette in the presence of the redox mediators flavin mononucleotide ($E_{m,7}-219$ mV), 2-hydroxy-1,4-naphthoquinone ($E_{m,7}-152$ mV), duroquinol ($E_{m,7}+5$ mV), phenazine ethosulfate ($E_{m,7}+55$ mV), phenazine methosulfate ($E_{m,7}+80$ mV) and diaminodurol ($E_{m,7}+240$ mV) (all 20 μM). Reductive titration was carried out using NADH in the presence of NADH cytochrome c reductase (0.5 mg, Sigma). Successive spectra were recorded after adjustment to an isobestic point of 544 nm and the redox potential of the solution was measured simultaneously. Selected values of the redox potential corresponding to individual spectra are shown. B Values of log (total ox)/(total red) (●) calculated from the titration are plotted against the measured redox potential (E_h). The presence of a component with positive midpoint potential and with α_{max} near 555 nm is evident from the initial stages of the reductive titration and was estimated to contribute 19% to the total absorbance change on the basis of the position of the sigmoidal portion of the curve. Points corresponding to this component (I) were replotted using the new value of fully reduced leaving a second component (II) which was also replotted using the new value for fully oxidised. The *broken line* of component I has a slope of 60 mV indicating a good fit to a single heme. The *broken line* of component II has a steeper slope (100 mV), indicating the presence of more than one heme contribution. Further arithmetical analysis allows the construction of a theoretical curve (*solid line*) containing contributions from three heme components – component I (19%, E_m+80 mV), component IIa (26%, E_m-150 mV) and component IIb (55%, E_m-230 mV). Thus component II contains contributions from two components in proportions of 2:1 and separated by 80 mV. Published values for the purified cytochromes c from *Chloropseudomonas ethylica* are cytochrome c-555, +103 mV (Shioi et al. 1972); cytochrome c_3 (3 heme), −194 mV (average) (Meyer et al. 1971)


0.1 A
-204 mV
0 mV
73 mV
540
550
560
A
Wavelength (nm)


200
100
0
-100
-300
I
II
E_h
-1
1
2
B
Log OX/RED

α-peak is symmetrical at 553 nm, the initial stages of reduction produce an α-peak at 555 nm. This is consistent with prior reduction of the heme of most positive potential and is confirmed by the potentiometric analysis based on the Nernst relationship:

$$E_h = E_m + 0.06 \log \text{ox/red} \quad (n = 1,\ 30\,^\circ C).$$

The plot of E_h against log ox/red will yield a straight line of slope 60 mV for a single component but if two components are present a sigmoidal curve is obtained and more than two components yield more complex curves. The sigmoidal curve of Fig. 1.4B can be resolved arithmetically using the procedure described in the legend into three components: a cytochrome c-555 ($E_{m,7}+80$ mV) which contributes 19% of the absorbance change and two low potential components ($E_{m,7}-150$ and -230 mV) which contribute 26% and 55% of the absorbance change respectively. These results are consistent with the properties of the purified cytochromes. In the case of the triheme cytochrome c_3, two of the hemes have midpoint potentials 80 mV more negative than the third in agreement with the results of Fiechtner and Kassner (1979) on the purified protein.

The method is a powerful one but requires confirmation by independent approaches and can give rise to problems in interpretation. For example, the analysis will give the simplest reasonable fit to the data but many more complex solutions will fit equally well. Also, the method cannot distinguish between the presence of one component with a split α-peak and the presence of two spectroscopically distinct but potentiometrically identical components. Finally, the method simply defines what redox centres are present but does not discriminate between those attached to the same protein and those attached to different proteins.

1.2 Characterisation

There are a large number of physicochemical properties which could be used to distinguish different cytochromes but those that have proved the most useful are size, amino acid analysis and heme content, spectra and midpoint potential.

1.2.1 Size

The true molecular weight of a cytochrome can only be determined from the amino acid sequence but estimates of relative molecular weight (M_r) can be obtained from a variety of methods of which SDS gel electrophoresis and molecular exclusion chromatography are the most popular. These two methods should give concordant results for cytochromes composed of a single polypep-

tide chain but cytochromes are known which are aggregates of the same or different polypeptide chains and these will dissociate to the individual subunits in SDS but remain as the aggregate under the native conditions of molecular exclusion chromatography. For example, in Fig. 1.3, cytochrome cd_1 and c_5 appear as dimers on the molecular exclusion column while cytochrome c-551 is monomeric.

For both methods, there are instances of anomalous behaviour. For example, non-spherical molecules such as the cytochrome c′ dimer (Meyer and Kamen 1982), appear larger on molecular exclusion chromatography than their actual molecular weight. Also, a cytochrome may appear intermediate in M_r between monomer and dimer if a rapid equilibrium is present. In the case of SDS gel electrophoresis, anomalous mobilities are seen for hydrophobic membrane proteins and, in the case of cytochrome c_4, the holoprotein migrates more rapidly in SDS than the apoprotein (Pettigrew, G. W., unpublished results).

1.2.2 Amino Acid Analysis and Heme Content

If amino acid analysis is performed on a pure sample of known heme content, then a minimum molecular weight per mole heme can be obtained by summing the constituent amino acids. If this is then compared with the value of M_r obtained from SDS gel electrophoresis, the heme content per mole protein can be found by division. An example of such an analysis is shown in Table 1.2 and Fig. 1.5. On the basis of SDS gel electrophoresis, the apocytochrome c_4 of *Azotobacter vinelandii* has a M_r of 20400. A figure of 19347 is obtained from the amino acid composition associated with 2 mol heme indicating that the cytochrome is a diheme protein. In this case, the amino acid sequence is known (Ambler et al. 1984) with a molecular weight of 19652 thus confirming the analysis.

Cysteine is usually underestimated after hydrolysis of a holocytochrome and a more reliable figure can be obtained after removal of the heme group with mercuric chloride. Tryptophan is destroyed by hydrolysis in HCl and as with the results of Table 1.2 is often not reported in amino acid compositions. It can be determined after hydrolysis in mercaptoethane sulfonic acid (Penke et al. 1974) or by its contribution to the ultraviolet spectrum of the protein. The problem with the latter approach is that the heme absorbs in the same region and therefore a method such as that of Goodwin and Morton (1946) which analyses the dual contributions of tyrosine and tryptophan to the composite UV spectrum can only be performed on the apoprotein. Alternatively, an approximate estimate of tryptophan may be found by subtracting the tyrosine and heme contribution from the holoprotein extinction at 280 nm as shown in Table 1.2.

