Biochimica et Biophysica Acta, 975 (1989) 1–28
Elsevier

BBA 42977

Review

Structure, function and distribution of soluble bacterial redox proteins

T.E. Meyer and M.A. Cusanovich

Department of Biochemistry, University of Arizona, Tucson, AZ (U.S.A.)

(Received 29 July 1988)

Key words: Electron transfer protein; Bacterial protein; Protein structure; Redox protein

Contents

I. Introduction

Diverse is the best way to describe bacterial electron transfer. Whether one considers structure–function relationships at the level of individual proteins or of their organization into pathways, the overall impression is wonder at the variety of ways in which bacteria carry out simple oxidations and reductions of both organic and inorganic substrates. Although it is possible to find parallels between diverse species of bacteria at the level of individual proteins, the way in which pathways are assembled and the way these in turn are integrated into the whole makes it unlikely that any two species will be identical. An appreciation of this diversity may be obtained by examining what is currently known about electron transfer proteins and their interactions in examples of the most actively studied bacteria. The bacterial species to be discussed here are not necessarily the most diverse collection one could assemble and are by no means complete, but they are sufficient to illustrate the complexity to be expected of all bacteria and to point out areas of understanding and those where more information is needed.

Abbreviations: SDS-PAGE; sodium dodecyl sulfate-polyacrylamide gel electrophoresis; APS, adenosine phosphosulfate; 8-Fe-S, ferrodoxin, iron sulfur proteins with two four-iron-four-sulfur clusters; HiPIPs, iron-sulfur proteins with a high oxidation-reduction potential; SHP, *sphaeroides* heme protein; DMSO, dimethyl sulfoxide; TMAO, trimethylamine *N*-oxide; DCPIP, 2,6-dichlorophenolindophenol.

Correspondence: T.E. Meyer, Department of Chemistry, University of Arizona, Tucson, AZ 85721, U.S.A.

A number of characteristics of bacterial electron transfer do not generally apply to higher organisms. For example, the function of a particular protein may not be the same from one species to the next. Moreover, particular functions are not necessarily carried out by the same enzymes in all species. In many bacteria, the detailed mechanism of electron transfer is unknown and in many cases the composition of pathways is still incompletely described. That is, functions are not obvious, isozymes are relatively common and many proteins *appear* to be superfluous. The localization of electron transfer proteins cannot be taken for granted in cytoplasm, periplasm or membrane in the bacteria. In fact, the implication of Gram stain in the localization of electron transfer proteins and composition of pathways is not widely appreciated. Thus, Gram-positive bacteria do not have an outer membrane, and hence presumably they do not have a periplasmic space nor periplasmic proteins. Environmental factors such as pH, temperature and salinity have far-reaching mechanistic implications which go beyond the obvious question of protein stability. For example, many periplasmic redox proteins use electrostatic attraction or repulsion as an important factor in defining interaction domains. However, in extreme halophiles this cannot be the case although the same structural families of redox proteins are present, because electrostatic interactions are masked at high ionic strength. Many pathways and proteins are induced by specific growth conditions and, therefore, are not expressed under all growth conditions. Moreover, such expression is likely to vary from one species to the next. Some pathways or components of pathways are not under chromosomal control but, rather, are borne on plasmids and presumably are more easily transferred from one species to another. Indeed, gene transfer may be a common occurrence for bacterial electron transfer proteins, but this is a hotly debated issue and there is not sufficient evidence to make definitive statements at this time. Finally, structural studies of redox proteins from both bacteria and higher organisms have concentrated on soluble proteins, with the consequence that much less is known about membrane-associated enzymes.

The scope of this article includes the soluble electron transport proteins, generally consisting of cytochromes, ferredoxins and copper proteins. We include in this review some mention of those enzymes and membrane components which are known to interact with the soluble components and are themselves well characterized either structurally or genetically. We do not intend to review integral membrane proteins in depth or to include all enzymes which interact with the soluble proteins. Only a small fraction of the total protein of the cell is periplasmic, yet soluble electron transfer proteins are disproportionately represented in the periplasm. Although it may be fortuitous, Wood [1] proposed that *c*-type cytochromes are all likely to be found in the periplasm or bound to the outer surface of the cytoplasmic membrane of Gram-negative bacteria. This generalization was based on a small number of observations, but, to date, no exceptions have been found. In contrast, *c*-type cytochromes have not been found except in trace amounts in the soluble fraction of any Gram-positive bacterium to date. In those Gram-negative bacteria, which have an *a*-type oxidase, there is generally a periplasmic cytochrome found which is analogous to mitochondrial cytochrome *c* in function. Aerobic Gram-positive bacteria have no outer membrane and presumably no periplasmic space, and probably have no counterpart to mitochondrial cytochrome *c* or, if one exists, it will be bound to the cytoplasmic membrane.

A common thread that ties together the various soluble redox proteins is structural homology. That is, although a diversity of physical-chemical and functional properties is found, only a small number of basic structural motifs are known. This is best illustrated by the soluble *c*-type cytochromes which can be divided into three principal structural families as outlined by Ambler [2]. Class I *c*-type cytochromes have been the best studied and represent the largest family based on the number of examples known. These heme proteins in which the heme is bound near the N-terminus and which have histidine-methionine heme ligation, are widely distributed and are found in most but not all prokaryotes and eukaryotes. They typically have oxidation-reduction potentials in the range of 0–470 mV and are well characterized structurally. The Class I *c*-type cytochromes can be divided into eight or more distinct subclasses based on sequence homology and other properties. Nevertheless, they represent as a whole, an evolutionarily related class of cytochromes. A number of reviews summarize our understanding of Class I *c*-type cytochromes [3–7].

Class II *c*-type cytochromes, typically referred to as the cytochromes *c'*, are a distinct structural family. The heme is covalently bound near the C-terminus (in contrast to Class I *c*-type cytochromes) and has either histidine as a fifth ligand with the sixth position vacant or has histidine and methionine as fifth and sixth ligands. Those Class II *c*-type cytochromes with only histidine ligation are found with the iron in the high-spin state and have spectral properties quite distinct from Class I *c*-type cytochromes. The low-spin examples which have histidine-methionine ligation are less well studied, but clearly in Class II based on amino acid sequence homology [2]. The Class II *c*-type cytochromes typically have redox potentials in the range -10 to $+200$ mV.

Class III *c*-type cytochromes are generically referred to as the cytochromes c_3 and have bis-histidine ligation and low oxidation-reduction potentials (-150 to -400 mV). A variety of *c*-type cytochromes have been identi-

fied which cannot readily be classified at this time. In general, these cytochromes are not well characterized structurally, hence they may belong in existing classes. However, the possibility that additional structural families exist cannot be excluded.

In bacteria, the iron sulfur proteins generally have one or two four-iron-four-sulfur clusters. Two principle types exist. Those with low oxidation-reduction potentials (−300 to −500 mV) and two clusters which will be termed 8-Fe-S ferredoxins for the purpose of this review and those with high oxidation-reduction potentials (50–450 mV) which we will term HiPIPs. 8-Fe-S ferredoxins and HiPIPs represent two distinct structural classes based on amino acid sequences, three-dimensional structures and in terms of physico-chemical properties. In some bacteria, a third type of iron-containing protein, rubredoxin, is found which has one iron and typically has a midpoint potential around −50 mV.

Soluble copper proteins are not widely distributed in bacteria, and to date those which are well characterized appear to be structurally related with the prototypic example being azurin. They contain a single blue copper and have redox potentials of +150 to +350 mV.

We have chosen to consider nine groups of bacteria based on the diversity of electron transfer and metabolic pathways. Thus, at one extreme *Paracoccus denitrificans* is a bacterial analogue to the mitochondrion and at the other extreme *Clostridium* is a strict *anaerobe* devoid of *c*-type cytochromes. *Escherichia coli* represents an aerobe almost devoid of *c*-type cytochromes and *Bacillus* a Gram-positive aerobic organism which may have no *soluble* cytochromes. *Azotobacter*, an obligate aerobe, and *Pseudomonas*, a facultative aerobe, are organisms which have similar soluble redox proteins, but which differ metabolically. *Alcaligenes*, although metabolically similar to *Pseudomonas* and *Paracoccus denitrificans*, has a distinct redox protein composition. *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* are characteristic examples of the facultatively aerobic purple photosynthetic bacteria and, although different in detail, have many similarities. In contrast, *Rhodopseudomonas viridis* and *Chromatium vinosum*, although they are also purple photosynthetic bacteria, are anaerobes (or marginal microaerophiles) and have quite different soluble and membrane-bound redox systems as compared to *Rb. capsulatus* and *Rb. sphaeroides*. *Desulfovibrio*, although a strict anaerobe like *Clostridium*, is rich in low oxidation-reduction potential redox proteins quite distinct from those found in other bacteria.

The choice of bacterial species for this review, the proteins and the pathways are the minimum required to demonstrate diversity of structure at the protein level and to show how the proteins are variously assembled into pathways which are far from uniform. No attempt is made to treat any particular organism in great depth, rather we focus on illustrating characteristic features. Due to space limitations, we have ommitted some very interesting organisms such as blue-green algae, methanogens, halophiles and green sulfur bacteria. Importantly, we have attempted to avoid traditional thinking which confines certain proteins (e.g., cytochrome *c*) to certain pathways (e.g., respiration). It is well established that certain copper proteins and cytochromes on the one hand or ferredoxins and flavodoxins on the other are interchangeable. We would like to consider the possibility that high potential ferredoxins (HiPIP) could replace cytochromes and low potential cytochromes could replace 8-Fe-S ferredoxins in certain situations as well. Finally, we want to show that proteins which have a well-established role in one species can perform quite differently in another, or be superfluous or discarded in a third. To document these points requires knowledge of amino acid sequence, three-dimensional structure, spectroscopy, redox properties, enzymology and genetics. The various species to be discussed have been characterized in these various ways and therefore are amenable to detailed analysis designed to illustrate the complexity and diversity of bacterial electron transfer.

II. *Paracoccus denitrificans*

P. denitrificans is a versatile organism capable of growth with a variety of organic substrates and electron acceptors. It has, consequently, become a popular organism for a variety of studies. Table I summarizes the redox protein composition.

P. denitrificans has gained distinction as the bacterium which has the greatest similarity to mitochondria [8]. The organism has a membrane-bound cytochrome bc_1 complex and an *a*-type cytochrome oxidase presumably interacting with a soluble Class I *c*-type cytochrome. However, *P. denitrificans* is capable of both aerobic growth or anaerobic growth with nitrate as the terminal electron acceptor. The analogy between aerobically grown *P. denitrificans* and the eukaryotic mitochondrion is quite strong in many respects, but more recent studies make the analogy with regards to the soluble *c*-type cytochrome less certain (see below). The contrast between *P. denitrificans* and another facultatively aerobic bacterium, *Escherichia coli*, is pronounced. These two represent the extremes in bacterial aerobic metabolism in that *E. coli* completely lacks the above-mentioned proteins (see following section).

P. denitrificans cytochrome c_2 was first purified by Scholes et al. [9] and was given the generic designation cytochrome *c*-550. The amino acid sequence was investigated by Timkovich et al. [10] and corrected by Ambler et al. [11]. A low-resolution three-dimensional structure was reported by Timkovich and Dickerson [12]. These structural studies demonstrated that the *P.*

TABLE I

Paracoccus denitrificans soluble redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome c_2	14	14	250	complete	constitutive
Cytochrome *c*-551	n.d.	22	190	n.d.	induced by methylamine or methanol
Cytochrome *c*-553	n.d.	30	160	n.d.	induced by methylamine or methanol
Cytochrome *c*-549	n.d.	9	190	n.d.	induced by methylamine or methanol
Cytochrome *c*-555/*c*-551	n.d.	45	128, −163	n.d.	diheme, induced by low oxygen
Pseudoazurin	14	14	230	n.d.	induced by anaerobic growth
Cytochrome *cd* (nitrite reductase)	120	60	n.d.	n.d.	induced by nitrate, two hemes per monomer
Amicyanin	15	15	294	partial	induced by methylamine

denitrificans cytochrome c_2 is closely related to the cytochromes c_2 from purple phototrophic bacteria and particularly to that from *Rb. capsulatus* [13]. There are some cytochromes c_2 which are virtually indistinguishable from mitochondrial cytochrome *c*, such as that from *Rhodopseudomonas globiformis* [14], but the larger cytochromes from *P. denitrificans* and *Rb. capsulatus* contain specific three and eight residue insertions and one and two residue deletions, which indicate that they are not likely to be immediate precursors of the mitochondrial cytochromes *c*. Nevertheless, based on amino acid sequence, cytochromes c_2 as a whole are more similar to the mitochondrial cytochromes *c* than are most bacterial *c*-type cytochromes.

P. denitrificans cytochrome c_2 is typical of the Class I cytochromes *c* in its reactions with non-physiological substrates such as free flavin and flavodoxin semiquinones [15,16]. *P. denitrificans* cytochrome c_2 is an acidic protein ($pI = 4.5$), yet retains a positive charge at the site of reduction typical of mitochondrial cytochrome *c*. This positive charge localized near the exposed heme edge is a characteristic of all cytochromes c_2 and is believed to play an important role in their interaction with physiological donors and acceptors. A characteristic of most cytochromes c_2 is a high redox potential (about 350 mV), however, *P. denitrificans* cytochrome c_2 has a redox potential similar to mitochondrial cytochromes *c* (250 mV).

It has been presumed for some time that the *P. denitrificans* cytochrome c_2 mediates electron transfer between the cytochrome bc_1 complex and oxidase. Cytochrome c_2 is a good substrate for purified *P. denitrificans* oxidase, it is constitutive and present in relatively large amounts (typically 12–18 μmol per kg of wet cells), and it appears to be periplasmic as expected for a cytochrome which functions in both aerobic and anaerobic pathways. However, recent studies suggest that a membrane-bound *c*-type cytochrome, not *P. denitrificans* cytochrome c_2, serves the role of mediator in the aerobic pathway. Berry and Trumpower [17] isolated a quinol oxidase complex, which contained seven subunits. The complex contains both cytochrome bc_1 and cytochrome oxidase components, plus a 22 kDa cytochrome *c* subunit and an unidentified 57 kDa subunit. This complex showed quinol oxidase activity in the absence of cytochrome c_2 and added cytochrome c_2 did not enhance the activity. These results suggest that cytochrome c_2 is not required for efficient electron transfer between the cytochrome bc_1 complex and the *a*-type oxidase complex. The 22 kDa cytochrome subunit has not been extensively characterized, but it could conceivably be a membrane-bound form of the soluble cytochrome c_2. More work is necessary to exclude this possibility.

As mentioned above, cytochrome c_2 is found to be abundant in both aerobically and anaerobically grown cells, and is therefore thought to have a role in denitrification. Most enzymes of the denitrification pathway are incompletely characterized, but are generally induced by growth on nitrate as an electron acceptor. The nitrite reductase is the most thoroughly studied enzyme in this pathway in *P. denitrificans*. *P. denitrificans* nitrite reductase is of the cytochrome *cd* type [18] and was most recently purified by Timkovich et al. [19]. Cytochrome *cd* presumably interacts with the bc_1 complex via cytochrome c_2, which is a good substrate for the enzyme. If the 22 kDa cytochrome subunit mediates electron transfer between the bc_1 complex and oxidase, then one might consider the possibility that it also mediates between cytochrome bc_1 and cytochrome *cd* (nitrite

reductase) however, it does not copurify with the cytochrome *cd* and as yet there is no evidence that it is involved in anaerobic electron transfer. In fact Bosma et al. [20] isolated the ubiquinol oxidase complex from anaerobic cells and found that the 22 kDa cytochrome was present at much lower concentrations than in the complex from aerobic cells. As will be discussed later, *Pseudomonas* contains a cytochrome *cd*-type nitrite reductase. However, cytochrome *c*-551, the electron donor to *Pseudomonas* cytochrome *cd*, does not react readily with *P. denitrificans* cytochrome *cd* suggesting substantial differences in the interaction domains of the two cytochromes *cd*. Although cytochrome *cd* is abundant in nitrate grown cells, it is labile and easily loses the *d* heme. The structure of 'heme *d*' shows that it is actually an iron porphinedione with an acrylic acid substituent [21] and is, therefore, unrelated to the oxidase heme *d* in coliforms. It is sometimes called heme d_1 to distinguish it from the latter.

A small soluble copper protein has been isolated from anaerobically grown *P. denitrificans* in amounts comparable to the cytochrome c_2 [22]. It is a poor reductant for nitrite reductase although its redox potential (230 mV) is very similar to that of the cytochrome c_2. The redox and spectral properties of the *P. denitrificans* copper protein suggest a similarity to azurins, which are typical of *Pseudomonas* species. However, the low cysteine content (one instead of three) indicates that the *P. denitrificans* copper protein is more closely related to pseudoazurin such as found in *Pseudomonas* AM1 [23] and it will henceforth be referred to as such.

Yet another small copper protein was induced to a level 2–4-times that of cytochrome c_2 in *P. denitrificans* by growth on methylamine [24–26]. Electron transfer proteins specifically induced by growth on methylamine were originally described for *Pseudomonas* AM 1 (now called *Methylobacterium*) by Tobari and Harada [27]. They coined the name amicyanin for the methylamine-inducible copper protein and found that it was the preferred electron acceptor for methylamine dehydrogenase. The amino acid sequence of *Pseudomonas* AM 1 amicyanin was determined by Ambler and Tobari [23], who showed that it was distantly related to azurin, pseudoazurin and plastocyanin. However, the N-terminal ten residues of *P. denitrificans* amicyanin are not obviously similar to the *Pseudomonas* protein which indicates that they are not closely related or there is an N-terminal extension in the *P. denitrificans* protein [25]. The redox potential of *P. denitrificans* amicyanin (294 mV) is not significantly higher than that of pseudoazurin (230 mV) [26] and the activity of the latter has not been tested with methylamine dehydrogenase.

P. denitrificans amicyamin is not an electron acceptor for methanol dehydrogenase [24], which is as expected because amicyanin is not induced by growth on methanol.