Table 1.2. The amino acid composition and heme content of *Azotobacter vinelandii* cytochrome c_4

	nmol[a]	mol/2 mol heme	Sequence
ASP	48.3	23.6	21
THR	16.4[b]	8	9
SER	15.6[b]	7.6	10
GLU	38.5	18.8	18
PRO	21.7[c]	10.6	11
GLY	47.3	23.1	25
ALA	60.1	29.3	29
VAL	11	5.4	5
MET	11	5.4	6
ILE	12.3	6	6
LEU	26.6	13	14
TYR	11.9	5.8	6
PHE	8.3	4	4
HIS	8.7	4.2	4
LYS	20.7	10.1	11
ARG	14.7	7.2	7
CYS	8[c]	3.9	4
TRP	ND[d]	–	0[e]
		19347	19652

[a] This sample contained 4.1 nmol heme as determined by the pyridine hemochrome method. The sample was hydrolysed for 70 h in 6 M HCl at 105 °C.
[b] Not corrected for breakdown during the 70 h hydrolysis.
[c] Cysteine as cysteic acid and proline were estimated after removal of the heme group, performic acid oxidation and acid hydrolysis for 20 h.
[d] Tryptophan was not determined by amino acid analysis. An estimate of the tryptophan content can be made by subtracting the tyrosine and heme contribution from the absorbance of the holoprotein at 280 nm. This calculation assumes $E_{Tyr,\,280\,nm}$ 1.1 $mM^{-1}\,cm^{-1}$ and $E_{heme,\,280\,nm}$ 13.85 $mM^{-1}\,cm^{-1}$, the latter being calculated from cytochromes of known tryptophan content. This calculation for *A. vinelandii* cytochrome c_4 is

	$mM^{-1}\,cm^{-1}$ (280 nm)
Heme	27.7 (2 heme)
Tyr (6)	6.6
	34.3
Holocytochrome	36.7
Remainder	2.4
Trp	5.2 } Trp content 0.46

[e] The sequence analysis found no tryptophan. The fractional value obtained above may be due to differences in heme absorption at 280 nm between different cytochromes or to differences in tyrosine absorption due to protein environment. (G. W. Pettigrew, unpublished results)

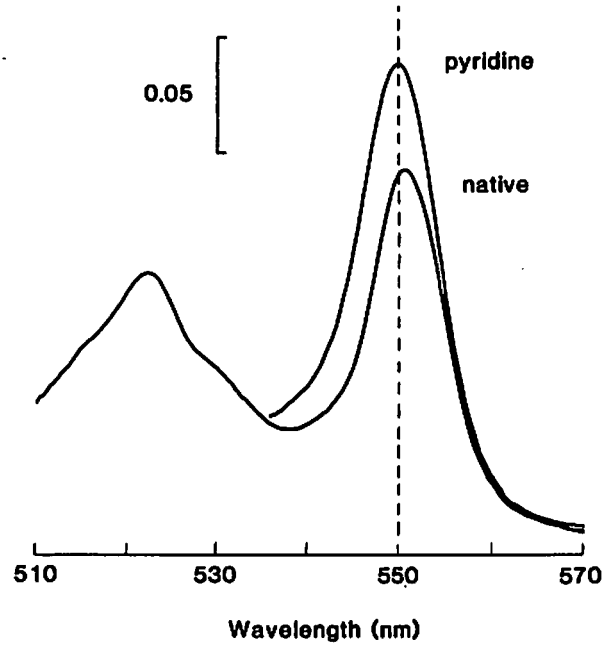


Fig. 1.5. The native and pyridine ferrohemochrome spectra of cytochrome c_4 from *Azotobacter vinelandii*. The native spectrum of ferrocytochrome c_4 in the region of the α- and β-bands was obtained by addition of solid sodium dithionite to the cytochrome in 0.1 M phosphate pH 7. The pyridine ferrohemochrome of the same concentration of cytochrome was formed in 0.15 M NaOH, 0.2 M pyridine with the addition of dithionite. Application of the extinction coefficient of the cytochrome c heme to the latter spectrum ($E_{550\,nm}$ 31.18 $mM^{-1}\,cm^{-1}$; Barstch 1971) allows the calculation of a heme concentration of 164 µM for the parent cytochrome c_4 solution. From this, 4.1 nmol heme were taken for amino acid analysis (Table 1.2)

1.2.3 Spectra

Cytochromes function by a reversible change in the redox state of their heme group between the levels of Fe II and Fe III, the only known exception being the peroxidatic centre of *Pseudomonas* cytochrome c peroxidase which can adopt an Fe IV state. Apart from the single thioether attachment of some protozoan cytochromes c (Vol. 2, Chap. 3), the heme group of cytochrome c is believed to be structurally constant and the visible spectrum is influenced only by the extraplanar ligands provided by the protein and also by the general protein environment.

The spectra of Figs. 1.6 and 1.7 illustrate the former effect. The cytochromes c' have a pentacoordinate heme giving rise to a high-spin spectrum (Figs. 1.6a and 1.7). Other cytochromes c have low-spin spectra (Fig. 1.6b, c and d) but a major subdivision of these is based on the presence of a band at 695 nm in the ferricytochrome (Fig. 1.7). This is present in those cyto-