When *P. denitrificans* is grown on methylamine or methanol, two or three additional soluble cytochromes are induced, whereas amicyamin is only found in methylamine grown cells [24,28,29]. Husain and Davidson [28] found an acidic, 22 kDa cytochrome *c*-551 and an acidic 30 kDa cytochrome *c*-553 with redox potentials of 190 and 148 mV, respectively [26]. Bosma et al. [29] found a pair of induced cytochromes with very similar properties to those found by Husain and Davidson [28] and, in addition, found a 9 kDa cytochrome *c*-549 which has a redox potential of 190 mV. Neither the cytochrome *c*-551 nor *c*-553 can act as electron acceptor for methylamine dehydrogenase, but amicyanin is reactive with the cytochrome *c*-551 which presumably is the ultimate electron acceptor in vivo. Unfortunately, none of the induced cytochromes were tested for activity with methanol dehydrogenase. In one study, the amount of methanol induced cytochromes was one quarter to one half the level of cytochromes c_2 [28], whereas in the other study utilizing slightly different growth conditions, there was more of the inducible cytochromes than of c_2 [29]. The 22 kDa and 9 kDa cytochromes have similar redox potentials and did not separate on DEAE-cellulose. Conceivably, the smaller could be a proteolytic fragment of the larger. If so, it might explain variable yields in the two studies. More work is necessary to clarify the structural and functional relationships of the methanol inducible cytochromes of *P. denitrificans*.

A 45 kDa diheme cytochrome was induced in *P. denitrificans* by oxygen-limited growth conditions [29]. This soluble protein was not seen in fully aerobic or anaerobic cells, but there was as much present in oxygen-limited cells as there was cytochrome c_2. The two hemes were spectrally and thermodynamically resolved into a *c*-555, $E_{m,7} = 128$ mV and *c*-551, $E_{m,7} = -163$ mV. Clearly, more detailed studies are required to characterize this *diheme* cytochrome.

In summary, *P. denitrificans* is capable of producing a wide variety of soluble redox proteins dependent on metabolic conditions. The following patterns are present: aerobic growth with succinate, cytochrome c_2; aerobic growth – low oxygen, cytochrome c_2 and cytochrome *c*-555/*c*-551; aerobic growth with methanol, cytochrome c_2, cytochrome *c*-551, cytochrome *c*-553 and cytochrome *c*-549; aerobic growth with methylamine, is the same as with methanol plus amicyanin; anaerobic growth with nitrate, cytochrome c_2, pseudoazurin and cytochrome *cd*. What is striking here is that among the myriad of redox proteins identified in *P. denitrificans* to date, only a few have clearly defined physiological donors and acceptors. A great deal of work is required to establish the exact function of each of the cytochromes and copper proteins discussed. Moreover, the possibility that different expression or even new soluble redox proteins will be found in the

future cannot be excluded. Nevertheless, *P. denitrificans* serves to illustrate the diversity of possibility in what on the surface would appear to be a simple bacterium when grown only one way.

III. *Escherichia coli*

E. coli is as interesting for its relative lack of *c*-type cytochromes as other bacteria are for their abundance (Table II). Although *E. coli* is an aerobic bacterium, there is no soluble cytochrome which might function in a manner analogous to mitochondrial cytochrome *c*. There is no cytochrome bc_1 complex and there is no *a*-type cytochrome oxidase. Instead, *E. coli* contains membrane-bound quinol oxidases of the *o* and *d* type (see Ref. 30 for a review). The *E. coli* aerobic pathway is thus a truncated version of the familiar respiratory pathway. As emphasized in other sections of this review, *E. coli* is unique among the bacteria considered herein in this respect. The two membrane-bound oxidases of *E. coli* have different affinities for oxygen and are expressed at different stages of growth although mutants lacking one or the other grow well under all conditions. Thus, the *d*-type oxidase has a greater affinity for oxygen and is present in older, denser cultures, whereas the *o*-type oxidase is constitutive [30].

The *d*-type oxidase has been isolated as a two-subunit (58 and 42 kDa) complex containing two *b*-type hemes in addition to two hemes *d* [31,32]. There is no copper present and the redox potentials of the hemes differ (160–180 mV for the *b*-type hemes, and 260 mV for heme *d*). The structure of the *d* heme has recently been determined [33] and shows no similarity with the '*d* heme' in the cytochromes *cd*, (nitrite reductase) [21]. Antibodies against the *E. coli* cytochrome *d* subunits cross-react with extracts of *Azotobacter*, but not of *Pseudomonas* [34]. The cytochrome *d* operon has been cloned and sequenced but there is no obvious similarity to other oxidases [35].

Cytochrome *o* is defined as a protoheme containing oxidase, which can be inhibited by carbon monoxide. In *E. coli*, the cytochrome *o* has been isolated as a two- or four-subunit complex (55 and 33 kDa or 66, 35, 22 and 17 kDa), which contains copper in addition to protoheme [36,37]. Perhaps there is some structural similarity to the *a*-type oxidases, which invariably contain copper.

Although *E. coli* can use nitrate as a terminal electron acceptor under anaerobic conditions, it is very different from those bacteria which are able to denitrify. The end products in *E. coli* are nitrite and ammonia, whereas denitrifiers evolve either nitrous oxide or nitrogen gas or both. The *E. coli* nitrate reductase is an integral membrane protein which contains three subunits, and the smallest subunit, which is a *b*-type cytochrome, anchors it to the membrane [38]. The larger subunits contain iron-sulfur centers and a molybdopterin cofactor. The nitrate reductase operon has been cloned and found to consist of four genes instead of the expected three [39]; the fourth possible gene product has not been identified. The enzyme presumably interacts directly with quinol rather than with a soluble electron carrier.

Nitrite reductase (cytochrome *c*-552) has been purified and is one of the few soluble cytochromes in *E.*

TABLE II

Escherichia coli soluble and particulate redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome *b*-562	12	12	113	complete	function unknown
Cytochrome *c*-552 (nitrite reductase)	72–136	69	−200	n.d.	contains six hemes, induced by NO_3^-
Bacterioferritin	large	15	−340	partial	contains heme *b*, non-stoichiometric
Cytochrome *c*-550	34	n.d.	low	n.d.	trace amounts
Cytochrome *o* complex	membrane bound	33, 55	125	n.d.	contains two protohemes, two coppers
Cytochrome *d* complex	membrane bound	43, 58	180, 160, 260	complete	contains two hemes *d* and two protohemes
Nitrate reductase complex	membrane bound	143, 60, 26, 85	n.d.	partial	contains molybdopterin, iron-sulfur clusters, and protoheme

coli, but it is not abundant [40]. The periplasmic cytochrome *c*-552 has six hemes and a molecular mass of 69 kDa. Although multiheme cytochromes with a single polypeptide chain are known, it is possible that cytochrome *c*-552 is a multimeric protein which cannot be dissociated until completely denatured such as by heme removal. This type of very strong association has been observed with a cytochrome from a halophilic *Paracoccus* sp. [41] and with a *Desulfovibrio* cytochrome [42]. The heme content, molecular mass and low redox potential of cytochrome *c*-552 are remarkably similar to the dissimilatory nitrite reductase purified from *desulfovibrio* by Liu and Peck [43] and from *Vibrio fischeri* by Liu et al. [44]. However, a heme content of 16 for a *Desulfovibrio* cytochrome which has a similar molecular mass (75 kDa) has recently been reported [45]. The heme content and subunit structure will have to await sequence determination before final resolution in both *E. coli* and *Desulfovibrio*. A cytochrome *c*-550 present in trace amounts in *E. coli* has half the molecular mass of the cytochrome *c*-552 [46] and no known function. The electron donor for the nitrite reductase in *E. coli* is an NADH dehydrogenase [47]. This is in contrast to the nitrite reductases of denitrifiers which are more abundant than in *E. coli* and use high redox potential cytochromes or copper proteins as electron donors.

Another soluble cytochrome in *E. coli* was originally described as a cytochrome b_1 [48]. It was shown by Yariv [49] that, in fact, the cytochrome b_1 was the same as a bacterioferritin previously characterized by Yariv et al. [50]. Bacterioferritin is a bacterial version of the familiar iron storage protein (ferritin) of eukaryotes. Ferritin has a molecular mass of 500 kDa and contains 24 subunits of two kinds, the L subunit is 19 kDa and the H subunit is 21 kDa [51,52]. *E. coli* bacterioferritin differs from ferritin in that it has a single 15 kDa subunits and in that is has protoheme which is low spin and which has a very low redox potential (−340 mV). Ferritin and bacterioferritin subunits form a cage around a ferric hydroxide core from which the iron can be mobilized for cellular metabolism by reduction. The heme in the bacterioferritins may be involved in the mobilization of iron through reduction. The electron donors are unknown, but are certain to have low redox potentials. In *E. coli*, the donor may be NADH, just as was found for the nitrite reductase. The partial amino acid sequence of *E. coli* bacterioferritin has been determined, but shows no similarity to either ferritin or to other *E. coli* cytochromes [53].

Cytochrome *b*-562 from *E. coli* was characterized by Itagaki and Hager [54] and Fujita [46] as a small (12 kDa) monoheme protein with redox potential 113 mV. It was found in both aerobic and anaerobic cells; its function is unknown. The amino acid sequence [55] and the three-dimensional structure [56] have been determined. Cytochrome *b*-562 is structurally related to cytochrome *c'* [57] in that both are composed of a four-alpha-helical bundle. There are also structural similarities to ferritin [58]. This suggests that cytochrome *b*-562 and bacterioferritin may also be related, although there is no obvious sequence similarity.

Clearly, *E. coli* has a redox protein composition quite distinct from that of *P. denitrificans* in that soluble copper proteins and high potential *c*-type cytochromes are absent. In fact, as an aerobe *E. coli* is quite unusual and not obviously related to any of the other bacteria to be discussed. Nevertheless, *E. coli* is capable of carrying out both aerobic electron transfer and nitrate reduction efficiently and serves, in contrast to *P. denitrificans*, as an excellent example of the diversity of redox components in bacteria that can provide the same function.

IV. *Bacillus* species

The aerobic *Bacillus* species are interesting in the present context because they are Gram-positive and, therefore, lack the outer membrane present in Gram-negative bacteria. Since it is generally thought that the outer membrane defines the periplasmic space it follows that Gram-positive bacteria lack periplasmic proteins. To our knowledge, it has not been shown that the cell wall alone cannot enclose a periplasmic space.

In *E. coli*, it has been shown that *c*-type cytochromes are not obligatory in the aerobic pathway and in *P. denitrificans*, where *c*-type cytochromes *are* involved, it is not certain that a *soluble* high potential cytochrome *c* is a necessary component. In *Bacillus* species, there are both *c* and *a*-type cytochromes present, which suggests that the aerobic pathway is more like that in *P. denitrificans* than that in *E. coli*. Only small amounts of two so-called soluble cytochromes *c* have been reported in *Bacillus subtilis*, cytochrome *c*-550 and cytochrome *c*-554, [59]. However, it is not known whether these cytochromes are truly soluble or artifacts of the isolation procedure. If they are soluble in vivo, then they are either present in the cytoplasm or they are extracellular. Given Wood's [1] generalization that all *c*-type cytochromes should be membrane-bound or periplasmic, but never cytoplasmic, and if the two *c*-type cytochromes are truly soluble, then *Bacillus* is an exception. Otherwise, the assumption that Gram-positive bacteria have no periplasm or analogous compartment should be reexamined.

The possibility that the *c*-type cytochromes of *Bacillus* are all membrane-bound in vivo has to be considered. Woolley [60] reported that trypsin in addition to lysozyme was necessary for release of three *c*-type cytochromes from *B. licheniformis*, although it was concluded that the cytochromes were not initially membrane-bound. Miki and Okunuki [59], on the other hand, included a proteinase inhibitor in their extraction

buffer. Nevertheless, their yield of soluble cytochrome was quite low (approx. 5% or less of what would be obtained from *Pseudomonas* or *Rhodopseudomonas* species). Woolley [60] obtained about the same amount of soluble cytochrome using trypsin as in the earlier study with proteinase inhibitor. Although the situation remains to be clarified, we are inclined to believe that the *Bacillus* cytochromes are normally membrane-bound and either partially soluble, like cytochrome c_4, or released by endogenous proteolysis, like cytochrome c_5 (see next section).

Both of Miki and Okunuki's [59] cytochromes are small monoheme proteins and cytochrome *c*-550 has a redox potential of 210 mV. Cytochrome *c*-554 has an unusual split alpha peak and the redox potential is −80 mV. Woolley [60] found small amounts of three cytochromes, however two were similar to one another and could be possibly related through proteolysis of about ten amino acid residues. Based on reported properties, Woolley's cytochromes *c*-551 and *c*-552 could be equivalent to the cytochrome *c*-550 of Miki and Okunuki [59] with cytochrome *c*-554 being the same in both studies.

Bacillus species contain an *a*-type oxidase, and this readily oxidizes *Bacillus* cytochrome *c*-550 [59]. Although cytochrome *c*-550 is unreactive with mitochondrial cytochrome oxidase, it is a good donor to *P. aeruginosa* cytochrome *cd* (nitrite reductase). *Bacillus* cytochrome *c*-550 is acidic and it seems, from in vitro assays, that it does not have basic residues near the site of oxidation apparently required for interaction with mitochondrial cytochrome oxidase. Either the *Bacillus* oxidase has very different specificity than the mitochondrial oxidase, or cytochrome *c*-550 is not the natural electron donor.

A seven-subunit quinol oxidase super complex was isolated from *Bacillus* PS3 [61] which is similar to that from *P. denitrificans*. It is composed of bc_1 complex plus oxidase complex and contains *a*, *b*-, and *c*-type hemes. There were three *c*-type subunits, but the heme *b* subunit was not identified. Added yeast cytochrome *c* did not stimulate quinol oxidase activity, which suggests that a soluble cytochrome *c* is not necessary in vivo. The 21 kDa cytochrome *c* subunit in the enzyme is presumably the same as the 22 kDa subunit in the *P. denitrificans* quinol oxidase super complex.

At the present time, there appears to be no compelling evidence for the existence of soluble redox proteins in *Bacillus*, consistent with the presumed lack of a periplasm. Thus, *Bacillus*, like *E. coli*, is an example of an aerobe which is capable of electron transfer in the absence of a soluble *c*-type cytochrome. However, unlike *E. coli* it contains a quinol oxidase similar to that from *P. denitrificans*. Taken together, the cytochrome compositions of *E. coli, Bacillus*, and *P. denitrificans* suggest that soluble high potential *c*-type cytochromes are not required for aerobic electron transfer in bacteria. The presence of a bound cytochrome *c* in *Bacillus* makes some sense in view of the absence of a periplasmic space. Nevertheless, these observations suggest that bacteria containing soluble *c*-type cytochromes (see following sections) must have physiological requirements for these cytochromes beyond that of aerobic electron transfer.

V. *Azotobacter vinelandii / Pseudomonas aeruginosa*

Azotobacter and *Pseudomonas* are aerobic bacteria which are generally thought to be very different from one another. *Azotobacter* is capable of fixing nitrogen and is very thoroughly characterized in this respect. *Pseudomonas*, on the other hand, is prototypic of bacteria which are able to convert nitrate to nitrogen gas (denitrification). However, it is becoming more obvious that these organisms are closely related in terms of their complement of electron transfer proteins, and that reported differences can be rationalized by the different modes of growth of the bacteria and methods of protein isolation and purification. In view of their similarities, these two organisms will be discussed together, with the properties of their soluble and peripherally membrane-bound redox proteins given in Tables III and IV.

The shared characteristics of the electron transport pathways of these two species are the simultaneous occurrence of three Class I cytochromes *c*-551, c_4 and c_5. In fact, these three proteins have been found in several denitrifying *Pseudomonas* species and some of these organisms will be introduced where aspects of electron transfer have been more thoroughly documented than in *Pseudomonas aeruginosa*. Interestingly, cytochromes *c*-551, c_4 and c_5 are homologous to one another and have similar redox potentials. However, as seen in the more detailed description to follow, cytochrome *c*-551 is the only truly soluble (and periplasmic) one of the three. Although the localization of cytochromes in the soluble or membrane fractions is an important distinction which must have functional ramifications, the detailed role of these proteins is unknown. There have been spectroscopic studies of whole cells and membranes which have ascribed functions to one or more of these three cytochromes, but wavelength maxima are so similar that they cannot be resolved, and there are other less well-characterized cytochromes present which make interpretation of such studies virtually impossible at this time.

In an historical sense, *Azotobacter vinelandii* is known as the source of cytochromes c_4 and c_5 and *Pseudomonas aeruginosa* as the source of cytochrome *c*-551. It is now clear that *A. vinelandii* contains significant amounts of cytochrome *c*-551, but it is easily denatured by the butanol isolation procedure originally developed

TABLE III

Azotobacter vinelandii soluble and peripheral membrane redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome *c*-551	10	10	280	complete	
Cytochrome c_4	24	21	317, 273	complete	contains two hemes
Cytochrome c_5	24	10	320	complete	has ragged N-terminus
Cytochrome c'	170	n.d.	n.d.	n.d.	
Ferredoxin I	14	14	−420	complete	contains 4-Fe-S and 3-Fe-S clusters
Flavodoxin	21	21	−250, −500	complete	
Ferredoxin II	24	n.d.	n.d.	n.d.	contains 2-Fe-S clusters, coinduced with nitrogenase
Bacterioferritin	415–660	17	−416	n.d.	contains one heme per two subunits
Nitrogenase, D, K subunits	245	56, 60	n.d.	n.d.	
Nitrogenase, H subunit	60	30	−240 −450	complete	

[a] Measured in the presence of MgATP.

for extracting cytochromes c_4 and c_5. Hence, its designation in the literature as 'c_4 minor'. *P. aeruginosa* produces cytochromes c_4 and c_5, but because they are predominantly membrane-associated, they were not recognized until the minor soluble components derived from the membrane-bound cytochromes were sequenced. *P. aeruginosa* cytochrome c_5 is also known as cytochrome *c*-554.

Swank and Burris [62] developed a procedure for purification of cytochromes from *A. vinelandii*. They noted that although some *c*-type cytochrome were extracted by buffer alone, substantially more could be obtained with butanol extraction. Not suspecting that the cytochromes isolated by buffer and by butanol might be different, they omitted the initial buffer extraction for the sake of simplicity and to optimize their yield of what turned out to be a mixture of cytochromes c_4 and c_5. This resulted in low yields of a cytochrome which was termed 'c_4 minor'. Ambler [63] showed that 'c_4 minor' was the principal soluble cytochrome from *A. vinelandii* and that the sequence was closely related to the cytochromes *c*-551 from *Pseudomonas* species. Thus, a specific cytochrome *c*-551 is common to *A. vinelandii* and to a number of *Pseudomonas* species.

TABLE IV

Pseudomonas aeruginosa soluble and peripheral membrane redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome *c*-551	10	10	260	complete	
Cytochrome c_5	n.d.	10	340	partial	ragged N-terminus
Cytochrome c_4	21	21	322, 268	partial	contains two hemes
Cytochrome *cd*	85–120	63	288, 218	partial	induced by NO_3^-
Cytochrome *c* peroxidase	43	43	320, −330	partial	contains two hemes
Azurin	14	14	327	complete	

The distribution and properties of *Pseudomonas* and *Azotobacter* cytochromes *c*-551 have been reviewed by Meyer and Kamen [3] and will not be discussed in detail here. The protein is homologous to mitochondrial cytochrome *c*, but contains only 82 amino acid residues and shows significant differences in three-dimensional structure. Unlike mitochondrial cytochrome *c*, *P. aeruginosa* cytochrome *c*-551 has a uniform distribution of charged amino acid side chains on its surface and there is no concentration of either positive or negative charge near the exposed heme edge, which is presumably where electron transfer most readily occurs. Meyer et al. [15] showed that *P. aeruginosa* cytochrome *c*-551 has only a weak negative charge at the site of reduction by FMN semiquinone and Tollin et al. [16] showed a similar effect with flavodoxin semiquinone reduction of the cytochrome. Thus, these in vitro studies suggest that electrostatic interactions between cytochromes *c*-551 and its reaction partners are not likely to have much of a role in its function, a result in sharp contrast with mitochondrial cytochrome *c* or bacterial cytochromes c_2, which have a large positive charge near the exposed heme edge and interact strongly with charged oxidants and reductants. Cytochrome *c*-551 has been shown to be a good electron donor for the cytochrome *cd* type of nitrite reductase in denitrifying *P. aeruginosa*, but a poor donor for *P. denitrificans* cytochrome *cd* [19]. In contrast, *P. denitrificans* cytochrome c_2 is a good donor for the *P. denitrificans* cytochrome *cd*, but is not effective with *P. aeruginosa* cytochrome *cd*. At least part of these differences in specificity must be due to the uniform surface charge distribution in the cytochromes *c*-551 as opposed to the cytochromes c_2. Importantly, *A. vinelandii* is unable to denitrify and a cytochrome *cd* has not been detected. Therefore, cytochrome *c*-551 must have a different physiological role in *A. vinelandii*. It is also likely that cytochrome *c*-551 is not the only electron donor to cytochrome *cd* in *P. aeruginosa* and its role is unlikely to be limited to anaerobic metabolism because it is present in aerobic as well as anaerobic cells.

Cytochrome c_5 is predominantly membrane-bound, but is also present as a minor component in the soluble fraction. Campbell et al. [64] improved the Swank and Burris [62] isolation procedure and showed that there was more than one form of cytochrome c_5, one isozyme of which was designated cytochrome *c*-555. Ambler and Taylor [65] showed that the principal isozyme in *Pseudomonas mendocina* had a ragged N-terminus and was probably a proteolytic fragment of a larger protein. Presumably the missing N-terminal fragment acts as a membrane anchor. Ambler [64] also showed that cytochrome c_5 commonly occurs in *Pseudomonas* species. The amino acid sequence of *A. vinelandii* c_5 is closely related to that of *P. mendocina* [66]. Cytochrome c_5 is recognized by the alpha peak in the visible absorption spectrum which is shifted slightly to the red (554–555 nm), by its dimeric nature, and by the presence of a disulfide bond. The dimer is about the same size as cytochrome c_4 and the monomer is about the same as cytochrome *c*-551 (9 kDa). The three-dimensional structure of cytochrome c_5 [66] shows that the protein is folded approximately the same as is cytochrome *c*-551. The protein crystallizes as a dimer and the dimer interface occludes the heme edge, which is solvent exposed in cytochrome *c*-551. However, the physiological significance of dimerization is unknown. In unpublished work, we have found that cytochrome c_5 behaves like a monomer in its reaction with non-physiological reductants (e.g., flavin semiquinones). There are also minimal electrostatic effects on kinetics of reduction of cytochrome c_5 as might be predicted from the crystal structure which indicates no significant charge clustering in the vicinity of the exposed heme edge. Thus, cytochrome c_5 may be functionally interchangeable with cytochrome *c*-551 in vitro. Goodhew et al. [67] found that cytochrome c_5 in *Pseudomonas stutzeri* was periplasmic and more abundant in aerobic than in anaerobic cells, whereas the periplasmic cytochrome *c*-551 was constitutive. Thus, cytochrome c_5 may be distinguished from cytochrome *c*-551 in a functional sense by its predominant membrane localization and by its apparent repression in anaerobic cells.

Cytochrome c_4 is membrane-bound, and when solubilized it does not aggregate. The molecular mass is about 18 kDa and it contains two hemes. The amino acid sequence of *A. vinelandii* cytochrome c_4 suggests that the protein is the product of a gene-doubling event [68]. N-terminal sequences indicate that cytochromes c_4 are found in several species of *Pseudomonas* [69]. A preliminary report of the three-dimensional structure of *P. aeruginosa* cytochrome c_4 shows that the two halves of the protein are folded in separate domains, each of which is similar in overall folding to cytochromes *c*-551 and c_5 [70]. There is no apparent stretch of hydrophobic residues in the amino acid sequence which might anchor the cytochrome to the membrane suggesting that it may be bound by electrostatic forces, although Pettigrew and Brown [71] propose that the hydrophobic interface between the two domains may be the site of attachment to the membrane. The two hemes in cytochrome c_4 have different redox potentials and there is no indication that they interact [72]. Goodhew et al. [67] found cytochrome c_4 in membranes of both aerobic and anaerobic cells. Pettigrew and Brown [71] quantitated membrane-bound and soluble cytochrome c_4 in several species and concluded that it was predominantly membrane-bound in all bacteria and present at similar levels under all growth conditions examined.

To summarize to this point, there are three small cytochromes in *A. vinelandii* and various *pseudomonas* species which are closely related structurally and which

have not been functionally resolved. We have emphasized similarities among these species, but there are both real and apparent differences in their complement of other electron transfer proteins.

In membranes of *Pseudomonas aeruginosa*, Wood and Willey [73] found a cytochrome *c*-551, which had a redox potential of 300 mV and molecular mass 32 kDa. They equated this protein with cytochrome c_1, which is the electron donor to cytochrome *c* in mitochondria or to cytochrome c_2 in *Rb. capsulatus*. Matsushita et al. [74] obtained similar results and observed additional cytochromes which were not characterized. Goodhew et al. [67] reported a membrane-bound 31 kDa cytochrome in *Pseudomonas stutzerii* but it was only present in aerobic cells. If the 32 kda cytochrome from *P. stutzeri* is a component of the bc_1 complex, we would expect it to be present in anaerobic as well as aerobic cells, because the denitrifying enzyme, cytochrome *cd* (see below), interacts with soluble *Pseudomonas* cytochrome *c*-551, which also should interact with a cytochrome c_1, unless there is coupling at the level of quinone. Thus, more work is required to establish whether the *P. stutzerii* 32 kDa protein is cytochrome c_1. Moreover, more convincing evidence is necessary before we conclude that cytochrome c_1 is present in other *Pseudomonas* species or in *Azotobacter* species.

Also relevant to the question of whether there is a bc_1 complex in *Pseudomonas* and *Azotobacter* is 'what type of oxidase do these bacteria utilize?' Branched oxidase pathways are common in bacteria and are typically either quinol or cytochrome oxidases. As already discussed for *E. coli*, there are only quinol oxidases, which neither require nor utilize *c*-type cytochromes or the bc_1 complex. The principal oxidase in *A. vinelandii* is a cytochrome *d*, which cannot oxidize the dye TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride) [75] suggesting that it is a quinol oxidase rather than a cytochrome oxidase because TMPD oxidation is supposedly mediated by cytochrome *c*. A *d*-type oxidase has been thoroughly characterized in *E. coli* as a quinol oxidase and presumably all *d*-type oxidases are related [31,32]. *A. vinelandii* mutants completely lacking cytochrome *c* still contain cytochrome *d* [76], however, wild-type cells can oxidize TMPD and thus presumably contain an alternate cytochrome oxidase. Yang [77] purified an *o*-type oxidase from *A. vinelandii* which had a single subunit (28 kDa) and a redox potential of −30 mV. TMPD oxidase activity with this preparation is dependent on the presence of *c*-type cytochrome, which itself is not a substrate. Presumably this oxidase also uses quinol as substrate and is not the cytochrome oxidase postulated to be present.

Cytochrome *cd* (nitrite reductase) is found in various *Pseudomonas* species but not in *A. vinelandii*. Goodhew et al. [67] showed that it is abundant and periplasmic in nitrate grown anaerobic cells, but virtually absent in aerobic cells of *P. stutzerii*. The properties of cytochrome *cd* have been reviewed elsewhere [3] and will not be repeated here. Physiologically, it appears that soluble cytochrome *c*-551 is the immediate electron donor to cytochrome *cd*.

Azurin is homologous to the plant copper protein plastocyanin both in amino acid sequence [78] and three-dimensional structure [79,80], with the copper-binding sites being essentially the same. Features unique to azurin are the presence of a disulfide bond and the uniform charge distribution on the molecular surface. The presence of cysteines beyond those required for copper ligation allow one to distinguish azurin from the copper protein found in *P. denitrificans* (pseudoazurin) which contains only one cysteine as already discussed. Azurin is a good substrate for cytochrome *cd* and, like the latter, is found in various *Pseudomonas* species but not *A. vinelandii*. There is rapid electron transfer between cytochrome *c*-551 and azurin [81,82] and they may be considered interchangeable in their interactions with cytochrome *cd*. The redox potential of azurin is similar to that of cytochrome *c*-551 and the site of reduction by free flavin semiquinones shows very small electrostatic effects [83]. Therefore, in a physico-chemical sense, azurin is very similar to cytochrome *c*-551.

Nitrous oxide reductase was purified from *Pseudomonas stutzerii* and characterized as a labile copper protein [105], with four coppers per subunit. The gene sequence yields a subunit of molecular mass 71 kDa, and a 12-residue segment shows homology to the copper-binding subunit II of cytochrome oxidase [85]. All denitrifying *Pseudomonas* species are expected to produce this enzyme.

There are two additional cytochromes found in *Pseudomonas* species, and both are diheme. *P. aeruginosa* cytochrome *c* peroxidase has two heme groups per 43 kDa monomer. It utilizes both cytochrome *c*-551 and azurin as substrate and in this sense resembles cytochrome *cd* (see Ref. 3 for a relatively recent review). Redox and EPR properties have been characterized with the active form of the enzyme which is the half-reduced species [86]. One heme is high-spin at room temperature, but becomes low-spin at liquid nitrogen temperature. One heme has a redox potential of 320 mV and the other has one of −330 mV.

Cytochrome *c′* is a common protein in purple phototrophic bacteria such as *R. capsulatus* and *R. spheroides*, but is also found in some aerobes and denitrifiers (such as *Alcaligenes*). Cytochrome *c′* has been found in *A. vinelandii* [87], but not in any of the *Pseudomonas* species. *A. vinelandii* cytochrome *c′* has the distinction of being the largest in its class, with a molecular mass of 170 kDa. Neither subunit structure nor sequence has been determined to date, but the cytochromes *c′* in other species are generally dimers of 14 kDa subunits. By analogy, the *A. vinelandii* protein might therefore

contain as many as 12 subunits. A functional role has not been established for any of the cytochromes *c'*.

A. vinelandii contains bacterioferritin similar in properties to that described already for *E. coli*. Although not reported to date in *Pseudomonas*, bacterioferritin has a large molecular mass, low solubility and unsatisfactory chromatographic behavior all of which make isolation difficult and its presence in new species cannot easily be excluded.

Nitrogen fixation is a pathway found in virtually all purple and green phototrophic bacteria, but very few coliforms or Pseudomonads are known to have this capability. This may partly be due to the fact that the former are predominantly anaerobic and the latter are primarily aerobic bacteria. Nitrogenase is typically oxygen-labile and nitrogen fixation is generally thought to be a strictly anaerobic process. *Azotobacter* species are, thus, interesting organisms in that they can only grow aerobically and yet are able to fix nitrogen. It is apparent that the enzyme must somehow be protected from oxygen, perhaps through compartmentalization, although the precise mechanism is unknown. A small, 2-Fe-S ferredoxin has been shown to afford some protection to nitrogenase as will be discussed below. Another requirement of nitrogenase is for a low redox potential reductant. In *Azotobacter*, the reductant has not been identified in vivo. In *Clostridium*, this role is filled by an 8-Fe-S ferredoxin, but in *Klebsiella* (one of the coliforms) a flavodoxin is induced along with nitrogenase and shown to be the obligate electron donor [88]. To complicate matters, three separate nitrogenase isozymes have been found in *A. vinelandii* [89]. The nitrogenase usually expressed contains molybdenum in addition to iron, but under conditions of molybdenum deficiency, an alternative vanadium-containing isozyme [89–92], or one which has no metal other than iron is expressed [89]. The redox potentials of the different nitrogenases were found to be similar [90]. It is possible that they might all use the same reductants, but one should leave open the possibility that these may also be different.

Flavodoxin is abundant in nitrogen-fixing *A. vinelandii*, and Scherings et al. [93] have shown that it is the best in vitro electron donor of those tested for nitrogenase. It had previously been shown that flavodoxin is induced in *C. pasteurianum* only under conditions of iron deficiency [94], and substitutes for ferredoxin. In contrast, the flavodoxin gene in *Klebsiella* is tightly linked to those for nitrogen fixation and it is the sole reductant for the enzyme [95]. This is not the case for *A. vinelandii*. Flavodoxin is constitutive although more plentiful under nitrogen-fixing conditions, and when the gene is deleted, *A. vinelandii* is able to produce alternative electron donors to nitrogenase [96]. Not only are there three nitrogenase isozymes in *Azotobacter*, but Klugkist et al. [97] found three flavodoxin isozymes as well. They differ in amino acid composition and redox potential. It is possible that a flavodoxin isozyme could serve as alternate electron donor, but it is equally probable that one or more 8-Fe-S ferredoxins could fill that role. The sequence of one of the *A. vinelandii* flavodoxin isozymes has been determined and it is homologous to that from *C. pasteurianum* [96] for which a three-dimensional structure has been determined [98]. There is no leader sequence, which suggests that flavodoxin resides in the cytoplasm. In vitro kinetics of the interaction of *A. vinelandii* and *C. pasteurianum* flavodoxins with non-physiological donors and acceptors have been studied [99]. They were for the most part similar, but there were also notable steric differences, which may have some bearing on the function. Thus, the FMN in *C. pasteurianum* flavodoxin appears to be more exposed. The most notable characteristic of flavodoxin from both species is the large negative charge surrounding the exposed edge of the flavin, reminiscent of the positive charges surrounding the exposed edge of the heme in some cytochromes *c*. This undoubtedly has mechanistic consequences in vivo as well as in vitro. With regard to interaction with nitrogenase, it suggests a positively charged site of reduction.

A. vinelandii ferredoxin I has been thoroughly characterized in terms of gene sequence [100] and three-dimensional structure [101,102]. There are 107 amino acid residues which is twice the size of typical *Clostridial* ferredoxins. There is no leader sequence, which suggests cytoplasmic localization. It has been shown to be an effective electron donor to nitrogenase in vitro, but when the gene was deleted, there was no effect on nitrogen fixation [100]. It is a single copy gene, therefore alternate electron donors for nitrogenase must exist, just as is found for deletion of the flavodoxin gene. It is necessary to construct a double mutant lacking both flavodoxin and ferredoxin to determine whether a third possible donor exists.

A ferredoxin gene was discovered in *A. chrococcum* when the gene for one of the nitrogenase isozymes was cloned and sequenced [103]. A ferredoxin gene was discovered at another nitrogen fixation locus in *A. vinelandii* and *Bradyrhizobium japonicum* [104]. It is not known what conditions effect expression of these genes and deletion mutants have not yet been constructed. These small ferredoxins are homologous to those from phototrophic bacteria, and in fact they are closer to the latter in amino acid sequence than either is to *A. vinelandii* ferredoxin I. The presence of a five-residue insertion in these proteins plus an extra cysteine links them in an evolutionary sense. *Rhizobium* contains a ferredoxin gene labeled fix X which is apparently required for nitrogen fixation [105] and which hybridizes to *A. vinelandii* DNA. The sequence is related to *Azotobacter* ferredoxin I. The implication is that there

may be additional ferredoxin isozymes in *Azotobacter* species.

As mentioned above, nitrogenase is extremely oxygen-sensitive and *Azotobacter* and other aerobic nitrogen-fixing bacteria must have mechanisms to protect it from inactivation. In most cyanobacteria, protection is afforded by differentiation of special nitrogen-fixing anaerobic cells called heterocysts. In *Azotobacter* one could imagine that nitrogen-fixation takes place in the cytoplasm and that oxygen reduction takes place on the periplasmic side of the cell membrane to prevent oxygen interacting with the labile nitrogenase. However, this is contrary to the usual situation, where oxidases reduce oxygen on the cytoplasmic side of the membrane, thus contributing to the proton gradient necessary for energy conservation. Perhaps the *Azotobacter* oxidase uniquely reduces oxygen on the periplasmic side of the membrane. Data are presently unavailable that address this point.