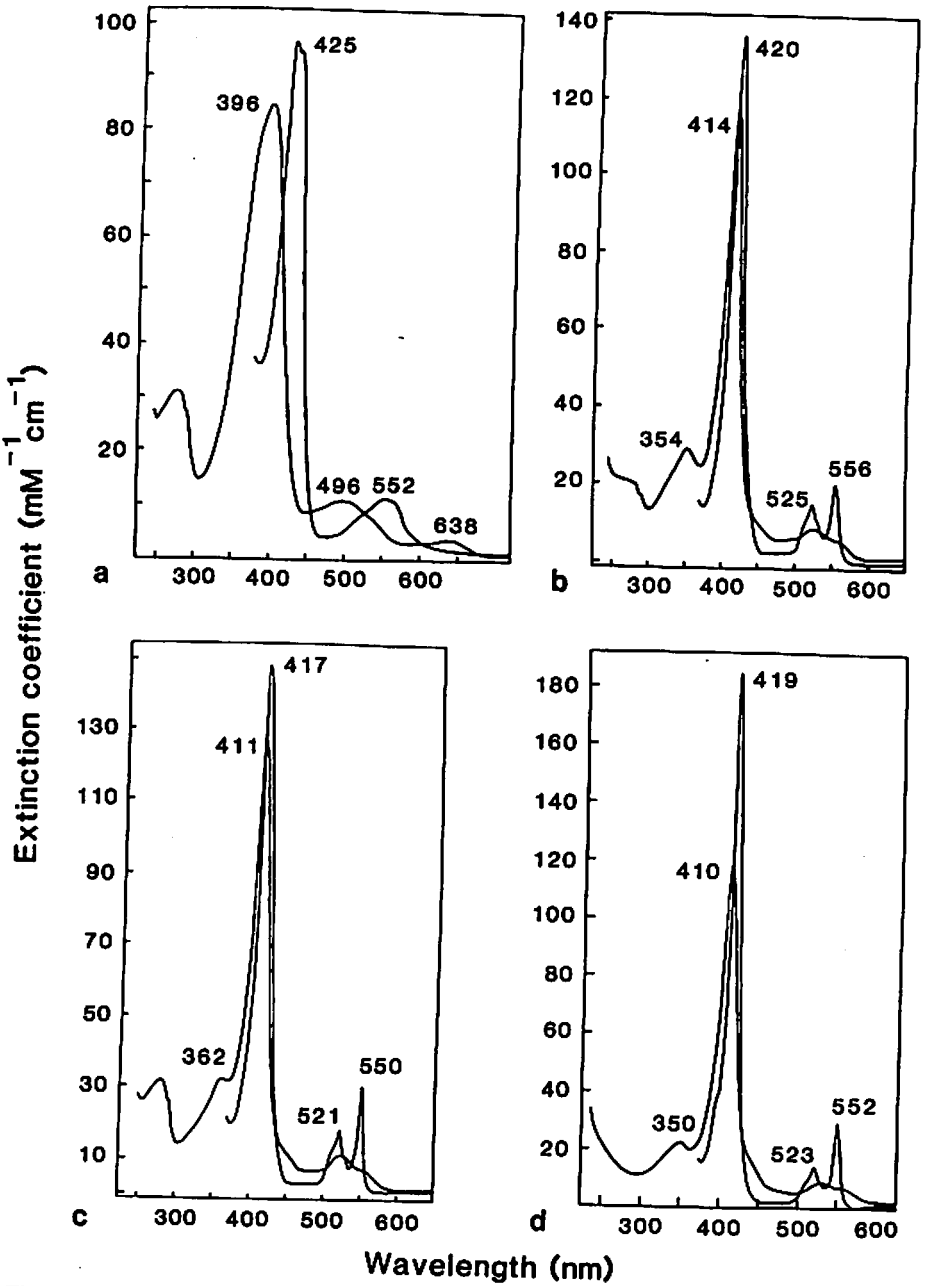


Fig. 1.6a–d. UV-visible absorption spectra of selected c-type cytochromes. **a** cytochrome c' from halotolerant *Paracoccus* sp.; **b** cytochrome c-556 from *Rhodopseudomonas palustris*; **c** cytochrome c_2 from *Rhodopseud. sphaeroides;* **d** cytochrome c_3 from *Desulfovibrio vulgaris.* In each case the ferri- and ferrocytochrome spectra are obtained by addition of potassium ferricyanide and sodium dithionite respectively to solutions of the cytochrome in 0.1 M phosphate pH 7. (Meyer and Kamen 1982). The ferrocytochrome spectra are those showing a band near 550 nm which is often used to designate a particular cytochrome

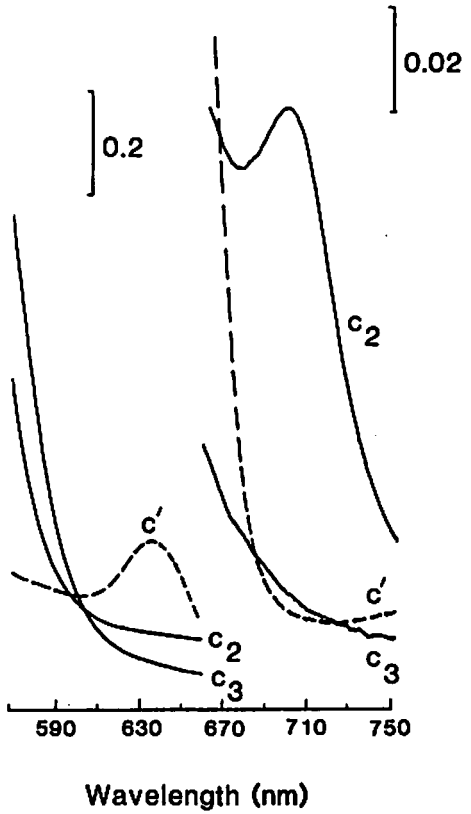


Fig. 1.7. Near-infrared absorption spectra of selected c-type ferricytochromes. Cytochrome c_2 is from *Rhodopseud. viridis* (118 μM), cytochrome c' from *Rhodopseud. capsulata* (100 μM) and cytochrome c_3 from *Desulfovibrio gigas* (153 μM)

chromes with a histidinyl-methionyl-Fe coordination while those with a bis-histidinyl coordination lack a band at this wavelength.

Rather more subtle spectroscopic differences further distinguish the different low-spin cytochromes c. These are presumably based on variations in the general environment provided by the protein but the detailed structural basis for these minor spectroscopic changes is not known. Illustrative differences in the symmetry and wavelength maximum of the α-band and the α/β ratio are shown in Fig. 1.8. Asymmetry in the α-band can be an intrinsic property of a single heme as in the case of *Porphyra* cytochrome c-553 (Fig. 1.8F) or it may be due to the presence of two hemes as in the case of *Pseudomonas stutzeri* cytochrome c_4 (Fig. 1.8I). With both cytochrome c-551 of *Ps. aeruginosa* (Fig. 1.8G) and cytochrome c-555 of *Chlorobium limicola* (Fig. 1.8H), the asymmetry is dependent on pH, being more pronounced above pH 7. An extreme case of asymmetry is found in the split α-band of cytochrome c-554 (548) from halotolerant *Paracoccus* (Fig. 1.8J).

The position of the α-band can vary between 549 and 556 nm. The exceptions are certain protozoan cytochromes (e.g. *Crithidia oncopelti* cytochrome

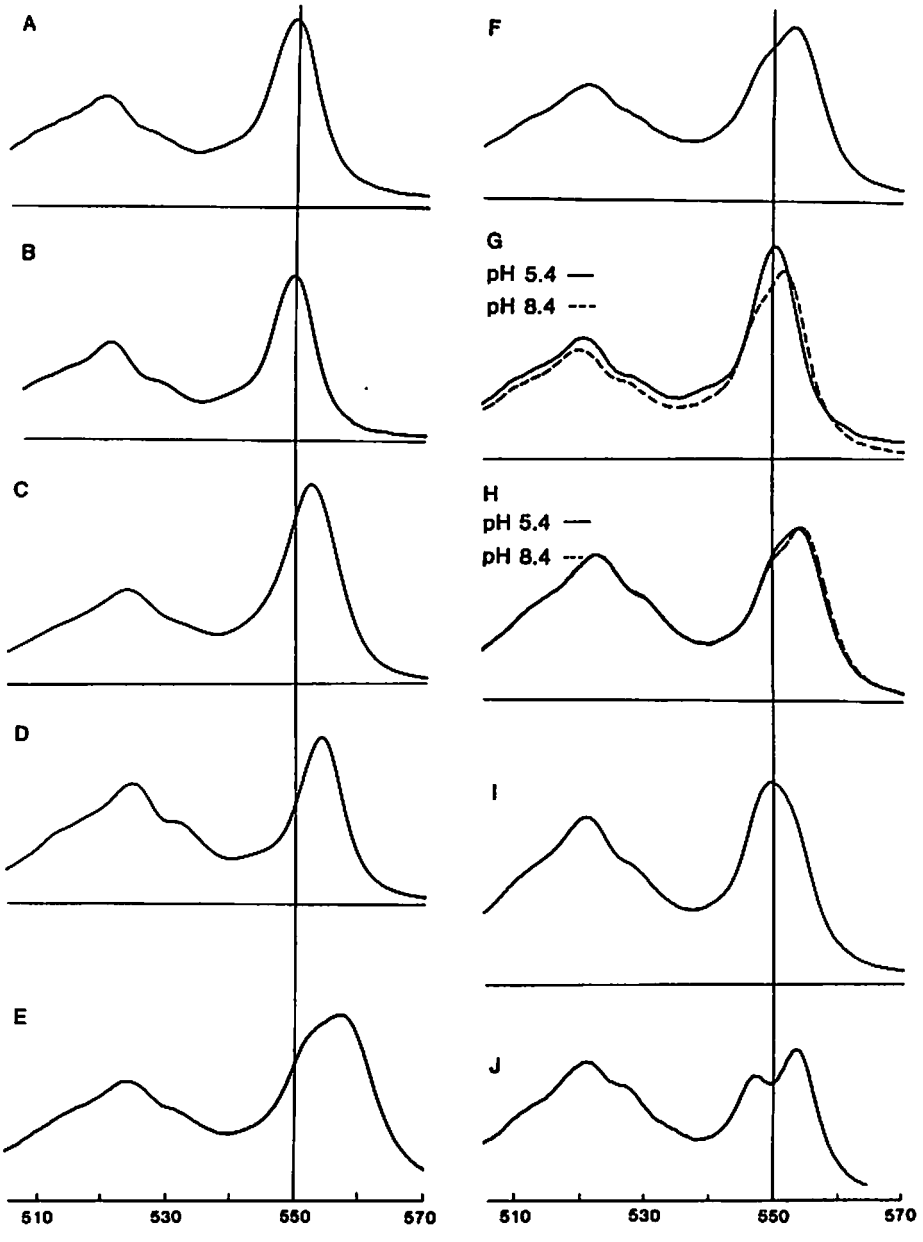


Fig. 1.8A–J. Absorption spectra of selected ferrocytochromes c in the region of the α- and β-bands. Spectra were recorded after addition of sodium dithionite for **A**, horse heart cytochrome c; **B** *Rhodopseudomonas capsulata* cytochrome c_2; **C** *Desulfovibrio gigas* cytochrome c_3; **D** *Ps. mendocina* cytochrome c_5; **E** *Crithidia oncopelti* cytochrome c-557; **F** *Porphyra tenera,* cytochrome c-553; **G** *Ps. aeruginosa* cytochrome c-551; **H** *Chlorobium*

Table 1.3. Quantitative features of the absorption spectra of selected c-type cytochromes

	α_{max}	$E_{\alpha max}$ (mM^{-1})	β_{max}	$E_{\beta max}$ (mM^{-1})	α/β	Reference
Horse heart c	550	29	521	15.5	1.87	Pettigrew et al. 1975
Rps. capsulata c_2	550	29	521	17.2	1.69	This work
D. gigas c_3 (4 heme)	552.5	124.4	524	58.4	2.13	This work
Ps. mendocina c_5	554	26.7	524	19.7	1.36	This work
C. oncopelti c-557	557	24.7	524	15	1.65	Pettigrew et al. 1975
P. tenera c-553	553	23	521	15.2	1.51	This work
Ps. aeruginosa c-551						
pH 5.4	550.5	31.8	521	17.7	1.79	This work
pH 8.4	551.5	28.1	520	15.9	1.77	This work
Chl. limicola c-555						
pH 5.4	555	19.8	523	16.5	1.2	Meyer (1970)
Ps. stutzeri c_4	550	44.4	521	36.6	1.21	This work
Paracoccus sp. c-554 (548)	554	18.1	521	16.6	1.09	This work

Spectra in 0.1 M phosphate pH 7 were recorded after addition of dithionite. Extinction coefficients were calculated on the basis of pyridine hemochrome spectra ($E_{mM,\,550\,nm}$ 31.18, Bartsch 1971).

c-557, Fig. 1.8E) which contain a single thioether linkage. The α/β peak ratio can be as low as 1.1 (Fig. 1.8J) and as high as 2.1 (Fig. 1.8C) (Table 1.3).

Together, these spectroscopic differences are an important basis for the identification and classification of cytochromes discussed in Section 1.3.

1.2.4 Midpoint Oxidation Reduction Potentials

As noted earlier in Section 1.1.3, the Nernst plot will give a slope of 60 mV for a pure monoheme system. This is illustrated in Fig. 1.9 for the redox titration of cytochrome c-554 (548) of halotolerant *Paracoccus* sp. Such titrations can be performed either with the aid of a redox electrode and mediators to report the ambient potential of the solution or by the method of mixtures with a known redox couple. They should be performed in both the oxidative and reductive directions to demonstrate reversibility.

Redox titrations of cytochromes having more than one heme can be complex and difficult to analyse and interpret (Fig. 1.4). A discussion of this problem is reserved for Vol. 2, Chap. 7.

limicola, f. *thiosulfatophilum*, cytochrome c-555; I *Ps. stutzeri* cytochrome c_4; J halotolerant *Paracoccus* sp. cytochrome c-554 (548). Cytochromes were dissolved in 0.1 M phosphate pH 7 except in the cases of *Ps. aeruginosa* cytochrome c-551 and *Chlorobium limicola* cytochrome c-555 where 0.1 M acetate pH 5.4 and 0.1 M Tris Cl pH 8.4 were used

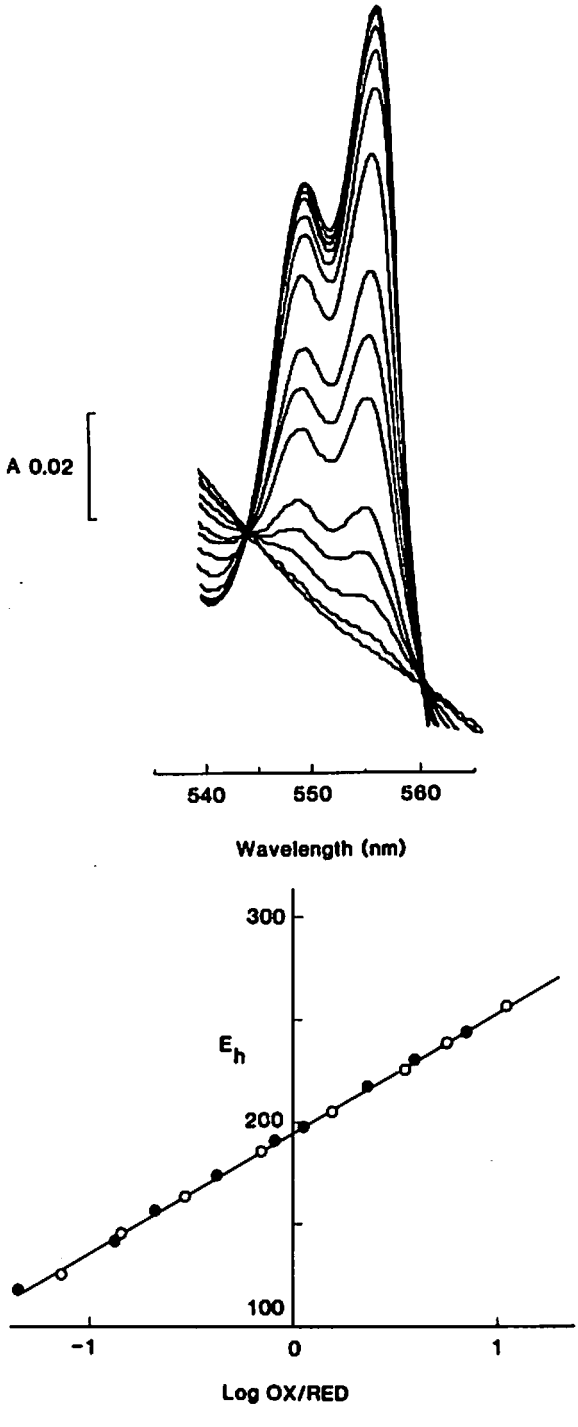


Fig. 1.9. The redox titration of cytochrome c-554 (548) from halotolerant *Paracoccus* sp. Cytochrome spectra were recorded in a stirred anaerobic cuvette containing 20 μM phenazine methosulfate, diaminodurol and ferric ammonium sulfate with 0.4 mM EDTA. Each spectrum corresponds to a particular ambient potential (not shown) and is used to calculate $\log \text{cyt.}_{ox}/\text{cyt.}_{red}$. ○, ● Experimental points from oxidative and reductive titrations respectively. The *line* has a slope of 60 mV