Less speculatively, it has been shown that an abundant *A. vinelandii* 2-Fe-S ferredoxin (labelled ferredoxin II) confers protection to nitrogenase against oxygen inactivation [93]. The amino acid sequence of a related protein from *C. pasteurianum* has been determined [106]. This ferredoxin has no obvious homology to any other known ferredoxin. No genetic experiments have been done with this protein, but they are clearly necessary to determine whether this may be the sole or even principal mechanism of oxygen protection.

Central observations with *Azotobacter* and *Pseudomonas* are the similarity of cytochrome *c*-551, c_4 and c_5. Unfortunately, the functions of the proteins are unclear, hence the metabolic roles, the same or different, have not been defined. Equating functionally similar *P. aeruginosa* cytochrome *c*-551 to *P. denitrificans* cytochrome c_2, and *P. denitrificans* pseudoazurin to *P. aeruginosa* azurin and remembering that both *P. aeruginosa* and *P. denitrificans* contain cytochrome *cd*, then some intrinsic similarities between these two denitrifying organisms are obvious. In contrast, *A. vinelandii* is quite distinct from *P. aeruginosa* and *P. denitrificans* in that it contains a variety of ferredoxins and flavodoxins which are apparently related to its ability to fix nitrogen. Moreover, *A. vinelandii* contains both bacterioferritin and cytochrome *c′* which have not been found in *P. aeruginosa* and *P. denitrificans*. Presumably, the cytochrome *c′* plays some distinct metabolic role, although what role is not clear. Nevertheless, it is relatively easy to rationalize the similarities and differences among *P. denitrificans*, *P. aeruginosa* and *A. vinelandii* based on their metabolism, but with overlapping components for overlapping functions. That is, these three organisms have similarities which cannot be noted when comparing them to *E. coli* or *Bacillus* species.

VI. *Alcaligenes* sp.

Alcaligenes sp. NCIB 11015 grows in much the same manner as *P. aeruginosa* or *P. denitrificans*. However, the electron-transfer proteins present in this organism provide an interesting contrast to those of the other denitrifiers (Table V). The predominant electron-transfer proteins in the soluble fraction of *Alcaligenes* grown under denitrifying conditions are azurin, cytochrome *c′*, and a copper-containing nitrite reductase [107,108]. There are no significant amounts of small, soluble, high redox potential cytochromes, which are so typical of *Pseudomonas* species and *Paracoccus denitrificans*, although the bright red membranes indicate large amounts of particulate cytochrome. In fact, Shidara [109] isolated cytochromes *c*-556 and *c*-551 from the membranes using a butanol extraction procedure similar to that used to prepare cytochromes c_4 and c_5 from *A. vinelandii*. The diheme cytochrome *c*-551 has molecular weight 29000 and redox potential 262 mV, whereas the monoheme cytochrome *c*-556 has a molecular weight of

TABLE V

Alcaligenes sp. soluble and peripheral membrane redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Azurin	16	16	230	complete	contains one copper
Nitrite reductase	70	37	260	n.d.	contains one copper per subunit
Cytochrome *c′*	27	14	90	complete	
Cytochrome *c*-556	18	n.d.	291	n.d.	one heme
Cytochrome *c*-551	29	n.d.	262	n.d.	two hemes
Cytochrome *c*-553	45	n.d.	0, −90	n.d.	two hemes

18 000 and redox potential of 291 mV [109]. However, there is insufficient information available to equate Shidara's cytochromes with cytochromes c_4 and c_5. Another diheme cytochrome (cytochrome *c*-553) was isolated from the soluble fraction of aerobic cells [110], but it has a larger molecular weight (45 000) and low redox potential.

The amino acid sequence of *Alcaligenes* azurin has been determined and it is closely related to those from *Pseudomonas* (Ambler, quoted by Ryden and Lundgren [111]) as is the three dimensional structure [112]. Azurin appears to be the sole electron donor to nitrite reductase, which is also a copper protein. In contrast, in *P. aeruginosa*, azurin is an alternate electron donor to a cytochrome *cd* type of nitrite reductase. Azurin appears to be the only electron-transfer protein common to *Alcaligenes* and *P. aeruginosa*.

The amino acid sequence of *Alcaligenes* cytochrome c' has been determined [113] and it is as similar to the phototrophic bacterial proteins as they are to one another suggesting a common evolutionary origin. Cytochrome c' is not generally found in other denitrifiers such as *Pseudomonas* species and is a minor component of *A. vinelandii*. There is no known function for cytochrome c' in *Alcaligenes* where it is abundant.

Alcaligenes has evolved a distinctive motif for electron transfer proteins as compared to *P. aeruginosa* and *P. denitrificans* using a copper containing nitrite reductase and no soluble high-potential *c*-type cytochromes. Nevertheless, azurin or pseudoazurin is common to all three organisms. In contrast, *Alcaligenes* has cytochrome c' in common with *A. vinelandii* but no other obvious similarities in terms of soluble redox proteins.

VII. *Rhodobacter capsulatus / Rhodobacter sphaeroides*

Rb. capsulatus and *Rb. sphaeroides* are both phototrophic purple bacteria, which have a number of features in common with regard to electron transfer yet have distinct differences. (See Tables VI and VII for redox protein composition.) Both organisms can be anaerobically grown in the light or aerobically grown in the dark. The principal soluble proteins in both cases are cytochrome c_2 (related to cytochrome c_2 from *Paracoccus denitrificans*) and cytochrome c' (also found in *Azotobacter, Alcaligenes* and *Chromatium*). It is generally believed that cytochrome c_2 serves as electron donor to both cytochrome oxidase in aerobic growth and to reaction centers in photosynthetic growth [114]. However, a number of interesting observations have been made in recent years which make the situation less clear. In *Rb. capsulatus*, it is well established that cytochrome c_2 is a periplasmic protein from both direct measurements [115] and from the gene sequence which indicates an amino terminal extension of 21 amino acids which is related to known signal sequences in other proteins and necessary for transport across the cytoplasmic membrane [116]. *Rb. sphaeroides* cytochrome c_2 gene also has a 21 residue leader sequence [117]. However, mutants of *Rb. capsulatus* in which the cytochrome c_2 gene has been deleted are still capable of photosynthetic growth, with cell yields typical of the wild type when grown on a rich medium [116]. In the

TABLE VI

Rb. sphaeroides soluble redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome c_2	13	13	356	complete	essential for photosynthetic growth
Cytochrome c'	23	13	30	complete	
Sphaeroides heme protein	13	13	−22	partial	binds O_2
Cytochrome *c*-551.5	21	16	−254	n.d.	
Cytochrome *c*-554	44	14	203	complete	induced by O_2
Cytochrome *c*-552	13.5	n.d.	250	n.d.	induced by nitrate
Nitrate reductase	60–112	n.d.	250	n.d.	contains Mo, hence *c*, induced by nitrate
Nitrite reductase	80	38, 40	n.d.	n.d.	contains two Cu, induced by nitrate
N_2O reductase	75	75	n.d.	n.d.	contains four Cu
DMSO reductase	82	82	n.d.	n.d.	contains Mo, induced by DMSO
Ferredoxin	n.d.	n.d.	n.d.	n.d.	

TABLE VII

Rb. capsulatus soluble redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome c_2	13	13	358	complete	not required for photosynthetic growth
Cytochrome c'	26	14	51	complete	
Nitrate reductase	n.d.	90	n.d.	n.d.	contains Mo, induced
N_2O reductase	n.d.	76	n.d.	n.d.	contains Cu
TMAO reductase/ DMSO reductase	n.d.	46	n.d.	n.d.	induced by TMAO or DMSO
Ferredoxin	12	n.d.	n.d.	n.d.	contains two 4-Fe-S clusters

deletion mutants, it has been shown that there is relatively rapid direct electron transfer from cytochrome c_1 of the bc_1 complex to the reaction center which apparently supports photosynthetic growth [118]. In sharp contrast, cytochrome c_2 deletion mutants of *Rb. sphaeroides* are unable to grow photosynthetically [119]. These results suggest that the architecture of the *Rb. capsulatus* and *Rb. sphaeroides* membranes are quite different with the bc_1 complex in *Rb. capsulatus* able to approach and to donate efficiently an electron to the reaction center, but not in *Rb. sphaeroides*.

Cytochrome c_2 is apparently directly oxidized by photosynthetic reaction centers and then reduced in turn by a cytochrome bc_1 complex. The three-dimensional structure of *Rb. sphaeroides* reaction center has been solved [120] and the gene sequence for *Rb. sphaeroides* and *Rb. capsulatus* reaction centers has been determined [121,122]. In both cases the reaction center is similar to that of *Rps. viridis* [123] except that the latter has a tetraheme cytochrome subunit which is absent in *Rb. sphaeroides* and *Rb. capsulatus*. The interaction of cytochrome c_2 with reaction center was modeled by Allen et al. [120]. The proteins are proposed to interact via complementary charge interactions with the heme and chlorophyll 1.1 nm apart in the hypothetical transient complex. On the other hand the reaction center cytochrome subunit in *Rps. viridis* is close to the chlorophyll and is bound by hydrophobic interactions to the other subunits. It should be emphasized that the cytochrome bc_1 complex, the presumed reductant for cytochrome c_2 has only been demonstrated in *Rb. sphaeroides* and *Rb. capsulatus* where the gene sequence of the bc_1 operon has been determined [124–126]. The three subunits are homologous to those from *Paracoccus*. Although we expect all phototrophic bacteria to contain a cytochrome bc_1 complex, detailed evidence is lacking in all species but these two. Davidson and Daldal [126] have not published the *Rb. sphaeroides* cytochrome *b* gene sequence, but report that it is closely related to that from *Rb. capsulatus*. A cytochrome *b*-562 gene was cloned from *Rb. sphaeroides* and sequenced by Iba et al. [127]. This cytochrome was supposedly derived from the cytochrome bc_1 complex, but there is no similarity to the *Rb. capsulatus* cytochrome *b* either in size (17 kDa vs. 40 kDa, respectively) or sequence.

The cytochrome *b* and c_1 genes were deleted in *Rb. capsulatus*, which resulted in complete loss of the cytochrome bc_1 complex [128]. As expected this mutant was unable to grow photosynthetically, but it was found that there were 26 and 33 kDa cytochromes *c* ($E_{m\,7} = 312$ mV) remaining in the membrane. Cytochrome bc_1-minus, cytochrome c_2-minus mutants were unable to reduce photooxidized reaction center [128], which demonstrates that cytochrome c_1 is the electron donor in mutants which only lack cytochrome c_2. The newly discovered membrane-bound cytochromes in *Rb. capsulatus* are inactive in coupling bc_1 to reaction center and no role has yet been assigned for them.

It has been shown that *Rb. capsulatus* has a branched terminal oxidase pathway and that mutants in one or the other oxidase still allow aerobic growth [129] while double mutants which lack both oxidases will not grow. In *Rb. capsulatus*, there is a cytochrome *o*-type quinol oxidase and a cytochrome *o*-type cytochrome oxidase [130,131]. The cytochrome oxidase purified from aerobic cells [132] contains a single subunit of 65 kDa and has a redox potential of 385 mV. Interestingly, a *c*-type cytochrome (cytochrome *c*-552) accompanied this oxidase until the final stages of purification [133]. This *c*-type cytochrome appears to have a native size of 25 kDa with a subunit molecular weight of 12000 and a redox potential of 234 mV. This situation is similar in many respects to that detailed earlier for the *Paracoccus* oxidase [17]. Most importantly, the *Rb. capsulatus c*-type

cytochrome did not cross-react with antibodies against cytochrome c_2. Presumably this membrane-bound cytochrome *c* is intimately associated with the oxidase in vivo. Daldal [134] combined the oxidase mutants with cytochrome c_2 minus mutants to show that cytochrome c_2 is not necessary for aerobic growth when the quinol oxidase is absent. Presumably, the cytochrome *c* accompanying the oxidase during purification [133] is functional in mediating electron transfer between the cytochrome bc_1 complex and the oxidase. Alternatively, Daldal [134] proposes that the cytochrome c_1 may interact directly with oxidase when cytochrome c_2 is absent. In the case of *Rb. sphaeroides*, an *a*-type oxidase has been purified [135]. This oxidase is highly active with yeast and horse cytochrome *c* but its activity with *Rb. sphaeroides* cytochrome c_2 has not been examined.

The cytochromes *c'* from both *Rb. sphaeroides* and *Rb. capsulatus* appear to be typical of this class of *c*-type cytochrome. Both *Rb. capsulatus* and *Rb. sphaeroides* cytochromes *c'* are quite acidic (pI = 5.1). However, there is little or no charge at the site of reduction by non-physiological electron donors [136]. As is typical of the cytochromes *c'*, the physiological electron donors and acceptors are unknown.

In contrast to *Rb. capsulatus, Rb. sphaeroides* contains a number of soluble cytochromes in addition to cytochrome c_2 and cytochrome *c'* [137]. A soluble high-spin *c*-type cytochrome capable of oxygen binding has been found. This cytochrome, termed *sphaeroides* heme protein (SHP), is spectrally similar to cytochrome *c'*, but based on available partial amino acid sequence data [137] it apparently constitutes a distinct structural class with no detectable homology to any known cytochrome *c*. *Rb. sphaeroides* also yields a low-potential cytochrome *c*-551.5 ($E_{m,7} = -254$ mV). Based on the very low midpoint potential as well as absorption spectra typical of cytochromes c_3, this cytochrome presumably has bis-histidine ligation and may be a Class-III *c*-type cytochrome. However, an amino acid sequence is needed before any conclusions can be drawn as to its evolutionary relationships. Finally, a cytochrome *c*-554 has been isolated from *Rb. sphaeroides* which is a low-spin member of the Class-II *c*-type cytochromes based on amino acid sequence homology (Ambler et al., unpublished results). At the present time the function of the *sphaeroides* heme protein, cytochrome *c*-551.5 and cytochrome *c*-554 are unknown.

Ferredoxin has been purified from *Rb. capsulatus* [138] and has properties similar to those of *Clostridium* 8-Fe-S ferredoxin. However, *Rb. capsulatus* ferredoxin is inactive with *Clostridial* hydrogenase and only half as effective as *Clostridium* ferredoxin when assayed as an electron donor for *Rb. capsulatus* nitrogenase [138]. *Rb. sphaeroides* also has a ferredoxin, presumably similar to the *Rb. capsulatus* ferredoxin; however, no detailed studies have been carried out.

Both *Rb. capsulatus* and *Rb. sphaeroides* are metabolically diverse with a number of redox enzymes inducible when grown on the appropriate substrates. A copper-containing nitrous oxide reductase is present in *Rb. sphaeroides* [139] as well as in *Rb. capsulatus* and is apparently widely distributed in the purple phototrophs [140] (the properties of this enzyme appear to be very similar to a nitrous oxide reductase from *Pseudomonas stutzerii* [84,85]). Both *Rb. capsulatus* and *Rb. sphaeroides* contain distinct nitrate reductases. The *Rb. capsulatus* nitrate reductase is periplasmic; it contains a molybdopterin cofactor but no heme, with a subunit molecular weight of 90 000 [141]. In contrast, the nitrate reductase from *Rb. sphaeroides* contains molybdenum and a *c*-type cytochrome, total molecular weight 60 000–112 000 [142]. The midpoint potential of the *c*-type cytochrome in the *Rb. sphaeroides* nitrate reductase complex is 250 mV [143]. Thus, it is apparently not cytochrome c_2. The *E. coli* nitrate reductase appears to be unrelated because it is membrane bound and has a different subunit composition including a *b*-type cytochrome [38].

Both *Rb. capsulatus* and *Rb. sphaeroides* can be induced by growth in the presence of dimethylsulfoxide to produce a DMSO reductase. This is a soluble, periplasmic protein [141,144]. The DMSO reductase from *Rb. sphaeroides* is a monomer with a molecular weight of 82 000 and contains a molybdopterin cofactor. *Rb. spheroides* and *Rb. capsulatus* DMSO reductases also use trimethylamine *N*-oxide (TMAO) and methionine sulfoxide as electron acceptors. When grown on dimethylsulfoxide, *Rb. sphaeroides* produces a pair of membrane-bound *c*-type cytochromes (molecular weights 20 000 and 45 000) [145]. Only the 44 kDa cytochrome is induced in *Rb. capsulatus* [146]. Deletion mutants of the cytochrome bc_1 complex in *Rb. capsulatus* are still able to grow on DMSO, but mutants completely lacking *c*-type cytochromes no longer grow on DMSO [124]. This suggests that the 44 kDa cytochrome probably interacts with quinone and bypasses the bc_1 complex and cytochrome c_2.

When grown on nitrate, a denitrifying strain of *Rb. sphaeroides* produces a soluble high redox potential cytochrome *c*-552 [147] with a molecular weight of 13 500. This may be the same cytochrome as found associated with the nitrate reductase complex [142] as the two cytochromes have similar redox potentials. In addition, a nitrite reductase is induced. This enzyme has two coppers, a molecular weight of 80 000 and two similar sized subunits of 38 000 and 40 000 [148,149]. Cytochrome c_2 is an effective electron donor for nitrite reductase in vitro and is probably the natural electron donor in vivo.

In summary, *Rb. capsulatus* and *Rb. sphaeroides* provide an interesting contrast in that they are similar metabolically and in terms of soluble redox proteins in

that both produce cytochrome c_2, cytochrome c' and ferredoxin in abundance. Also, in both organisms additional redox proteins are induced by nitrate, DMSO and TMAO. Nevertheless, in terms of minor components these bacteria are quite distinct. Thus *Rb. sphaeroides*, but not *Rb. capsulatus*, contains SHP, cytochrome *c*-551 and cytochrome *c*-554 under normal growth conditions. The significance of this will have to await elucidation of the function of the minor components but suggests that *Rb. sphaeroides* has distinct metabolic pathways in addition to those of *Rb. capsulatus*.