1.3 Classification

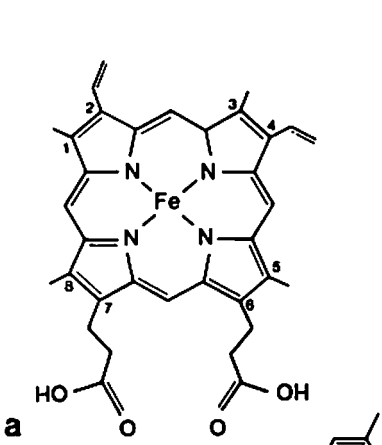
Classification in biology is both a fascinating and a frustrating exercise. Definitions are based on incomplete knowledge and so must be regarded as temporary and subject to modification (if not complete change) with the appearance of new information. In spite of this, many biologists feel that a good classification scheme approaches a natural order – a phylogeny – which has a historical validity and which maps the evolutionary relationships of the defined groups.

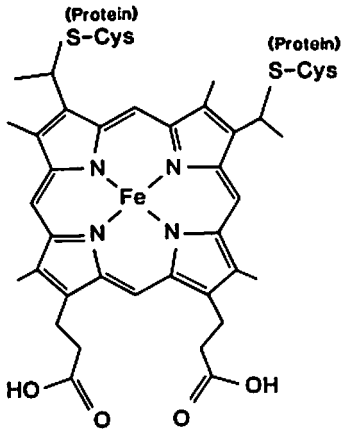
With whole organisms we have no difficulty in exclusively defining a group such as the insects (although we have more difficulty with a group such as the Pseudomonads). Such classification has only been achieved by the accumulation of observations on species, thus building up the natural history of the group. But with proteins we are just at the beginning of defining their natural history (Ambler 1977) and because information is sparse, our present minimum definition of a group will become inadequate with future discoveries.

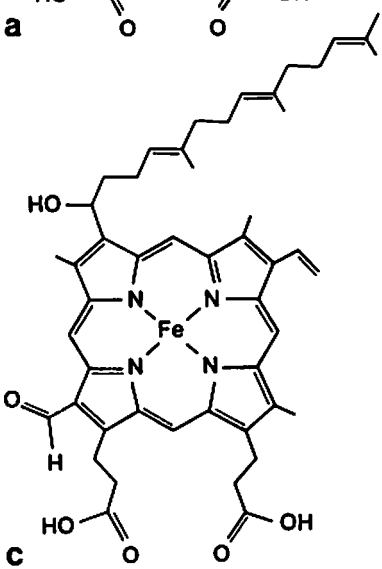
The classification of cytochromes into the types a, b, c and d is, however, a quite straightforward and accepted procedure. The associated prosthetic heme groups (Fig. 1.10) are sufficiently different to give rise to distinctive optical absorption spectra (although those of the b- and c-type cytochromes do overlap). In Fig. 1.11 we have illustrated these spectral distinctions by showing three complex systems each of which has a c-type cytochrome tightly associated with another heme. C-type cytochromes are further distinguished by the covalent attachment of their heme and can be quantified in complex mixtures after extraction of the non-covalently bound hemes of other cytochromes into acidified acetone.

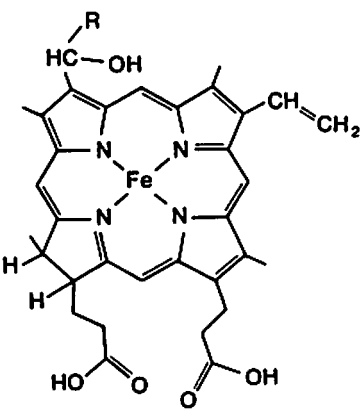
Further subdivision of the c-type cytochromes is important if we are to understand the relatedness of the electron transport chains and organisms of which they are a part. It might be argued that we could use existing whole organism groups to classify their constituent cytochromes. However, this assumes congruence of the history of a single gene with that of the whole genome. As we shall see, present evidence suggests that this may be valid for eukaryotes but not necessarily so for prokaryotes. Thus mitochondrial cytochrome c appears to be a sensible grouping but *Pseudomonas* cytochrome c-551 is more of a problem. Not all Pseudomonads have this cytochrome and some non-Pseudomonads have structurally close relatives of the cytochrome (Ambler 1977). The latter observation, with others, suggests that cross-species gene transfer may have given rise to bacterial genomes which are historical mosaics rather than of single divergent origin (Ambler et al. 1979a; Vol. 2, Chap. 6).

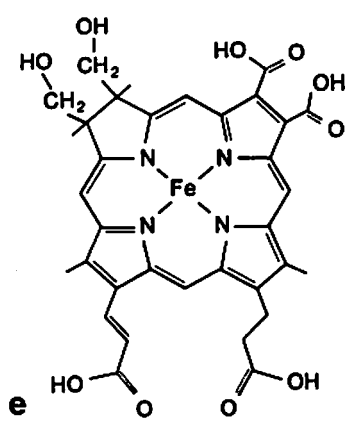
For the following three reasons we are on no stronger ground if we attempt classification on the basis of function. Firstly, we often have very sketchy information on the function of bacterial electron transport proteins. This is partly because of the complexity of their terminal electron transfer processes in comparison to those of the mitochondrion. For example, in the case of *Pseudo-*

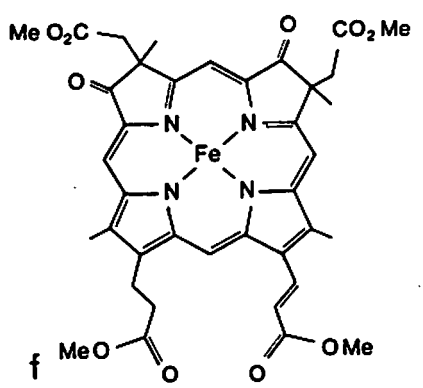

a
HO
OH
O
O
Fe
N


b
(Protein)
S-Cys
(Protein)
S-Cys
HO
OH
O
O
Fe
N


c
HO
O
H
HO
OH
O
O
Fe
N


d
R
HC—OH
CH=CH2
H
H
HO
OH
O
O
Fe
N


e
OH
HO
CH2
CH2
HO
O
OH
O
HO
OH
O
O
Fe
N


f
Me O2C
O
CO2 Me
O
MeO
OMe
O
O
Fe
N

monas aeruginosa, we find cytochrome c-551, c_4 and c_5 as well as the copper protein azurin which, as a group, are presumed to act as e^- donors to the terminal reductases for oxygen, hydrogen peroxide and the oxides of nitrogen.