VIII. *Rhodopseudomonas viridis / Chromatium vinosum*

Rb. viridis and *C. vinosum*, although quite distinct in most respects, have one principal common feature, namely the presence of a multiheme membrane-bound cytochrome which is tightly associated with the photosynthetic reaction center. This is in sharp contrast to many purple phototrophic bacteria, for example, *Rb. sphaeroides* and *Rb. capsulatus*, which have reaction centers which do not contain bound cytochromes.

Rps. viridis is an unusual purple phototrophic bacterium in several ways. First, it contains bacteriochlorophyll *b*, which is found in only four other species (*Thiocapsa pfennigii, Ectothiorhodospira halochoris, Ectothiorhodospira abdelmalekii*, and *Rhodopseudomonas sulfoviridis*). Second, it produces an abundant cytochrome c_2 which is more closely related to mitochondrial cytochrome *c* than are many of those from bacteria which can grow fully aerobically and have an *a*-type oxidase [150]. However, *Rps. viridis* is a microaerophile and grows better as a phototroph than in the air. Third, *Rps. viridis* has a large, membrane-bound, tetraheme cytochrome which is tightly bound to the reaction center. Fourth, *Rps. viridis* apparently has no soluble cytochromes other than cytochrome c_2.

In contrast, *C. vinosum*, a purple-sulfur photosynthetic bacterium, does not have an abundant, high redox potential soluble cytochrome. The principle soluble electron-transfer proteins in *C. vinosum* are HiPIP, cytochrome c', flavocytochrome *c*, and bacterial ferredoxin (see Table VIII). However, *C. vinosum* does contain a reaction-center-associated membrane cytochrome, similar to that found in *Rps. viridis* (see next section).

The *Rps. viridis* reaction center complex was the first such three-dimensional structure to be solved [123]. It is composed of four subunits: L, M and H similar to those of *Rb. sphaeroides* and other photosynthetic bacteria, and a heme-containing subunit (not found in *Rb. sphaeroides*). The L and M subunits span the membrane and bind the reaction center pigments including 4 bacteriochlorophylls, 2 bacteriopheophytins, 1 iron and 2 quinones. The H subunit is located on the cytoplasmic side of the membrane, whereas the cytochrome subunit is on the periplasmic side. The *Rps. viridis* reaction center cytochrome gene is adjacent to those for the L and M reaction center subunit genes and all three genes have been cloned and sequenced [151,152]. It must be stressed that the L, M and H subunits of *Rps. viridis* are homologous with those of the *Rb. sphaeroides* and *Rb. capsulatus* reaction centers. The *Rps. viridis* reaction center cytochrome has an unusual structure in that three hemes have His–Met coordination and one has

TABLE VIII

Chromatium vinosum soluble and peripheral membrane redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
HiPIP	10	10	360	complete	
Cytochrome c'	28	14	18	complete	dissociates upon binding CO
Flavocytochrome *c*	72	21, 48	15	n.d.	sulfide dehydrogenase
Bacterial ferredoxin	10	10	−490	complete	
Hydrogenase	98	50	n.d.	n.d.	contains Ni and a 4-Fe-S cluster
Cytochrome *c*-553 (550)	20–30	13	330	n.d.	membrane associated
Cytochrome *c*-551	16	16	240	n.d.	minor component
Cytochrome *c*-551	18	18	−299	n.d.	minor component
Heme protein	12	12	−110	n.d.	binds O_2
Cytochrome *c*-550	12	12	−82	n.d.	minor component

His–His ligation. Presumably, the latter heme has a low redox potential and the heme nearest to the reaction center special pair chlorophylls has the highest redox potential. The redox potentials of the individual hemes have been measured by Alegria and Dutton [153] as 340, 225, 90 and −90 mV. A similar measurement by Dracheva et al. [154] resulted in potentials of 380, 310, 20 and −60 mV. Kinetics of photooxidation of the cytochrome were measured by Dracheva et al. [154] and by Shopes et al. [155]. Interestingly, it has been reported that reduction of the light-oxidized reaction center cytochrome in membranes is very slow unless cytochrome c_2 is added [156]. This result suggests that cytochrome c_2 could be the electron donor, to at least the high potential heme of the reaction center cytochrome. *Rps. viridis* cytochrome c_2 may also serve as an electron acceptor for the postulated *Rps. viridis* cytochrome bc_1 complex [156].

The predominant membrane cytochrome in *C. vinosum* is that which is copurified with the reaction center. It was isolated and characterized by Kennel and Kamen [157,158] and has four hemes and a minimum molecular weight of 45 000, hence is similar if not homologous to the *Rps. viridis* reaction center cytochrome (the amino acid sequence is not yet known). Spectral and redox properties of the *C. vinosum* reaction center cytochrome are apparently unchanged by solubilization, but some proteolytic cleavage of the peptide chain has been noted [157,158]. The hemes absorbing at 556 nm have an average redox potential of 325 mV and the hemes absorbing at 552 nm have an average redox potential of 8 mV. However, the redox properties should be re-examined in light of the recent results with the *Rps. viridis* reaction center cytochrome where four different heme potentials were resolvable [153,154].

From physico-chemical and structural standpoints, flavocytochrome *c* is one of the most interesting soluble electron-transfer proteins found in *C. vinosum*. It has been characterized by Bartsch et al. [159] as a diheme, 72 kDa protein, which contains a covalently bound flavin. Flavocytochrome *c* functions as a sulfide dehydrogenase presumably coupling the oxidation of sulfide to the reduction of metabolically important electron carriers [160]. Unfortunately, the specific electron acceptor for *C. vinosum* flavocytochrome *c* has not been identified to date. It should be pointed out that not all purple-sulfur bacteria using sulfide contain flavocytochrome *c* (for example, *T. pfennigii*). Meyer and Bartsch [161] showed that the flavin moiety of flavocytochrome *c* uniquely reacts with cyanide, sulfite, thiosulfate, and mercaptans to form adducts, which are characterized by a charge-transfer band in the red (600–700 nm). The kinetics of adduct formation have been studied [162]. Moreover, the reduction of holoflavocytochrome *c*, of the isolated heme subunit, and the flavocytochrome-*c* complex with mitochondrial cytochrome *c* have been reported [163,164]. An interesting result of the kinetic studies carried out to date is that oriented electron flow occurs. That means that flavin reduction is followed by intracomplex electron transfer to the heme moiety, with the heme inaccessible to exogenous reductant in the holoenzyme. The heme subunit can then reduce added electron acceptors (for example, horse cytochrome *c* and the dye DCPIP).

C. vinosum contains a cytochrome *c′* with prototypic properties as it is one of the most extensively studied examples. The molecular weight is 28 000 and there are two identical subunits (14 000 each) which do not dissociate under physiological conditions but do form monomers in the presence of carbon monoxide [165]. The structure of one example of cytochrome *c′* from *R. molischianum* [166] is known and amino acid sequence homology [167] among the cytochromes *c′* suggest the same structural motif for all examples. Although the function of this class of cytochromes is not yet clear, the kinetics of reduction by free flavins and flavodoxin semiquinones indicates free access to the heme by small reactants, but sterically hindered access to larger protein reactants [136]. These observations suggest that cytochrome *c′* may be designed to interact with small molecules.

Of the bacteria discussed in this review, only *C. vinosum* contains HiPIP, a small soluble high-redox-potential ferredoxin. Although the function of HiPIP is not known, it is found in many species of phototrophic bacteria (it has been isolated from 15 different species), but not all (for example, it is absent in *Rb. spheroides, Rb. capsulatus* and *Rps. viridis*). The three-dimensional structure of *C. vinosum* HiPIP has been determined [168] and the in vitro kinetics have been extensively characterized [169].

C. vinosum also contains a number of minor soluble cytochromes; however, it is important to note that these cytochromes have not been extensively studied and it is not yet clear which growth conditions favor their expression. Cytochrome *c*-553 (550) has been solubilized from *C. vinosum* by extraction with 50% acetone [170]. It has a high redox potential and a minimum molecular weight of 13 000 and is present in whole cells at a level of approx. 5% of the level of the principal soluble electron-transfer proteins (flavocytochrome *c*, cytochrome *c′* and HiPIP). Once solubilized, cytochrome *c*-553 (550) is water soluble although it does show a tendency to aggregate.

A soluble cytochrome *c*-550 or *c*-551 was reported to be involved in cyclic electron flow in *C. vinosum* [171]. Knaff et al. [172] showed that horse cytochrome *c* could substitute for the *C. vinosum* protein, suggesting that they might bear structural similarities. *C. vinosum* cytochrome *c*-551 was purified by Tomiyama et al. [173] and Gray et al. [174] and has an apparent molecular weight of 16 000 and a redox potential of 240 mV.

However, cytochrome *c*-551 is labile and present only in very small quantities, at least an order of magnitude less than the concentration of principle components of the electron-transfer chain. To date we have been unable to identify cytochrome *c*-551 in *C. vinosum* extracts (50 preparations of 500 or more grams of wet weight cells). Thus, the possibility exists that cytochrome *c*-551 results from a contamination in the culture or it is a breakdown product of one of the more abundant cytochromes. Moreover, light-induced difference spectra of *C. vinosum* chromatophores do not suggest the presence of a high potential cytochrome with an α peak at 551 nm [175]. Clearly more work is required to resolve the question of the presence and role of cytochrome *c*-551.

Three other soluble *c*-type cytochromes have been reported at various times although no detailed studies have been carried out and only small quantities are present. Gray et al. [174] reported a cytochrome *c*-551 with a molecular weight of 18 000 and a redox potential of −299 mV. This protein is apparently quite labile and could possibly be a breakdown product of the reaction-center cytochrome. A cytochrome *c*-550 [176] with a molecular weight of 12 000 and a redox potential of −82 mV has also been reported. The absorption spectra of this protein indicate a mixture of high- and low-spin heme suggestive of denaturation. Finally, a high-spin heme protein which binds oxygen [176] has been reported and appears to be similar to the *Rb. sphaeroides* heme protein discussed earlier. The *Chromatium* oxygen binding protein has an apparent molecular weight of 12 000 and a redox potential of −110 mV. To date no amino acid sequence data are available for any of these minor components and further studies are required to establish their role, if any.

A cytochrome c_1 was reported in *C. vinosum* by Gaul and Knaff [177]. The detergent solubilized cytochrome had an apparent molecular weight of 31 000 with redox potential 245 mV. Although it is generally assumed that all purple bacteria have a cytochrome bc_1 complex as part of the cyclic photosynthetic pathway, it has only been proven in *Rb. sphaeroides* and *Rb. capsulatus* where the complex is easily observed spectroscopically and the genes have been cloned and sequenced. The presence of an abundant tetraheme reaction-center cytochrome in *Rps. viridis* and *C. vinosum* makes it difficult, if not impossible, to identify a cytochrome bc_1 complex spectroscopically in membrane fractions. Kennel and Kamen [157,158] showed that pure reaction-center cytochrome, separated from a reaction-center membrane preparation, ran as three heme-containing bands on SDS-PAGE (45, 29, 23 kDa), whereas the membrane-bound protein ran as a single band (45 kDa). This is similar to the peptide pattern observed by Gaul and Knaff [177] for their cytochrome c_1 preparation (44, 31, 24 kDa) and is probably the result of proteolysis in both instances. Kennel and Kamen [157,158] also noted that prolonged exposure to detergent caused progressive denaturation of the long-wavelength high-potential heme. Thus, all of the essential characteristics of the reported impure cytochrome c_1 preparation were present in the pure reaction center cytochrome preparation from the earlier study. We have to conclude that the presence of cytochrome c_1 has not yet been proven in *C. vinosum*. Perhaps a genetic approach might be more fruitful. In a similar analysis by Wynn et al. [178], a cytochrome bc_1 complex was reported in *Rps. viridis*. Protoheme was detected in the membrane, a 31 kDa cytochrome *c* (along with 38 kDa reaction-center cytochrome) was observed and an EPR signal typical of Rieske iron-sulfur protein suggested the presence of a cytochrome bc_1 complex. Stronger evidence for the presence of a cytochrome bc_1 complex in *Rps. viridis* was obtained by Kampf et al. [179]. Semiaerobically grown cultures of *Rps. viridis* contained only low amounts of chlorophyll and essentially none of the interfering reaction-center cytochrome. The membranes contained mainly *b*- and *d*-type cytochrome, but heme-stained SDS-polyacrylamide gels showed the presence of a 31 kDa *c*-type cytochrome. *Rb. sphaeroides* cytochrome c_1 antibodies cross-reacted with the 31 kDa band and the cytochrome was also present in photrophically grown cells. It thus appears likely that *Rps. viridis* does contain a cytochrome bc_1 complex in both growth modes.

Ferredoxin has been isolated from *C. vinosum* and it contains two 4-Fe-S clusters and is homologous to *Clostridial* and the green-sulfur bacterial ferredoxins [180]. Its low redox potential (−490 mV) precludes a role as an electron acceptor for hydrogenase, which functions to oxidize hydrogen rather than to evolve H_2 as a byproduct of fermentation as in *Clostridium*. *C. vinosum* hydrogenase has been purified [181] and is a membrane-bound 4-Fe-S protein which apparently is a dimer with a molecular weight of 50 000 per subunit and contains nickel [182]. The pure protein is inactive with *Clostridial* ferredoxin, but its activity with *Chromatium* proteins has not been reported.

Although apparently similar in terms of reaction-center architecture, *Rps. viridis* and *C. vinosum* have quite different soluble redox-protein compositions. *Rps. viridis* is quite unusual among the purple photosynthetic bacteria in that the only soluble redox protein identified to date is a high-potential *c*-type cytochrome with no HiPIP or cytochrome *c′* present. In contrast, *c. vinosum* has no abundant high potential *c*-type cytochromes but does contain HiPIP and cytochrome *c′*. The minor soluble redox proteins in *C. vinosum* require much more study before their roles can be established. *C. vinosum* does contain flavocytochrome *c*, a protein apparently functioning in sulfur metabolism. However, flavocy-

tochrome *c* is not widely distributed and its specific role in *C. vinosum* is not well defined in spite of its interesting structure and kinetic properties.

IX. *Desulfovibrio vulgaris*

Desulfovibrio species are strictly anaerobic bacteria which oxidize lactate and other organic components and reduce sulfate to sulfide. (Related species are able to use elemental sulfur as electron acceptor). *Desulfovibrio* are gramnegative bacteria which obtain energy via electron-transfer-coupled proton translocation as recently reviewed by Odom and Peck [183]. There are many electron-transfer proteins in *Desulfovibrio* species, some of which have also been observed in other bacteria, but the low redox-potential tetraheme cytochrome c_3 has not conclusively been found elsewhere to date. The pattern of redox proteins observed in *D. vulgaris* is typical of the genus (Table IX) but there are some species differences which will be noted in the description which follows.

Desulfovibrio species all seem to produce an abundant, periplasmic, multiheme cytochrome c_3. The structure of *D. desulfuricans* and *D. vulgaris* cytochromes c_3 have been solved by X-ray diffraction [184,185]. Although the cytochromes c_3 contain four hemes, there is no relationship to the tetra-heme, membrane-bound, reaction-center cytochrome from *Rps. viridis* [123]. Spatially, the heme groups in cytochrome c_3 are roughly perpendicular to one another and all have some portion exposed to solvent. Each heme has two histidine ligands, which accounts in part for their low redox potentials as bis-histidine ligation always results in lower redox potentials as compared to His-Met ligation. The redox potentials of the four hemes in cytochrome c_3 have been resolved [186,187] and when interactions between hemes are taken into account a total of 32 midpoint potentials are required [188]. However, typically the four heme potentials range from −290 mV to −375 mV [187]. Attempts have been made to assign potentials to individual hemes as defined in the structure but have not been convincing to date [189].

There have been several studies of kinetics of reduction of cytochrome c_3 [190,191]. The cytochromes c_3 appear to be readily reduced by a variety of compounds consistent with partially exposed hemes and there appears to be rapid intramolecular electron transfer. Cytochrome c_3 can function as an electron acceptor for hydrogenase [192] and may be an obligate intermediate in the reduction of sulfate as discussed below [193]. Both cytochrome c_3 and a hydrogenase were reported to be periplasmic [194,195] and the gene sequence of cytochrome c_3 has a signal peptide necessary for transport across the cytoplasmic membrane [196]. There appear to be substantial differences among the cytochromes c_3 as the amino acid sequences of different species are difficult to align and require numerous insertions and deletions [3]. Moreover, at least one cytochrome c_3, isolated from *Desulfuromonas acetoxidans*, possesses only three hemes in a peptide chain of 68 amino acid residues [197]. These observations imply that not all four hemes are required for function and that there is likely to be a significant variation in three-dimensional structures as well.

There are other low-redox-potential cytochromes in *Desulfovibrio* species [42], but it is not clear how many

TABLE IX

Desulfovibrio vulgaris soluble redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
Cytochrome c_3	13	13	−336	complete	contains four hemes
Cytochrome c_3	75	n.d.	low	n.d.	contains 16 hemes
Cytochrome *c*-553	9	9	10	complete	contains one heme
Flavodoxin	15	15	−150, −400	complete	contains one FMN
Rubredoxin	6	6	−60	complete	contains one iron
Sulfite reductase	226	42, 50	n.d.	n.d.	contains two sirohemes + iron sulfur clusters
Hydrogenase	60	46, 13	n.d.	complete	contains three 4-Fe-S clusters
APS reductase	220	70, 20	n.d.	n.d.	contains FAD + iron-sulfur clusters
Ferredoxin	12	6	−300	partial	contains two 4-Fe-S clusters

and how they might be related to cytochrome c_3. *D. gigas, D. vulgaris*, and *D. desulfuricans* have been reported to have a 26 000 molecular weight cytochrome c_3, which has eight hemes. This cytochrome was originally reported to be monomeric, but after heme removal the *E. desulfuricans* protein was found to dissociate into 13 kDa subunits [42]. The subunit size, redox potential, and heme content all suggest that the 26 kDa cytochrome may be an isozyme of cytochrome c_3. We have not observed a 26 kDa cytochrome in *D. vulgaris*, but we have seen a protein having a molecular weight of 85 000. Yagi [198] reported a 60 000 molecular weight cytochrome and Higuchi et al. [45] found a 16-heme, 75 kDa cytochrome which crystallized in a hexagonal space group. Although the 75 kDa cytochrome appeared to be monomeric, it is the right size to be a hexamer of a cytochrome c_3 isozyme. Based on available evidence we suggest that these are all the same protein, which because of insolubility at low ionic strength aggregates to varying degrees. Clearly, amino-acid sequence data on the large cytochromes *c* would clarify the situation.