Secondly, there is a relative lack of specificity of electron transfer in vitro. Thus *Pseudomonas* nitrite reductase (cytochrome cd_1) will react with a wide variety of bacterial cytochromes c and will accept electrons with similar efficiency from c-551, c_4, c_5 and azurin (Yamanaka 1972, 1975). The small cytochromes themselves show cross-reactivity, for example mitochondrial cytochrome c will quite rapidly reduce *Pseudomonas* cytochrome c-551, so that to deduce electron transport sequences and functional homology from the reactivity of purified components is of doubtful validity. This lack of specificity in electron transport between the small c-type cytochromes themselves can lead to problems in the use of oxidase particle preparations where some endogenous c-type cytochrome may still be present. In such cases the endogenous protein may catalyse an electron transfer to the oxidase which would not otherwise occur.

Thirdly, there are clear examples of close structural relatives performing different functions and of distant relatives performing the same function. Thus cytochrome c_2 of *Rhodopseudomonas capsulata* is the e^- donor to the photooxidised reaction centre, yet is very similar in amino acid sequence to cytochrome c-550 from *Paracoccus denitrificans,* a cytochrome which donates electrons to a cytochrome oxidase. On the other hand, cytochrome c-553 of *Rhodospirillum tenue* probably performs the same function as cytochrome c_2 but is not closely related in structure (Ambler et al. 1979a, b).

1.3.1 Cytochrome Classes I, II and III

The amino acid sequence work on the bacterial cytochromes, principally by R. P. Ambler and colleagues, has revolutionised our understanding of the relationships between different cytochromes and between the organisms of which they are a part. The ubiquitous small bacterial cytochromes divide into three groups on the basis of amino acid sequence (Ambler 1980) and the distinct and exclusive structure of these groups has been confirmed by X-ray crystallography (Almassy and Dickerson 1978; Korszun and Salemme 1977; Weber et al. 1980; Haser et al. 1979). We call these groups class I, II and III after Ambler (1980) and define them in Table 1.4.

Apart from the Cys-X-Y-Cys-His heme attachment, there is no sequence similarity between the three classes and thus no ambiguity in assigning a particular sequence to a particular class. However, not all amino acid sequences

Fig. 1.10a–f. The hemes of cytochromes. **a** protoheme IX; **b** substituted protoheme IX (heme c); **c** heme a (Caughey et al. 1975; Thompson et al. 1977); **d** proposed structure of heme d (Barrett 1956; Lemberg and Barrett 1973); **e** proposed structure of heme d_1 (Timkovich et al. 1984); **f** proposed structure of heme d_1 (methylester derivative) (Chang 1985)

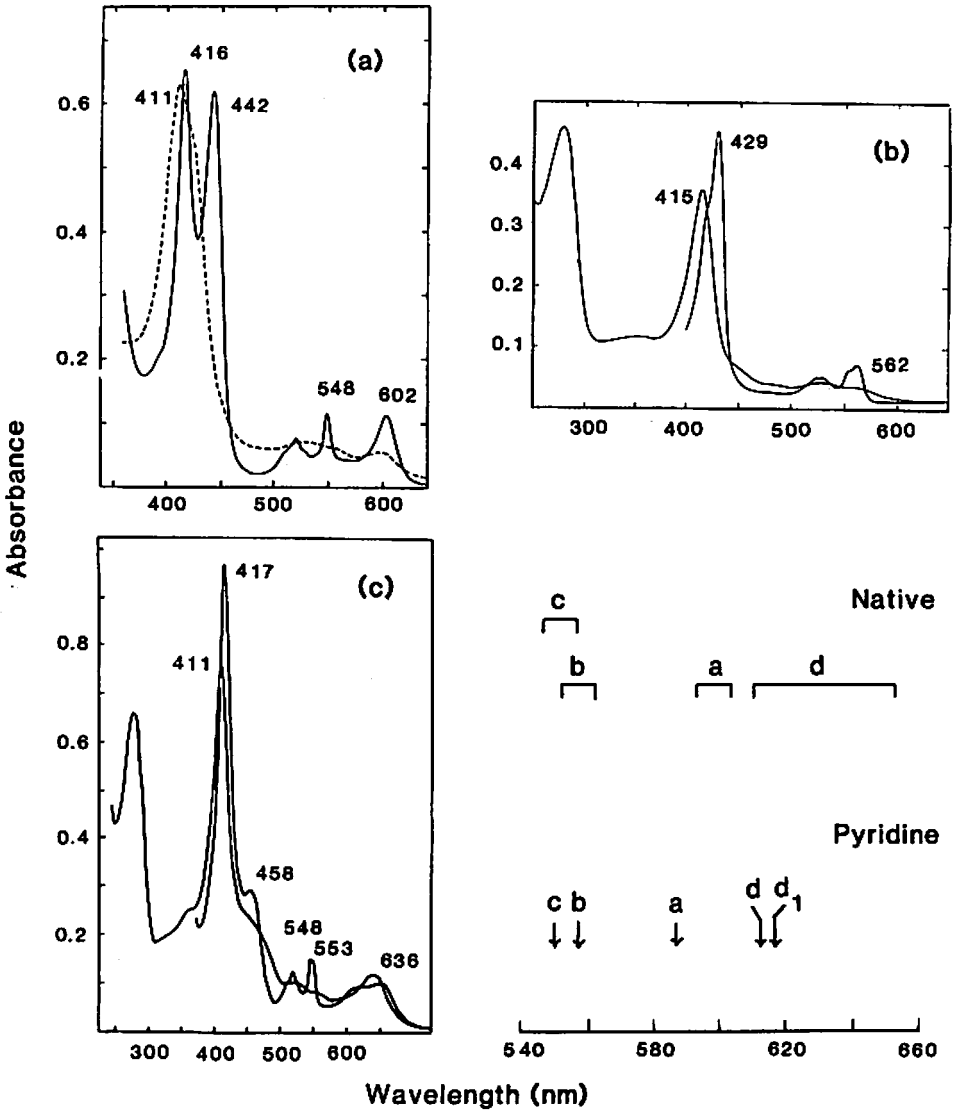


Fig. 1.11a–c. UV-visible absorption spectra of complex cytochromes containing heme c. The ferrocytochrome spectra are those showing a band near 550 nm. **a** The caa_3 complex of *Thermus thermophilus* (Hon-nami et al. 1980); **b** the cytochrome bc_1 complex of mitochondria (Yu et al. 1974); **c** cytochrome cd_1 of *Ps. aeruginosa* (Meyer and Kamen 1982). The *lower right* part of the figure shows the range of the α-peak maximum for native ferrocytochromes (┌──┐) and for the alkaline pyridine ferrohemochromes (↓) (Yamanaka and Okunuku 1974; Falk 1964; Lemberg and Barrett 1973). The range for the native ferrocytochrome d_1 is large because the α-band maximum is dependent on the nature of the reductant (635 nm with dithionite, 655 nm with ascorbate) (Parr et al. 1974). A few protozoan cytochromes have only one thioether linkage to the heme group and show pyridine ferrohemochrome spectra intermediate between cytochromes b and c (Pettigrew et al. 1975)

Table 1.4. Classification of c-type cytochromes: major structural divisions

Class	Features	Subdivision	Features	Examples
I	Low-spin, His + Met heme coordination, heme near N-terminus, 80 – 120 amino acids	(a) Large	Loop of residues closes bottom of heme crevice	Mitochondrial cyt.c, cyt.c_2
		(b) Small	Lacks loop of (a). Left side folds downward to close bottom of heme crevice	*Pseudomonas* cyt.c-551 *Chlorobium* cyt.c-555 (Algal cyt.c-553) Cyt.c_4, Cyt.c_5 (*Desulfovibrio* cyt.c-553)
II	Heme near C-terminus	(a) High-spin	His only heme coordination	Cyt.c′
		(b) Low-spin	His + Met heme coordination	(Cyt.c-556)
III	Multi-heme, one heme per 30 – 40 amino acids bis-Histidyl coordination	On basis of heme content		[Cyt.c_3 (3 heme)] Cyt.c_3 (4 heme) [Cyt.c_3 (8 heme)]

Cytochrome groups in parentheses have not yet been fully characterised by X-ray crystallography although preliminary studies are in progress in most cases.
The original class IV of Ambler (1980) included complex proteins with prosthetic groups other than heme c. Other c-types, such as cytochrome c_1 and cytochrome c peroxidase were unclassified. We suggest that this is not a useful arrangement and propose that class IV and subsequent classes be reserved for homogeneous structural groups. In Volume 2, Chapter 5 we propose that the reaction centre cytochrome c of *Rhodopseudomonas viridis* be the prototype of a new class IV. It is possible that complex cytochromes such as cytochrome cd_1 and flavocytochrome c may be shown in future to have domains resembling existing classes such as class I. If this is so, we propose that these domains be included in the existing class rather than given the status of a new class.*

will be determined and it is useful to ask whether exclusive definitions can be formulated on the basis of more immediately accessible properties of the molecules such as spectra, molecular size, redox potential and reactivity with model oxidase and reductase preparations. An important point to bear in mind when attempting classification on these bases is that for a given cytochrome, a property is not an absolute parameter but depends on the conditions of measurement. We cannot solve the problem by measuring the property under "physiological conditions" because usually we have little or no reliable information about what these conditions are. Thus the reactivity of different mitochondrial cytochromes c with cytochrome oxidase was originally claimed to be highly variable (Yamanaka and Okunuki 1968), then constant (Smith et al. 1973) and more recently variable (Ferguson-Miller et al. 1978), the results depending on the exact conditions used. Similarly, midpoint redox potentials are often pH-dependent and, because the cytochromes are located at the acid side of bioenergetic membranes, it is not at all clear that pH 7 affords the most

* See appendix note 1

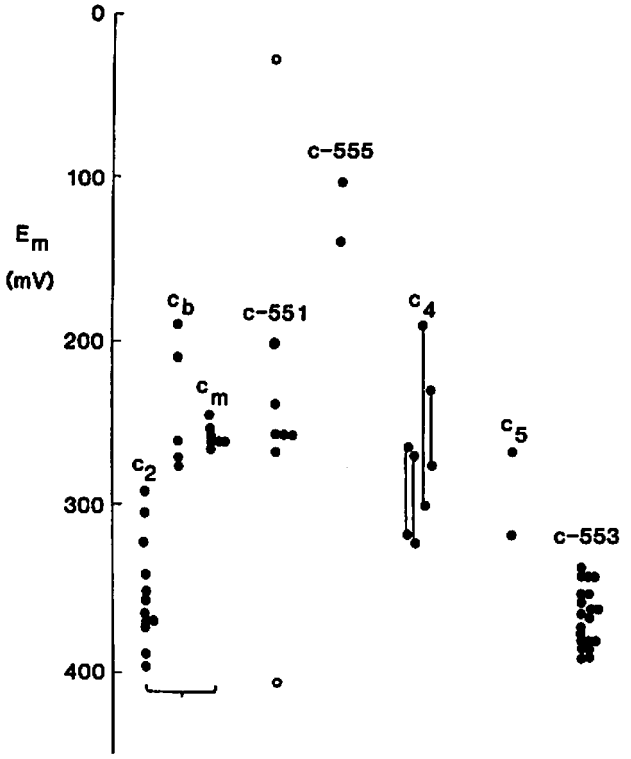


Fig. 1.12. The midpoint oxidation reduction potentials of the class I cytochromes c. Midpoint potentials are compiled from values given elsewhere in the book. Each *circle* represents a measurement of the midpoint potential of one member of the cytochrome group named above the columns. The *brackets* indicate that cytochrome c_2 (c_2), mitochondrial cytochromes c (c_m) and the bacterial cytochromes c, known to be donors to bacterial cytochrome aa_3 (c_b), are structurally homologous. The *open circles* of the cytochrome c-551 group correspond to measurements for *Rps. gelatinosa* cytochrome c-550 (+20 mV) and *Rsp. tenue* cytochrome c-553 (+405 mV). These proteins show remote but convincing sequence similarity to the *Pseudomonas* c-551 group (Vol. 2, Chap. 3). c-555 are the cytochromes c-555 of the Chlorobiaceae; c-553 are the cytochromes c-553 from algae. The *lines* joining values for cytochrome c_4 indicate the presence of two potentiometrically distinct hemes per molecule

valuable and valid point of comparison. Nonetheless physicochemical properties do greatly assist classification.

The class I cytochromes all have the 695-nm band in the ferric state which is dependent on the presence of a histidinyl-methionyl-Fe coordination as noted in Section 1.2.3. A further spectroscopic indicator of such coordination is the pronounced upfield shift of the methionine methyl resonance in ^{1}H-NMR spectra (Vol. 2, Chap. 2). Class I cytochromes contain 1 heme per 70–120 residues (a definition that includes the diheme cytochrome c_4 of M_r 20 K). They act as immediate electron donors to a terminal oxidising centre

which may be a photo-oxidised chlorophyll species or a terminal respiratory enzyme. As a consequence they tend to exhibit redox potentials near the positive end of the biological scale, but since the terminal oxidising centres show considerable variation in operating potential, the range covered by their cytochromes c is correspondingly large (0 to +500 mV) (Fig. 1.12).

The class II cytochromes originally included only the cytochromes c' which were characterised by high-spin absorption spectra (Fig. 1.6a). The discovery of low-spin homologues has complicated any definition of the group, particularly as some of these have a 695-nm band and contain methionine as a sixth heme ligand. These low-spin cytochromes have pronounced red shifts in most of their absorption bands (Meyer and Kamen 1982; Fig. 1.6b) but it is doubtful whether they can be reliably identified on this basis. This, so far, small subgroup probably constitutes the main risk of misclassification of a structurally uncharacterised cytochrome.

Cytochromes of class III are distinguished by their multi-heme nature and very low redox potentials. Even in the absence of analytical information of this sort they can be identified by the presence of a distinctive shoulder on the trailing edge of the reduced Soret peak, a high Soret (red.)/Soret (ox.) ratio and the absence of a 695-nm band in the oxidised form. These features serve to distinguish the class III cytochromes from those of other groups (Figs. 1.6d and 1.7).

Thus the three main structural groups of c-type cytochromes can be defined on exclusive lines in terms of their properties, and a novel cytochrome can usually be reliably designated. Problems arise when subdivision of class I is attempted.

1.3.2 Subdivision of Class I

Attempts to subdivide the cytochromes of class I on the basis of size (Ambler 1980) and three-dimensional structure (Dickerson 1980) have been made and will be discussed more fully in Vol. 2, Chaps. 3 and 4. We feel that some differences in size may reflect rather minor genetic changes in otherwise related proteins. For example, the cytochromes c_2 form a quite homogeneous group of proteins with greater than 30% identity in amino acid sequence (Meyer and Kamen 1982), yet subdivision on the basis of size splits this group into two.