An additional low-redox-potential cytochrome has been isolated from *D. desulfuricans* [183], an inducible nitrite reductase, which has six hemes and a molecular weight of 66 000. This strain of *Desulfovibrio* can grow using nitrate as an alternate terminal electron acceptor, and nitrite reductase is then induced 10-fold over the level in sulfate-grown cells. There is some similarity between the *Desulfovibrio* nitrite reductase and that found in *E. coli* [40]. However, there is no relationship between the *Desulfovibrio* dissimilatory nitrite reductase and the assimilatory nitrite and sulfite reductases of many bacteria and eucaryotes or the dissimilatory sulfite reductase in *Desulfovibrio* which contain siroheme (see below).

Several, but not all, *Desulfovibrio* species synthesize a small soluble cytochrome *c*-553 containing a single heme, which is unrelated to cytochrome c_3 [199]. The amino acid sequence of *D. vulgaris* Miyazaki strain cytochrome *c*-553 [200] suggests that it is distantly related to the cytochromes c_4 of *Azotobacter* and *Pseudomonas* [68] and has His–Met heme ligation. The reported sequence of the Hildenborough strain of cytochrome *c*-553 appears to be quite different from that from the Miyazaki strain [201]. However, because there were insufficient peptide overlaps reported in the latter study for complete analysis of the sequence and because the tryptic peptides are very similar to those from the Miyazaki strain, the reported differences should be reinvestigated. Since cytochromes c_3 from these two strains are quite closely related (85% identity), it is likely that there are errors in the Hildenborough cytochrome *c*-553 sequence and the two cytochromes *c*-553 are more closely related than published results indicate. Alignment of the tryptic peptides with the sequence of strain Miyazaki cytochrome *c*-553 suggests 75% identity. There are various reports of the redox potential of cytochromes *c*-553, most in the region of 10 mV [202]. However, Yagi [198] reported that a cytochrome *c*-553 was the electron acceptor for formate dehydrogenase in the Miyazaki strain and that it had a redox potential of −260 mV. This is clearly outside the range of cytochromes having His/Met ligands (0–450 mV) and also much lower than other reports for what appear be the same protein.

Sulfite reductase and adenosine phosphosulfate reductase are the principal terminal enzymes in *Desulfovibrio*. The sulfite reductase from *Desulfovibrio* contains siroheme and iron-sulfur clusters in a 226 kDa enzyme, composed of both 42 and 50 kDa subunits [203] and is cytoplasmic. Sulfite reductases from various sulfate-reducing bacteria have widely differing absorption spectra, but all show high-spin-heme EPR spectra and similar carbon monoxide complexes [204]. There appears to be a strong similarity with the assimilatory sulfite and nitrite reductases from spinach and *E. coli* [205] and to the *Desulfovibrio* assimilatory sulfite reductase. The spinach proteins have a single subunit of 61–69 kDa and have one siroheme and a single 4-Fe-S cluster, with strong magnetic interaction between the siroheme and the iron-sulfur cluster [206]. In *E. coli* sulfite reductase, one of the cysteines, bound to the iron-sulfur cluster, is also a heme ligand [207]. Sulfite reductase requires a low-potential reductant, which in *E. coli* is NADPH, and in spinach is the 2-Fe-S ferredoxin. The electron donor in *Desulfovibrio* has not been established, but is expected to be ferredoxin or flavodoxin.

Adenosine phosphosulfate (APS) reductase has a native molecular weight of 220 000 and contains 70 000 and 20 000 molecular weight subunits [208]. There are both iron-sulfur clusters and FAD in APS reductase. As with sulfite reductase, APS reductase requires a low-redox-potential reductant, presumably ferredoxin or flavodoxin and is cytoplasmic.

Ferredoxin has not been characterized in *E. vulgaris* Hildenborough because it is present in too small a quantity [183]. In this species, flavodoxin is abundant and is apparently functionally interchangeable with ferredoxin. However, other *Desulfovibrio*, species, such as *D. gigas* and *D. desulfuricans*, produce several ferredoxin isozymes and much less flavodoxin than does *D. vulgaris*. Based on the available sequence data *Desulfovibrio* ferredoxins appear to be degenerate forms of the *Clostridial* 8-Fe-S ferredoxins [209]. Most appear to bind a single 4-Fe-S cluster and have additional cysteines which may be involved in aggregation [210]. Only *D. desulfuricans* Norway iso-2 ferredoxin has binding sites for two 4-Fe-S clusters. Unlike *D. vulgaris* Hildenborough, *D. vulgaris* Miyazaki produces appreciable quantities of ferredoxin [211]. The sequence is most similar to that of *D. africanus* [212]. In the various species, *Desulfovibrio* ferredoxin is cytoplasmic and is

probably the reductant for sulfite and APS reductases.

Flavodoxin is induced by growth in iron-limited media in many bacterial species and generally substitutes for an 8-Fe-S ferredoxin. The first example was characterized by Knight and Hardy [94] in *Clostridium pasteurianum*. Flavodoxin typically has a molecular weight of about 15 000 and has one FMN at the active site. The protein can be reduced in two one-electron steps with redox potentials about −150 and −400 mV and the semiquinone is stable. It is the second step of reduction, which allows it to substitute for ferredoxin. *D. vulgaris* flavodoxin was first isolated by Dubourdiu and LeGall [213] and is apparently constitutive. This is perhaps not surprising because the sulfide produced by *Desulfovibrio* precipitates iron-sulfide from the medium, thus creating an iron deficiency. The amino acid sequence of the *D. vulgaris* flavodoxin was determined by Dubourdiu and Fox [214] and the gene sequence by Krey et al. [215] and Curley and Voordouw [216]. The three-dimensional structure [217] is generally similar to that of flavodoxin from *Clostridium* and *Anacystis nidulans* [218]. Flavodoxin is cytoplasmic, as expected if it is to substitute for ferredoxin, which is also cytoplasmic [183].

Rubredoxin is found in many species of anaerobic bacteria and apparently in all species of *Desulfovibrio*. Rubredoxin binds a single iron in a protein of 6 kDa size and the redox potential is about −50 mV. The amino acid sequence is known for three *Desulfovibrio* species [219,220], and the gene sequence is known for *D. vulgaris* [221]. Abberant proteins related to rubredoxin were found in *D. gigas* and *D. vulgaris* and named desulforedoxin [222] and rubrerythrin [223]. Three-dimensional structures for three *Desulfovibrio* rubredoxins have been determined [224–226]. There is a specific reductase for rubredoxin in *Desulfovibrio* [227] but evidence concerning its role in metabolism is lacking. Rubredoxin is apparently cytoplasmic [183].

Hydrogenase is one of the most interesting enzymes in *Desulfovibrio* and provides a means for creating a proton gradient coupled to reduction of sulfate by lactate [193]. In this pathway, oxidation of lactate is coupled to hydrogen evolution in the cytoplasm. Hydrogen gas diffuses across the membrane and is oxidized by a periplasmic hydrogenase. Electron transport across the membrane is followed by reduction of APS and sulfite to complete the process. It would be far simpler to oxidize the lactate in the periplasmic space, but Odom and Peck [193] report that lactate dehydrogenase is cytoplasmic. One problem with the hydrogen cycle just described is that coupling between lactate dehydrogenase and cytoplasmic hydrogenase must be very specific so that there is no direct reduction of sulfate. If ferredoxin and flavodoxin mediate electron transfer from membrane carriers to APS and sulfite reductases, then they cannot also mediate between lactate dehydrogenase and cytoplasmic hydrogenase. Although there is more than one hydrogenase in *Desulfovibrio* [228] the specific enzyme which supposedly functions to evolve hydrogen in the cytoplasm has not been identified nor has it been shown how it is coupled to lactate dehydrogenase. Lastly, it should be noted that the postulated membrane carriers have not been identified. Despite problems with the hydrogen cycle, it remains an attractive idea worthy of further consideration.

There are two well-characterized hydrogenases in *Desulfovibrio* species. *D. vulgaris* Hildenborough hydrogenase was the first to be cloned and sequenced [229]. The enzyme is remarkable in that residues 25–84 of the 420 residue protein are homologous to small 8-Fe-S ferredoxins such as found in *Clostridium*. Four of the remaining ten cysteines presumably bind the other 4-Fe-S cluster which comprises the site of oxidation and reduction of hydrogen. Also remarkable about this hydrogenase is the absence of a signal peptide in the principal subunit which is generally believed to be required for transport across the membrane, leading Voordouw and Brenner [229] to conclude that the enzyme is cytoplasmic. However, the principal hydrogenase was reported to be periplasmic [194,195]. Menon et al. [230] cloned and sequenced a nickel-containing hydrogenase from *D. baculatus* and found that the principal subunit also lacks a signal peptide. However, they noted that both *D. vulgaris* and *D. baculatus* small subunits contained signal peptides and concluded that this was sufficient to transport the whole enzyme across the membrane. Substantial corrections to the sequence were subsequently reported, but do not affect these conclusions [231]. This has not previously been observed to our knowledge and if true has important ramifications for the study of membrane transport of enzymes. Comparison of the amino acid sequence of *D. vulgaris* hydrogenase with that from *D. baculatus* shows no similarity at all. Although both enzymes contain iron-sulfur clusters, *D. baculatus* hydrogenase also contains nickel and selenium. Nickel is a common constituent of hydrogenases, and only *Clostridium* and *D. vulgaris* hydrogenases have been found to lack this cofactor. *D. vulgaris* strains Hildenborough and Miyazaki are closely related (based on cytochrome c_3 sequences), but the hydrogenase purified from strain Hildenborough is soluble and that from Miyazaki is membrane-bound [232]. Miyazaki hydrogenase has been crystallized [45] following solubilization with trypsin. Cytochrome c_3 is the preferred donor/acceptor for all *Desulfovibrio* hydrogenases which have been characterized to date. Both cytochrome c_3 and the soluble hydrogenases are periplasmic and based on this observation one might suspect that the Miyazaki enzyme is bound to the periplasmic side of the membrane.

Desulfovibrio presents an interesting contrast to other bacteria containing soluble *c*-type cytochromes. Al-

though *Desulfovibrio* contains multiple *c*-type cytochromes, all of them have low oxidation-reduction potentials consistent with the redox environment in which *Desulfovibrio* normally lives (rich in sulfide). The lack of structural and sequence homology of *Desulfovibrio* cytochrome c_3 with high-potential cytochromes and the unproven occurrence of Class-III *c*-type cytochrome outside of *Desulfovibrio* suggests an independent evolution of this class of cytochromes. Although more work is required, the role of the soluble redox proteins in *Desulfovibrio* is much more clearly defined than in many bacteria. Thus, as the genetics of *Desulfovibrio* redox proteins are worked out, a detailed understanding of the control and function of electron transfer in these organisms can be expected.

X. *Clostridium pasteurianum*

Clostridium is gram-positive and all soluble proteins are expected to be cytoplasmic although this has not been rigorously proven. *Clostridium* is also a strictly fermentative bacterium, nevertheless it contains a variety of electron-transfer proteins excluding cytochromes and copper proteins (see Table X). The 8-Fe-S ferredoxin, rubredoxin, and flavodoxin from *Clostridium* have been well-characterized and serve as prototypes for these classes of proteins (see Yoch and Carithers [233] for a review of structure and function).

The 8-Fe-S ferredoxin gene has been cloned [234] and there is no leader sequence in the 55-residue protein, which is consistent with cytoplasmic location. Moreover, there appears to be only one gene, unlike many other bacteria which have several related 8-Fe-S ferredoxins. The redox potentials of the two 4-Fe-S clusters in ferredoxin are the same (−412 mV) and do not vary with pH or ionic strength [235]. Ferredoxin functions as electron acceptor for a number of enzymes in clostridia, but in *C. pasteurianum* a principal interaction is with pyruvate dehydrogenase. Ferredoxin is strongly negatively charged and is expected to interact with positively charged residues of its reaction partners.

Although rubredoxin is well characterized structurally, much less is known of its functional roles in *Clostridium*. Rubredoxin has a single iron in its 53-residue peptide chain and has a much higher redox potential than the 8-Fe-S ferredoxin, $E_{m,7} = -57$ mV [236] thus it is not expected to participate in the same reaction as ferredoxin. In a nitrate reducing strain of *C. perfringens*, rubredoxin has been found to be necessary for NAD(P)H dependent reduction of nitrate [237]. Rubredoxin also seems to be the preferred electron acceptor for carbon monoxide dehydrogenase in *C. thermoaceticum* [238]. The three-dimensional structure of rubredoxin [239] shows that the iron is exposed at one end of the molecule with nearby hydrophobic residues at the surface which would define the principal

TABLE X

Clostridium pasteurianum soluble redox proteins

n.d., not determined.

	Native molecular mass (kDa)	Subunit molecular mass (kDa)	Redox potential (mV)	Protein sequence	Comments
8-Fe-S ferredoxin	6	6	−412	complete	contains two 4-Fe-S clusters
Rubredoxin	6	6	−57	complete	contains one Fe
Hydrogenase I	60	60	−400, −420	n.d.	contains three 4-Fe-S clusters
Hydrogenase II	53	53	−400, −180	n.d.	contains two 4-Fe-S clusters
2-Fe-S ferredoxin	25	12	−300	complete	
Nitrogenase DK subunit complex	220	56, 60	−20 or −70	complete	contains four 4-Fe-S clusters and two Mo Fe cofactors per complex
Nitrogenase reductase H subunit	60	30	−240 or −294 −380 [a] −400 [a]	complete	contains one 4-Fe-S cluster per two subunits and binds ATP
Flavodoxin	15	15	−132, −419	complete	contains one FMN

[a] In the presence of Mg (ADP) or Mg (ATP).

site of oxidation and reduction. The protein is strongly negatively charged, but the acidic residues are not very near the partially exposed iron and they may orient the protein to inhibit unwanted interactions with ferredoxin reaction partners.

Flavodoxin is a 138-residue FMN-containing protein which, in *Clostridium*, is only synthesized under iron-deficiency conditions [94] and substitutes for the 8-Fe-S ferredoxin. The 3-dimensional structure [98] shows that a portion of the FMN is exposed at one end of the protein and surrounded by negatively charged amino acids which dominate in interactions with nonphysiological substrates [16] and are expected to be important in defining interactions with the normal ferredoxin reaction partners such as pyruvate dehydrogenase, nitrogenase and hydrogenase. Flavodoxin is also able to substitute for ferrodoxin because the flavin semiquinone is stabilized, allowing the protein to be reduced in two one-electron steps. The redox potential of the second step of reduction, from semiquinone to fully reduced protein, $E_{m,7} = -419$ mv is comparable to the midpoint potential of ferredoxin [240].

Both hydrogenase and nitrogenase are also well characterized in *Clostridium*. Ferredoxin is the natural electron donor to both enzymes, but under iron-deficiency conditions flavodoxin is synthesized and substitutes for ferredoxin [94]. The structural genes for nitrogenase have been cloned, and it was found that the D and K subunits are present as single copies, but there are as many as six separate H-like genes [241]. The H subunit serves two functions, as electron donor to the DK subunit complex and it is necessary for synthesis of the molybdenum cofactor (it is possible that there is a different isozyme for each of these roles). It is also possible that different isozymes are functional when flavodoxin substitutes for ferredoxin although no available data bear on this point. Redox potentials of the nitrogenase DK subunit complex were reported by Walker and Mortenson [242] and Zumft et al. [243] but these redox transition are at too high a potential to be functional in nitrogen fixation. The redox potential of the H subunit changes to lower values in the presence of ATP or ADP [243].

In *Azotobacter*, it was shown that a small 2-Fe-S ferredoxin protects nitrogenase against oxygen inactivation [93]. A similar 2-Fe-S ferredoxin is found in *Clostridium* [244]. The amino acid sequence of this *Clostridium* 2-Fe-S ferredoxin has been determined and there is no obvious relation to any other known iron-sulfur protein [106] (there are no sequence data for the *Azotobacter* 2-Fe-S ferredoxin which may be related). *Clostridium* is a strict anaerobe and there presumably is no need to protect against oxygen; therefore the function of this 2-Fe-S ferredoxin must be different from that in *Azotobacter* or other aerobic bacteria.

Clostridium often evolves large quantities of hydrogen as the end product of fermentation, but hydrogen is also the undesired byproduct of nitrogen fixation. Similar to what is found in other bacteria, there are hydrogenase isozymes in *Clostridium* [245,246]. One isozyme (hydrogenase I) is responsible for hydrogen evolution using the 8-Fe-S ferredoxin as electron donor and a second isozyme (hydrogenase II) is functional in hydrogen uptake, presumably to recover reducing equivalents lost via nitrogenase. These different roles for hydrogenase are possible because the iron-sulfur clusters of the two isozymes have very different redox potentials (-420 mV for hydrogenase I vs. -180 mV for hydrogenase II). The 8-Fe-S ferredoxin does not interact with hydrogenase II, but rubredoxin has a redox potential which would be compatible for the postulated electron acceptor (-57 mV).

Hydrogenase isozymes, located on opposite sides of the cytoplasmic membrane, are thought to function in establishing a proton gradient in *Desulfovibrio* [193]. However, because the two *Clostridium* isozymes are soluble, and *Clostridium* is gram-positive, it may be assumed that they are both cytoplasmic and cannot function in the same manner as in *Desulfovibrio*.

In summary, as is apparently typical of gram-positive anaerobic bacteria, *Clostridium* is devoid of soluble *c*-type cytochromes. However, in contrast to aerobic *Bacillus* species which do not yield any significant soluble redox proteins, *Clostridium* does produce abundant soluble iron-sulfur proteins of several varieties.

XI. Conclusion

It is clear from the foregoing that diversity best describes the soluble redox protein content of bacteria. Although we have attempted to be as comprehensive as possible in this review it should be apparent that our understanding of the distribution and expression of soluble redox proteins is incomplete. Clearly, minor components isolated from any particular species of bacteria could result from contaminants in the culture. This is a difficult point to deal with experimentally and can only be clearly resolved if the same proteins are found independently or the necessary genetic information becomes available. Moreover, it appears that proteolytic fragmentation during purification (or in vivo) can result in multiple soluble redox proteins (for example, *Azotobacter*). Before a minor component can be considered present for certain some amino acid or DNA sequence is required. Most importantly it is clear that different metabolic conditions can have a dramatic effect on the soluble redox protein composition. This makes comparison of studies from different laboratories difficult unless the bacteria are grown under exactly the same conditions. In addition, this variable leaves open the possibility that additional soluble redox proteins may be expressed under metabolic conditions which

have not been tested to date. This is shown most dramatically in *Paracoccus* where aerobic, anaerobic and partial oxygenation conditions yield different *c*-type cytochromes.

The most distressing fact in bacterial electron-transfer studies is our lack of a clear metabolic role for many of the soluble redox proteins. Thus, whole classes of proteins, for example Class-II *c*-type cytochromes and HiPIP, have no known functions. Morover, even in instances where we can relate the presence of a particular redox protein to a metabolic pathway, it is not always clear what are the physiological electron donors and acceptors for the particular protein (e.g., flavocytochrome *c*). However, one can be optimistic that these generic questions will be resolved in the future. The available techniques of molecular biology by which gene sequences of operons can be determined will help to define specific pathways. More importantly, the ability to delete specific genes should more clearly define functional roles. Nevertheless, even genetic approaches will not always be definitive if the anomalous role of *R. capsulatus* cytochrome c_2 proves to be a typical example.

The most important conclusion that can be drawn from studies to date is that the same metabolic function can be carried out utilizing different redox proteins. The strict anaerobes *Desulfovibrio* and *Clostridium* provide an interesting example, with *Desulfovibrio* having abundant *c*-type cytochromes and *Clostridium* devoid of *c*-type cytochromes. Similarly, the aerobes *E. coli* and *Bacillus* have no abundant soluble high-potential *c*-type cytochromes yet *Paracoccus* and *Rb. sphaeroides*, when grown under appropriate conditions, have multiple *c*-type cytochromes. However, even in *Paracoccus*, the precise role of the constitutive high-potential cytochrome c_2 is unclear (see also *R. capsulatus* above).

In view of the large number of laboratories presently involved in the study of bacterial electron transfer, continued progress and new insights can be expected. It is clear that as our understanding of bacterial electron-transfer evolves, substantial information on the adaptation and evolution of proteins will be obtained. In addition, new redox carriers and enzymes will be characterized and our understanding of metabolic pathways improved and most importantly the details of mechanisms which control electron transfer between redox proteins will be obtained.

References

1 Wood, P.M. (1983) FEBS Letts 164, 223–226.
2 Ambler, R.P. (1977) in The Evolution of Metalloenzymes, Metalloproteins and Related Materials (Leigh, G.J., ed.), pp. 100–118, Symposium Press, London.
3 Meyer, T.E. and Kamen, M.D. (1982) Advances in Protein Chemistry 35, 105–212.
4 Cusanovich, M.A., Meyer, T.E. and Tollin, G. (1988) in Advances in Inorganic Biochemistry (Eichhorn, G.L., Marzilli, L.G., eds.), pp. 37–91, Elsevier, New York.
5 Pettigrew, G.W. and Moore, G.R. (1987) Cytochromes *c* – Biological Aspects, Springer-Verlag, New York.
6 Adam, E.T. (1979) Biochim. Biophys. Acta 549, 107–144.
7 Dreyer, J.L. (1984) Experientia 40, 653–776.
8 John, P. and Whatley, F.R. (1975) Nature 254, 495–498.
9 Scholes, P.B., McLain, G. and Smith, L. (1971) Biochemistry 10, 2072–2076.
10 Timkovich, R., Dickerson, R.E. and Margoliash, E. (1976) J. Biol. Chem. 251, 2197–2206.
11 Ambler, R.P., Meyer, T.E., Kamen, M.D., Schichman, S.A. and Sawyer, L. (1981) J. Mol. Biol. 147, 351–356.
12 Timkovich, R. and Dickerson, R.E. (1976) J. Biol. Chem. 251, 4033–4046.
13 Ambler, R.P., Daniel, M., Hermoso, J., Meyer, T.E., Bartsch, R.G. and Kamen, M.D. (1979) Nature 278, 659–660.
14 Ambler, R.P., Meyer, T.E., Cusanovich, M.A. and Kamen, M.D. (1987) Biochem. J. 246, 115–120.
15 Meyer, T.E., Watkins, J.A., Przysiecki, C.T., Tollin, G. and Cusanovich, M.A. (1984) Biochemistry 23, 4761–4767.
16 Tollin, G., Cheddar, G., Watkins, J.A., Meyer, T.E. and Cusanovich, M.A. (1984) Biochemistry 23, 6345–6349.
17 Berry, E.A. and Trumpower, B.L. (1985) J. Biol. Chem. 260, 2458–2467.
18 Newton, N. (1969) Biochim. Biophys. Acta 185, 316–331.
19 Timkovich, R., Dhesi, R., Martinkus, K.J., Robinson, M.K. and Rea, T.M. (1982) Arch. Biochem. Biophys. 215, 47–58.
20 Bosma, G., Braster, M., Stouthamer, A.H. and Van Verseveld, H.W. (1987) Eur. J. Biochem. 165, 657–663.
21 Chang, C.K. and Wu, W. (1986) J. Biol. Chem. 261, 8593–8596.
22 Martinkus, K., Kennelly, P.J., Rea, T. and Timkovich, R. (1980) Arch. Biochem. Biophys. 199, 465–472.
23 Ambler, R.P. and Tobari, J. (1985) Biochem. J. 232, 451–457.
24 Husain, M. and Davidson, V.L. (1985) J. Biol. Chem. 260, 14626–14629.
25 Husain, M., Davidson, V.L. and Smith, A.J. (1986) Biochemistry 25, 2431–2436.
26 Gray, K.A., Knaff, D.B., Husain, M. and Davidson, V.L. (1986) FEBS Lett. 207, 239–242.
27 Tobari, J. and Harada, Y. (1981) Biochem. Biophys. Res. Commun. 101, 502–508.
28 Husain, M. and Davidson, V.L. (1986) J. Biol. Chem. 261, 8577–8580.
29 Bosma, G., Braster, M., Stouthamer, A.H. and Van Verseveld, H.W. (1987) Eur. J. Biochem. 165, 665–670.
30 Anraku, Y. and Gennis, R.B. (1987) Trends Biochem. Sci. 12, 262–266.
31 Miller, M.J. and Gennis, R.B. (1983) J. Biol. Chem. 258, 9159–9165.
32 Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259, 3375–3381.
33 Timkovich, R., Cork, M.S., Gennis, R.B. and Johnson, P.Y. (1985) J. Am. Chem. Soc. 107, 6069–6075.
34 Kranz, R.G. and Gennis, R.B. (1985) J. Bacteriol. 161, 709–713.
35 Green, G.N., Fang, H., Lin, R.J., Newton, G., Mather, M., Georgiou, C.D. and Gennis, R.B. (1988) J. Biol. Chem. 263, 13138–13143.
36 Kita, K., Konishi, K. and Anraku, Y. (1984) J. Biol. Chem. 259, 3368–3374.
37 Matsushita, K., Patel, L. and Kaback, H.R. (1984) Biochemistry 23, 4703–4714.
38 MacGregor, C.H. (1976) J. Bacteriol. 126, 122–131.
39 Sodergren, E.J. and DeMoss, J.A. (1988) J. Bacteriol. 170, 1721–1729.

40 Kajie, S. and Anraku, Y. (1986) Eur. J. Biochem. 154, 457–463.
41 Ambler, R.P., Daniel, M., McLellan, L., Meyer, T.E., Cusanovich, M.A. and Kamen, M.D. (1987) Biochem. J. 248, 365–371.
42 Guerlesquin, F., Bovier-Lapierre, G. and Bruschi, M. (1982) Biochem. Biophys. Res. Commun. 105, 530–538.
43 Liu, M.C. and Peck, H.D., Jr. (1981) J. Biol. Chem. 256, 13159–13164.
44 Liu, M.C., Bakel, B.W., Liu, M.Y. and Dao, T.N. (1988) Arch. Biochem. Biophys. 262, 259–265.
45 Higuchi, Y., Yasuoka, N., Kakudo, M., Katsube, Y., Yagi, T. and Inokuchi, H. (1987) J. Biol. Chem. 262, 2823–2825.
46 Fujita, T. (1966) J. Biochem. 60, 329–334.
47 Liu, M.C., Peck, H.D., Jr., Abou-Jaoude, A., Chippeaux, M. and LeGall, J. (1981) FEMS. Microbiol. Lett. 10, 333–337.
48 Deeb, S.S. and Hager, L.P. (1964) J. Biol. Chem. 239, 1024–1031.
49 Yariv, J. (1983) Biochem. J. 211, 527.
50 Yariv, J., Kalb, A.J., Sperling, R., Bauminger, E.R., Cohen, S.G. and Ofer, S. (1981) Biochem. J. 197, 171–175.
51 Aisen, P. and Listowsky, I. (1980) Annu. Rev. Biochem. 49, 357–393.
52 Theil, E.C. (1987) Annu. Rev. Biochem. 56, 289–315.
53 Tsugita, A. and Yariv, J. (1985) Biochem. J. 231, 209–212.
54 Itagaki, E. and Hager, L.P. (1966) J. Biol. Chem. 241, 3687–3695.
55 Lederer, F., Glatigny, A., Bethge, P.H., Bellamy, H.D. and Mathews, F.S. (1981) J. Mol. Biol. 148, 427–448.
56 Mathews, F.S., Bethge, P.H. and Czerwinski, E.W. (1979) J. Biol. Chem. 254, 1699–1706.
57 Weber, P.C., Salemme, F.R., Mathews, F.S. and Bethge, P.H. (1981) J. Biol. Chem. 256, 7702–7704.
58 Weber, P.C. and Salemme, F.R. (1980) Nature 287, 82–84.
59 Miki, K. and Okunuki, K. (1969) J. Biochem. 66, 831–854.
60 Woolley, K.J. (1987) Arch. Biochem. Biophys. 254, 376–379.
61 Sone, N., Sekimachi, M. and Kutoh, E. (1987) J. Biol. Chem. 262, 15386–15391.
62 Swank, R.T. and Burris, R.H. (1969) Biochim. Biophys. Acta 180, 473–489.
63 Ambler, R.P. (1973) Syst. Zoology, 22, 554–565.
64 Campbell, W.H., Orme-Johnson, W.H. and Burris, R.H. (1973) Biochem. J. 135, 617–630.
65 Ambler, R.P. and Taylor, E. (1973) Biochem. Soc. Trans. 1, 166–168.
66 Carter, D.C., Melis, K.A., O'Donnell, S.E., Burgess, B.K., Furey, W.F., Jr., Wang, B.C. and Stout, C.D. (1985) J. Mol. Biol. 184, 279–295.
67 Goodhew, C.F., Brown, V.R. and Pettigrew, G.W. (1986) Biochim. Biophys. Acta 852, 288–294.
68 Ambler, R.P., Daniel, M., Melis, K. and Stout, C.D. (1984) Biochem. J. 222, 217–227.
69 Ambler, R.P. and Murray, S. (1973) Biochem. Soc. Trans. 1, 162–164.
70 Sawyer, L., Jones, C.L., Damas, A.M., Harding, M.M., Gould, R.O. and Ambler, R.P. (1981) J. Mol. Biol. 153, 831–835.
71 Pettigrew, G.W. and Brown, K.R. (1988) Biochem. J. 252, 427–435.
72 Leitch, F.A., Brown, K.R. and Pettigrew, G.W. (1985) Biochim. Biophys. Acta 808, 213–218.
73 Wood, P.M. and Willey, D.L. (1980) FEMS Microbiol. Lett. 7, 273–277.
74 Matsushita, K., Shinagawa, E., Adachi, O. and Ameyama, M. (1982) J. Biochem. 92, 1607–1613.
75 Hoffman, P.S., Morgan, T.V. and DerVartanian, D.V. (1979) Eur. J. Biochem. 100, 19–27.
76 Hoffman, P.S., Morgan, T.V. and DerVartanian, D.V. (1980) Eur. J. Biochem. 110, 349–354.
77 Yang, T. (1986) Biochim. Biophys. Acta 848, 342–351.
78 Ambler, R.P. and Brown, L.H. (1967) Biochem. J. 104, 784–825.
79 Adman, E.T., Stenkamp, R.E., Sieker, L.C. and Jensen, L.H. (1978) J. Mol. Biol. 123, 35–47.
80 Norris, G.E., Anderson, B.F. and Baker, E.N. (1983) J. Mol. Biol. 165, 501–521.
81 Antonini, E., Finazzi-Agro, A., Avigliano, L., Guerrieri, P., Rotilio, G. and Mondovi, B. (1970) J. Biol. Chem. 245, 4847–4856.
82 Silvestrini, M.C., Brunori, M., Wilson, M.T. and Darley-Usmar, V.M. (1981) J. Inorg. Biochem. 14, 327–338.
83 Tollin, G., Meyer, T.E., Cheddar, G., Getzoff, E.D. and Cusanovich, M.A. (1986) Biochemistry 25, 3363–3370.
84 Coyle, C.L., Zumft, W.G., Kroneck, P.M.H., Korner, H. and Jakob, W. (1985) Eur. J. Biochem. 153, 459–467.
85 Viebrock, A. and Zumft, W.G. (1988) J. Bacteriol. 170, 4658–4668.
86 Ellfolk, N., Ronnberg, M., Aasa, R., Andreasson, L.E. and Vangard, T. (1983) Biochim. Biophys. Acta 743, 23–30.
87 Yamanaka, T. and Imai, S. (1972) Biochem. Biophys. Res. Commun. 46, 150–154.
88 Drummond, M.H. (1985) Biochem. J. 232, 891–896.
89 Chisnell, J.R., Premakumar, R. and Bishop, P.E. (1988) J. Bacteriol. 170, 27–33.
90 Bergstrom, J., Eady, R.R. and Thorneley, R.N.F. (1988) Biochem. J. 251, 165–169.
91 Hales, B.J., Case, E.E., Morningstar, J.E., Dzeda, M.F. and Mauterer, L.A. (1986) Biochemistry 25, 7251–7255.
92 Robson, R.L., Eady, R.R., Richardson, T.H., Miller, R.W., Hawkins, M. and Postgate, J.R. (1986) Nature 322, 388–396.
93 Scherings, G., Haaker, H. and Veeger, C. (1977) Eur. J. Biochem. 77, 621–630.
94 Knight, E., Jr. and Hardy, R.W.F. (1966) J. Biol. Chem. 241, 2752–2756.
95 Roberts, G.P., MacNeil, T., MacNeil, D. and Brill, W.J. (1978) J. Bacteriol. 136, 267–279.
96 Bennett, L.T., Jacobson, M.R. and Dean, D.R. (1988) J. Biol. Chem. 263, 1364–1369.
97 Klugkist, J., Voorberg, J., Haaker, H. and Veeger, C. (1986) Eur. J. Biochem. 155, 33–40.
98 Burnett, R.M., Darling, G.D., Kendall, D.S., LeQuesne, M.E., Mayhew, S.G., Smith, W.W. and Ludwig, M.L. (1974) J. Biol. Chem. 249, 4383–4392.
99 Cheddar, G., Meyer, T.E., Cusanovich, M.A., Stout, C.D. and Tollin, G. (1986) Biochemistry 25, 6502–6507.
100 Morgan, T.V., Lundell, D.J. and Burgess, B.K. (1988) J. Biol. Chem. 263, 1370–1375.
101 Ghosh, D., O'Donnell, S., Furey, W., Jr., Robbins, A.H. and Stout, C.D. (1982) J. Mol. Biol. 158, 73–109.
102 Stout, G.H., Turley, S., Sieker, L.C. and Jensen, L.H. (1988) Proc. Natl. Acad. Sci. USA 85, 1020–1022.
103 Robson, R., Woodley, P. and Jones, R. (1986) EMBO J. 5, 1159–1163.
104 Ebeling, S., Noti, J.D. and Hennecke, H. (1988) J. Bacteriol. 170, 1999–2001.
105 Earl, C.D., Ronson, C.W. and Ausubel, F.M. (1987) J. Bacteriol. 169, 1127–1136.
106 Meyer, J., Bruschi, M.H., Bonicel, J.J. and Bovier-Lapierre, G.E. (1986) Biochemistry 25, 6054–6061.
107 Masuko, M., Iwasaki, H., Sakurai, T., Suzuki, S. and Nakahara, A. (1984) J. Biochem. 96, 447–454.
108 Suzuki, H. and Iwasaki, H. (1962) J. Biochem. 52, 193–199.
109 Shidara, S. (1980) J. Biochem. 87, 1177–1184.
110 Iwasaki, H. and Shidara, S. (1969) J. Biochem. 66, 775–781.
111 Ryden, L. and Lundgren, J.O. (1976) Nature 261, 344–346.
112 Korszun, Z.R. (1987) J. Mol. Biol. 196, 413–419.
113 Ambler, R.P. (1973) Biochem. J. 135, 751–758.
114 Hochman, A., Fridberg, I. and Carmeli, C. (1975) Eur. J. Biochem. 58, 65–72.
115 Prince, R.C., Baccarini-Melandri, A., Hauska, G.A., Melandri,