In this case, size differences involve short loops of chain on the surface of the molecule. A more major size difference is observed between the cytochromes c_2/mitochondrial cytochrome c group on the one hand, and a group of smaller class I cytochromes, on the other. Here the size difference is due to the presence or absence of a large loop of chain which closes off the bottom of the heme crevice (Almassy and Dickerson 1978; Korszun and Salemme 1977) and it is possible that this represents a useful basis for subdivision. Implicit in this proposal is the belief that such a major change could have occurred only once in evolution and that it gave rise to two coherent lines of descent.

Table 1.5. Subdivision of class I cytochromes c; distinguishing features of subclasses

Subclass	Size	Spectra	$E_{m,7}$	Structure	Function	Comments and Anomalies
Mitochondrial cytochrome c	*103 – 112 aa* Size variation at N-terminus	*α (550 nm), sym. α/β* 1.8	*244 – 264 mV*	40% minimum pairwise sequence identity	*Reduced by cytochrome c_1, oxidised by cyt.aa_3*	*Tetrahymena* cyt.c does not react with mammalian oxidase. Other protozoan cytochromes have red-shifted absorption bands due to a single cysteine heme attachment site
Rhodospirillum cyt.c_2	*97 – 124 aa* Size variation at surface loops	*α (550 nm), sym. α/β 1.6*	*290 – 390 mV*	30% minimum pairwise sequence identity	Probably reduced by cyt.c_1, *oxidised by reaction centre* and in some species by a cyt.c oxidase	By simple sequence comparison, cyt.c_2 cannot be distinguished from mitochondrial cyt.c yet no cytochrome c_2 reacts well with mammalian oxidase. *Paracoccus denitrificans* cyt.c-550 is a close homologue of *Rps. capsulata* cyt.c_2 (54% identity) yet occurs in a non-photosynthetic organism and has an $E_{m,7} = 260$ mV
Pseudomonas cyt.c-551	*81 – 82 aa*	*α (551 – 553 nm)* sym. or asym., *α/β = 1.7 – 2.1*	*200 – 265 mV*	64% minimum pairwise sequence identity within limited groups. *Lacks 13 residue loop at base of heme crevice*	Perhaps reduced by cyt.c_1, possibly oxidised by cyt.cd_1 and/or an o-type oxidase	A homologue with 64% identity is found in the aerobe *Azotobacter vinelandii*. More distant homologues are also found in the photosynthetic bacteria (*Rsp. tenue* cyt.c-553, 50% identity; *Rps. gelatinosa* cyt.c-550, 35% identity)
Chlorobiaceae cyt.c-555	*86,99 aa* Size variation mostly at N-ter-	*α (555 nm), asym., α/β = 1.2*	*154, 103 mV*	54% sequence identity. *Lacks loop at base of heme like*	Probably involved in electron transfer from sulfide and thiosulfate to reac-	

Algal cyt. c-553	*83 – 89 aa*	*α (552 – 554 nm), usually asym., $\gamma_t/\alpha = 5.7 - 7.7$*	*335 – 390 mV*	38% minimum pairwise sequence identity. *Lacks loop at base of heme like cyt.c-551*	*Interchangeable with plastocyanin in algae. Acts between cyt.f and photo-oxidised chlorophyll*	*Euglena* cyt.c-552 has a symmetrical α-peak and $\gamma_t/\alpha = 5.2$. It is also the most divergent sequence
Cytochrome c_4	*181, 190 aa*	*α (550 nm), sym. $\alpha/\beta = 1.2$. Low 260 – 280 nm absorption, no shoulder at 290 nm*	*190 – 320 mV*	77% sequence identity. *Two-domain structure* with one heme per domain, *gene doubling*, each domain lacks loop at base of heme. *No Trp*	Not known but reactive with *Pseudomonas* cyt.cd_1 in vitro	Hemes probably have different redox potentials with variable degree of separation. Cytochrome c-554 (548) from halotolerant *Paracoccus* is 50% identical to cytochrome c_4 and is isolated as a dimer of mol.wt.18000
Cytochrome c_5	*87 aa*	*α (554 nm) $\alpha/\beta = 1.4$*	*265, 320 mV*	*Contains a second Cys-X-Y-Cys site with no heme bound.* Ragged N-terminus	Not known, suggested to be a fragment of larger protein	
Desulfovibrio cyt.c-553	*82 aa*	*α (553 nm)*	*0 mV*	Little similarity to other sequences. *No Trp*	Not known	The amino acid sequence of the cytochromes c-553 from the Hildenborough and Miyazaki strains have been determined and show little resemblance in spite of the similarity in their cytochromes c_3 (4 heme). The E_m for the Miyazaki strain was quoted as −280 mV yet the cytochrome was reducible with ascorbate

Principal discriminating features are indicated by italic script

If cytochromes are found which lack this portion of chain and yet are close relatives of the large cytochromes on the basis of the rest of the amino acid sequence such a proposal would have to be discarded.

With this doubt about classification on the basis of detailed structural information it is not surprising that classifications on other bases are not rigorous. We offer in Table 1.5 some tentative definitions of subgroups of class I on the basis of size, spectrum, redox potential and reactivity with oxidase and reductase preparations. Together, these properties allow a composite definition which distinguishes one group of cytochromes from another but often there are anomalies which must be taken into account.

We have reached a point where the amount of available structural information should encourage a re-examination of the commonly used subdivisions of class I and we offer a partial reorganisation and renaming of the cytochrome subclasses in Table 1.6. In doing this two main problems have to be faced. One is whether to group together or to subdivide extensively, i.e. what degree of structural difference merits a separate grouping? The second is whether to

Table 1.6. A cytochrome c nomenclature

Names in common usage	Suggested[a] name
Cytochrome f Cytochrome c_1	c_1
Mitochondrial cyt.c Cytochrome c_2	c_2
Cytochrome c_3[b] Cytochrome cc_3 Cytochrome c_7	c_3 (4 heme) c_3 (8 heme) c_3 (3 heme)
Cytochrome c_4	c_4
Cytochrome c_5	c_5
Cytochrome c_6 Algal cytochrome f Algal cytochrome c-553	c_6
Pseudomonas cyt.c-551[c]	c_7
Cytochrome c'[d] Cytochrome c-556	c_8

[a] We should emphasise that these are personal recommendations. Cytochrome nomenclature remains in the hands of an International Committee.

[b] The cytochrome c_3 group has also been subdivided on the basis of M_r (c_3-14 K, c_3-26 K, c_3-9 K). We have not adopted this nomenclature because of variation of M_r within a subdivision.

[c] Cytochromes c from *Azotobacter vinelandii, Rhodospirillum tenue* and *Rhodopseudomonas gelatinosa* are homologous to the cytochromes c-551 of the Pseudomonads.

[d] The cytochromes c' were originally given a variety of names, e.g. cytochromoid, cryptocytochrome c, cytochrome cc', RHP which are no longer in use.

employ functional as well as structural criteria. Our revised scheme is conservative in its degree of subdivision and essentially ignores functional considerations. An alternative scheme based on such considerations could be constructed but would be sketchy through lack of knowledge and would incorporate both convergent and divergent effects.

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