B.A. and Crofts, A.R. (1975) Biochim. Biophys. Acta 387, 212–227.
116 Daldal, F., Cheng, S., Applebaum, J., Davidson, E. and Prince, R.C. (1986) Proc. Natl. Acad. Sci. USA 83, 2012–2016.
117 Donohue, T.J., McEwan, A.G. and Kaplan, S. (1986) J. Bacteriol. 168, 962–972.
118 Prince, R.C., Davidson, E., Haith, C.E. and Daldal, F. (1986) Biochemistry 25, 5208–5214.
119 Donohue, T.J., McEwan, A.G., Van Doren, S., Crofts, A.R. and Kaplan, S. (1988) Biochemistry 27, 1918–1925.
120 Allen, J.P., Feher, G., Yeates, T.O., Komiya, H. and Rees, D.C. (1987) Proc. Natl. Acad. Sci. USA 84, 5730–5734 and 6162–6166.
121 Youvan, D.C., Bylina, E.J., Alberti, M., Begusch, H. and Hearst, J.E. (1984) Cell 37, 949–957.
122 Williams, J.C., Steiner, L.A., Feher, G. and Simon, M.I. (1984) Proc. Natl. Acad. Sci. USA 81, 7303–7307.
123 Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 618–624.
124 Daldal, F., Davidson, E. and Cheng, S. (1987) J. Mol. Biol. 195, 1–12.
125 Davidson, E. and Daldal, F. (1987) J. Mol. Biol. 195, 13–24.
126 Davidson, E. and Daldal, F. (1987) J. Mol. Biol. 195, 25–29.
127 Iba, K., Morohashi, K., Miyata, T. and Takamiya, K. (1987) J. Biochem. 102, 1511–1518.
128 Prince, R.C. and Daldal, F. (1987) Biochim. Biophys. Acta 894, 370–378.
129 Marrs, B. and Gest, H. (1973) J. Bacteriol. 114, 1045–1051.
130 LaMonica, R.F. and Marrs, B.L. (1976) Biochim. Biophys. Acta 423, 431–439.
131 Zannoni, D., Melandri, B.A. and Baccarini-Melandri, A. (1976) Biochim. Biophys. Acta 423, 413–430.
132 Hudig, H. and Drews, G. (1982) FEBS Lett. 146, 389–392.
133 Hudig, H. and Drews, G. (1983) FEBS Lett. 152, 251–255.
134 Daldal, F. (1988) J. Bacteriol. 170, 2388–2391.
135 Gennis, R.B., Casey, R.P., Azzi, A. and Ludwig, B. (1982) Eur. J. Biochem. 125, 189–195.
136 Meyer, T.E., Cheddar, G., Bartsch, R.G., Getzoff, E.D., Cusanovich, M.A. and Tollin, G. (1986) Biochemistry 25, 1383–1390.
137 Meyer, T.E. and Cusanovich, M.A. (1985) Biochim. Biophys. Acta 807, 308–319.
138 Hallenbeck, P.C., Jovanneau, Y. and Vignais, P.M. (1982) Biochim. Biophys. Acta 681, 168–176.
139 Michalski, W.P., Hein, D.H. and Nicholas, D.J.D. (1986) Biochim. Biophys. Acta 872, 50–60.
140 McEwan, A.G., Greenfield, A.J., Wetzstein, H.G., Jackson, J.B. and Ferguson, S.J. (1985) J. Bacteriol. 164, 823–830.
141 McEwan, A.G., Wetzstein, H.G., Meyer, O., Jackson, J.B. and Ferguson, S.J. (1987) Arch. Microbiol. 147, 340–345.
142 Satoh, T. (1981) Plant Cell Physiol. 22, 443–452.
143 Yokota, S., Urata, K. and Satoh, T. (1984) J. Biochem. 95, 1535–1541.
144 Satoh, T. and Kurihara, F.N. (1987) J. Biochem. 102, 191–197.
145 Ward, J.A., Hunter, C.N. and Jones, O.T.G. (1983) Biochem. J. 212, 783–798.
146 Zsebo, K.M. and Hearst, J.E. (1984) Cell 37, 937–947.
147 Michalski, W.P., Miller, D.J. and Nicholas, D.J.D. (1986) Biochim. Biophys. Acta 849, 304–315.
148 Sawada, E., Satoh, T. and Kitamura, H. (1978) Plant Cell Physiol. 19, 1339–1351.
149 Michalski, W.P. and Nicholas, D.J.D. (1985) Biochim. Biophys. Acta 828, 130–137.
150 Ambler, R.P., Meyer, T.E. and Kamen, M.D. (1976) Proc. Natl. Acad. Sci. USA. 73, 472–475.
151 Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–158.
152 Weyer, K.A., Lottspeich, F., Gruenberg, H., Lang, F., Oesterhelt, D. and Michel, H. (1987) EMBO J. 6, 2197–2202.
153 Alegria, G. and Dutton, P.L. (1988) Biophys. J. 53, 615a.
154 Dracheva, S.M., Drachev, L.A., Konstantinov, A.A., Semenov, A.Y., Skulachev, V.P., Arutjunjan, A.M., Shuvalov, V.A. and Zaberezhnaya, S.M. (1988) Eur. J. Biochem. 171, 253–264.
155 Shopes, R.J., Levine, L.M.A., Holten, D. and Wraight, C.A. (1987) Photosynth. Res. 12, 165–180.
156 Shill, D.A. and Wood, P.M. (1984) Biochim. Biophys. Acta 764, 1–7.
157 Kennel, S.J. and Kamen, M.D. (1971) Biochim. Biophys. Acta 253, 153–166.
158 Kennel, S.J. and Kamen, M.D. (1971) Biochim. Biophys. Acta 234, 458–467.
159 Bartsch, R.G., Meyer, T.E. and Robinson, A.B. (1968) in Structure and Function of Cytochromes (Okunuki, Y., Kamen, M.D. and Sekuzu, I., eds.), pp. 443–451, University Park Press, Baltimore.
160 Fukumori, Y. and Yamanaka, T. (1979) J. Biochem. 85, 1405–1414.
161 Meyer, T.E. and Bartsch, R.G. (1976) in Flavins and Flavoproteins (Singer, T.P., ed.), pp. 312–317, Elsevier, New York.
162 Cusanovich, M.A. and Meyer, T.E. (1982) in Flavins and Flavoproteins (Massey, V. and Williams, C.H., Jr., eds.), pp. 844–848, Elsevier, Amsterdam.
163 Cusanovich, M.A., Meyer, T.E. and Tollin, G. (1985) Biochemistry 24, 1281–1287.
164 Meyer, T.E., Vorkink, W.P., Tollin, G. and Cusanovich, M.A. (1985) Arch. Biochem. Biophys. 236, 52–58.
165 Doyle, M.L., Gill, S.J. and Cusanovich, M.A. (1986) Biochemistry 25, 2509–2516.
166 Finzel, B.C., Weber, P.C., Hardman, K.D. and Salemme, F.R. (1985) J. Mol. Biol. 186, 627–643.
167 Ambler, R.P., Bartsch, R.G., Daniel, M., Kamen, M.D., McLellan, L., Meyer, T.E. and Van Beeumen, J. (1981) Proc. Natl. Acad. Sci. USA 78, 6854–6857.
168 Carter, C.W., Jr., Kraut, J., Freer, S.T., Xuong, N.H., Alden, R.A. and Bartsch, R.G. (1974) J. Biol. Chem. 249, 4212–4225.
169 Przysiecki, C.T., Cheddar, G., Meyer, T.E., Tollin, G. and Cusanovich, M.A. (1985) Biochemistry 24, 5647–5652.
170 Cusanovich, M.A. and Bartsch, R.G. (1969) Biochim. Biophys. Acta 189, 245–255.
171 Van Grondelle, R., Duysens, L.N.M., Van Der Wel, J.A. and Van De Wal, H.N. (1977) Biochim. Biophys. Acta 461, 188–201.
172 Knaff, D.B., Whetstone, R. and Carr, J.W. (1980) Biochim. Biophys. Acta 590, 50–58.
173 Tomiyama, Y., Doi, M., Takamiya, K. and Mishimura, M. (1983) Plant Cell Physiol. 24, 11–16.
174 Gray, G.O., Gaul, D.F. and Knaff, D.B. (1983) Arch. Biochem. Biophys. 222, 78–86.
175 Cusanovich, M.A., Bartsch, R.G. and Kamen, M.D. (1968) Biochim. Biophys. Acta 153, 397–417.
176 Gaul, D.F., Gray, C.O. and Knaff, D.B. (1983) Biochim. Biophys. Acta 723, 333–339.
177 Gaul, D.F. and Knaff, D.B. (1983) FEBS Lett. 162, 69–75.
178 Wynn, R.M., Gaul, D.F., Shaw, R.W. and Knaff, D.B. (1985) Arch. Biochem. Biophys. 238, 373–377.
179 Kampf, C., Wynn, R.M., Shaw, R.W. and Knaff, D.B. (1987) Biochim. Biophys. Acta 894, 228–238.
180 Hase, T., Matsubara, H. and Evans, M.C.W. (1977) J. Biochem. 81, 1745–1749.
181 Gitlitz, P.H. and Krasna, A.I. (1975) Biochemistry 14, 2561–2568.
182 Albracht, S.P.J., Kalkman, M.L. and Slater, E.C. (1983) Biochim. Biophys. Acta 724, 309–316.
183 Odom, J.M. and Peck, H.D., Jr. (1984) Annu. Rev. Microbiol. 38, 551–592.
184 Pierrot, M., Haser, R., Frey, M., Payan, F. and Astier, J.P. (1982) J. Biol. Chem. 257, 14341–14348.

185 Higuchi, Y., Kusunoki, M., Matsuura, Y., Yasuoka, N. and Kakudo, M. (1984) J. Mol. Biol. 172, 109–139.
186 DerVartanian, D.V., Xavier, A.V. and LeGall, J. (1978) Biochimie 60, 321–325.
187 Bruschi, M., Loutfi, M., Bianco, P. and Haladjian, J. (1984) Biochem. Biophys. Res. Commun. 120, 384–389.
188 Santos, H., Moura, J.J.G., Moura, I., LeGall, J. and Xavier, A.V. (1984) Eur. J. Biochem. 141, 283–296.
189 Dolla, A., Cambillan, C., Bianco, P., Haladjian, J. and Bruschi, M. (1987) Biochem. Biophys. Res. Commun. 147, 818–823.
190 Favaudon, V., Ferradini, C., Pucheault, J., Gilles, L. and LeGall, J. (1978) Biochem. Biophys. Res. Commun. 84, 435–440.
191 Haladjian, J., Bianco, P., Guerlesquin, F. and Bruschi, M. (1987) Biochem. Biophys. Res. Commun. 147, 1289–1294.
192 Bell, G.R., LeGall, J. and Peck, H.D., Jr. (1974) J. Bacteriol. 120, 994–997.
193 Odom, J.M. and Peck, H.D., Jr. (1981) FEMS Microbiol. Lett. 12, 47–50.
194 Odom, J.M. and Peck, H.D., Jr. (1981) J. Bacteriol. 147, 161–169.
195 Badziong, W. and Thauer, R.K. (1980) Arch. Microbiol. 125, 167–174.
196 Voordouw, G. and Brenner, S. (1986) Eur. J. Biochem. 159, 347–351.
197 Ambler, R.P. (1971) FEBS Lett. 18, 351–353.
198 Yagi, T. (1979) Biochim. Biophys. Acta 548, 96–105.
199 Fauque, G., Bruschi, M. and LeGall, J. (1979) Biochem. Biophys. Res. Commun. 86, 1020–1029.
200 Nakano, K., Kikumoto, Y. and Yagi, T. (1983) J. Biol. Chem. 258, 12409–12412.
201 Bruschi, M., LeGall, J. and Bovier-Lapierre, G. (1972) Biochim. Biophys. Acta 271, 48–60.
202 Bertrand, P., Bruschi, M., Denis, M., Gayda, J.P. and Mauca, F. (1982) Biochem. Biophys. Res. Commun. 106, 756–760.
203 Lee, J.P., LeGall, J. and Peck, H.D., Jr. (1973) J. Bacteriol. 115, 529–542.
204 Murphy, M.J., Siegel, L.M., Kamin, H., DerVartanian, D.V., Lee, J.P., LeGall, J. and Peck, H.D., Jr. (1973) Biochem. Biophys. Res. Commun. 54, 82–88.
205 Krueger, R.J. and Siegel, L.M. (1982) Biochemistry 21, 2892–2904.
206 Krueger, R.J. and Siegel, L.M. (1982) Biochemistry 21, 2905–2909.
207 McRee, D.E., Richardson, D.C., Richardson, J.S. and Siegel, L.M. (1986) J. Biol. Chem. 261, 10277–10281.
208 Bramlett, R.N. and Peck, H.D., Jr. (1975) J. Biol. Chem. 250, 2979–2986.
209 Bovier-Lapierre, G., Bruschi, M., Bonicel, J. and Hatchikian, E.C. (1987) Biochim. Biophys. Acta 913, 20–26.
210 Bruschi, M., Bovier-Lapierre, G., Bonicel, J. and Couchoud, P. (1979) Biochem. Biophys. Res. Commun. 91, 623–628.
211 Ogata, M., Kondo, S., Okawara, N. and Yagi, T. (1988) J. Biochem. 103, 121–125.
212 Okawara, N., Ogata, M., Yagi, T., Wakabayashi, S. and Matsubara, H. (1988) J. Biochem. 104, 96–199.
213 Dubourdiu, M. and LeGall, J. (1970) Biochem. Biophys. Res. Commun. 38, 965–972.
214 Dubourdieu, M. and Fox, J.L. (1977) J. Biol. Chem. 252, 1453–1463.
215 Krey, G.D., Vanin, E.F. and Swenson, R.P. (1987) in Flavins and Flavoproteins (Edmondson, D.E. and McCormick, D.B., eds.), pp. 315–318, Walter De Gruyter, New York.
216 Curley, G.P. and Voordouw, G. (1988) FEMS Microbiol. Lett. 49, 295–299.
217 Watenpaugh, K.D., Sieker, L.C., Jensen, L.H., LeGall, J. and Dubourdieu, M. (1972) Proc. Natl. Acad. Sci. USA 69, 3185–3188.
218 Ludwig, M.L., Pattridge, K.A., Smith, W.W., Jensen, L.H. and Watenpaugh, K.D. (1982) in Flavins and Flavoproteins (Massey, V. and Williams, C.H., Jr., eds.), pp. 19–27, Elsevier, Amsterdam.
219 Vogel, H., Bruschi, M. and LeGall, J. (1977) J. Mol. Evol. 9, 111–119.
220 Hormel, S., Walsh, K.A., Prickril, B.C., Titani, K., LeGall, J. and Sieker, L.C. (1986) FEBS Lett. 201, 147–150.
221 Voordouw, G. (1988) Gene 69, 75–83.
222 Moura, I., Bruschi, M., LeGall, J., Moura, J.J.G. and Xavier, A.V. (1977) Biochem. Biophys. Res. Commun. 75, 1037–1044.
223 LeGall, J., Prickril, B.C., Moura, I., Xavier, A.V., Moura, J.J.G. and Huynh, B.H. (1988) Biochemistry 27, 1636–1642.
224 Adman, E.T., Sieker, L.C., Jensen, L.H., Bruschi, M. and LeGall, J. (1977) J. Mol. Biol. 112, 113–120.
225 Sieker, L.C., Stenkamp, R.E., Jensen, L.H., Prickrill, B. and LeGall, J. (1986) FEBS Lett. 208, 73–76.
226 Frey, M., Sieker, L.C., Payan, F., Haser, R., Bruschi, M., Pepe, G. and LeGall, J. (1987) J. Mol. Biol. 197, 525–541.
227 Petitdemange, H., Marczak, R., Blusson, H. and Gay, R. (1979) Biochem. Biophys. Res. Commun. 91, 1258–1265.
228 Prickril, B.C., He, S.H., Li, C., Menon, N., Choi, E.S., Przybyla, A.E., DerVartanian, D.V., Peck, H.D., Jr., Fauque, G., LeGall, J., Teixeira, M., Moura, I., Moura, J.J.G., Patil, D. and Huynh, B.H. (1987) Biochem. Biophys. Res. Commun. 149, 369–377.
229 Voordouw, G. and Brenner, S. (1985) Eur. J. Biochem. 148, 515–520.
230 Menon, N.K., Peck, H.D., Jr., LeGall, J. and Przybyla, A.E. (1987) J. Bacteriol. 169, 5401–5407.
231 Menon, N.K., Peck, H.D., Jr., LeGall, J. and Przybyla, A.E. (1988) J. Bacteriol. 170, 4429.
232 Yagi, T. (1976) J. Biochem. 68, 649–657.
233 Yoch, D.C. and Carithers R.P. (1979) Microbiol. Rev. 43, 383–421.
234 Graves, M.C., Mullenbach, G.T. and Rabinowitz, J.C. (1985) Proc. Natl. Acad. Sci. USA 82, 1653–1657.
235 Prince, R.C. and Adams, M.W.W. (1987) J. Biol. Chem. 262, 5125–5128.
236 Lovenberg, W. and Sobel, B.E. (1965) Proc. Natl. Acad. Sci. USA 54, 193–199.
237 Seki, S., Ikeda, A. and Ishimoto, M. (1988) J. Biochem. 103, 583–584.
238 Ragsdale, S.W., Ljungdahl, L.G. and DerVartanian, D.V. (1983) J. Bacteriol. 155, 1224–1227.
239 Watenpaugh, K.D., Sieker, L.C. and Jensen, L.H. (1979) J. Mol. Biol. 131, 509–522.
240 Mayhew, S.G. and Ludwig, M.L. (1975) The Enzymes 12, 57–118.
241 Wang, S.Z., Chen, J.S. and Johnson, J.L. (1988) Nucleic Acids Res. 16, 439–454.
242 Walker, M. and Mortenson, L.E. (1973) Biochem. Biophys. Res. Commun. 54, 669–676.
243 Zumft, W.G., Mortenson, L.E. and Palmer, G. (1974) Eur. J. Biochem. 46, 525–535.
244 Cardenas, J., Mortenson, L.E. and Yoch, D.C. (1976) Biochim. Biophys. Acta 434, 244–257.
245 Adams, M.W.W. and Mortenson, L.E. (1984) Biochim. Biophys. Acta 766, 51–61.
246 Adams, M.W.W. (1987) J. Biol. Chem. 262, 15054–15061.