Biochimica et Biophysica Acta, 449 (1976) 275–284

BBA 47190

PURIFICATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF THREE FORMS OF FERREDOXIN FROM THE SULFATE-REDUCING BACTERIUM *DESULFOVIBRIO GIGAS*

MIREILLE BRUSCHI[a], E. CLAUDE HATCHIKIAN[a], JEAN LE GALL[a] and JOSÉ J. G. MOURA[b] and ANTÓNIO V. XAVIER[b]

[a]*Laboratoire de Chimie Bactérienne, CNRS, 13274 Marseille-Cédex 2 (France) and* [b]*Centro de Química Estrutural da Universidade de Lisboa I.S.T., Lisbon 1 (Portugal)*

(Received May 4th, 1976)

SUMMARY

Three forms of ferredoxin FdI, FdI', and FdII have been isolated from *Desulfovibrio gigas*, a sulfate reducer. They are separated by a combination of DEAE-cellulose and gel filtration chromatographic procedures. FdI and FdI' present a slight difference in isoelectric point which enables the separation of the two forms over DEAE-cellulose, while FdII is easily separated from the two other forms by gel filtration. The three forms have the same amino acid composition and are isolated in different aggregation states. Molecular weight determinations by gel filtration gave values of 18 000 for FdI and FdI' and 24 000 for FdII, whereas a value of 6000 is determined when dissociation is accomplished with sodium dodecyl sulfate. The electronic spectra are different and their ultraviolet-visible absorbance rations are 0.77, 0.87 and 0.68 respectively for FdI, FdI' and FdII. Despite these differences, the physiological activities of the three forms are similar as far as the reduction of sulfite by molecular hydrogen is concerned.

INTRODUCTION

Isolation, preliminary chemical and physical properties and amino acid sequence of a four-iron-four-sulfur ferredoxin from *Desulfovibrio gigas* have been previously reported [1, 2].

Other four-iron ferredoxins have been isolated and characterized in several organisms [3]: *Bacillus polymyxa* [4] *Spirochaeta aurantia* [5], *Bacillus stearothermophilus* [6] and *Desulfovibrio desulfuricans* [7]. *D. gigas* ferredoxin differs from these other four-iron ferredoxins in its cysteine (six residues) and methionine (two residues) content. Another class of four-iron-four-sulphur proteins includes those with positive redox potentials known as high potential iron proteins. They were isolated from *Chromatium* spp. [8], *Rhodopseudomonas gelatinosa* [9] and *Thiocapsa pfenngii* [10].

The presence of two types of ferredoxin has been reported in some organisms:

Bacillus polymyxa [11, 12], *Rhodospirillum rubrum* [13] and *Azotobacter vinelandii* [14]. These ferredoxins are, in all cases, two proteins with different amino acid composition.

In the present article, we report the isolation of three different forms of four iron-four-sulphur ferredoxin from *D. gigas* FdI, FdI′ and FdII with identical apoproteins and physiological activities but differing in their aggregation states, spectra-properties and thermal stabilities.

EXPERIMENTAL PROCEDURE

Isolation of the three forms of ferredoxin from D. gigas

The isolation of ferredoxins is largely based on the acidic characteristics of these proteins. A frozen paste (4 kg) of cells grown as previously described [15] was treated. All operations were carried at 0–4 °C unless otherwise stated. Tris · HCl and phosphate buffers pH 7.6 of appropriate molarity were used.

Step I. The acidic proteins extract, obtained as described elsewere [15], was absorbed on a large DEAE-cellulose column (50×300 mm). The proteins were eluted with a discontinuous gradient 0.1–0.5 M Tris · HCl buffer. This gradient enables the separation of a band mainly constituted by ferredoxin (eluted with 0.4 M Tris · HCl) from other components of the acidic extract (flavodoxin [16], cytochrome c_3, mol wt. 26 000 [17] and rubredoxin [1]. Fractions containing ferredoxin, recognized by their characteristic dark brown colour, were localized on the top of the column. A rough subdivision of this band is observed during the elution with 0.4 M Tris · HCl buffer. The fraction containing ferredoxin was diluted 3-fold with distilled water and readsorbed on a second DEAE-cellulose column (40×200 mm). Ferredoxin was eluted from this column with 0.4 M Tris · HCl as described above in a volume of 110 ml. The fractions containing ferredoxin presented a very high A_{280}/A_{405} ratio (approx. 60).

Step II. The protein solution was brought to 70 % saturation with ammonium sulfate and the precipitate was removed by centrifugation and discarded

A ratio of $A_{280}/A_{405} \simeq 7.0$ (purity index) was obtained. The ferredoxin fraction was then dialyzed against 20 l of 0.01 M Tris · HCl buffer, concentrated on a small DEAE-cellulose column (20×60 mm) eluted with 0.6 M Tris · HCl giving a volume of 35 ml.

Step III. The concentrated ferredoxin fraction was passed over a Sephadex G-75 column (50×1000 mm) equilibrated with 0.01 M Tris · HCl buffer. Two main fractions were obtained the first containing mainly FdII and the second a mixture of FdI and FdI′. The purity indices were then approx. 4.5.

Step IV. The separation of FdI and FdI′ was achieved using a DEAE-Sephadex A-50 column (40×200 mm) equilibrated with 0.45 M Tris · HCl. The fraction containing FdI and FdI′ was brought to approximately the same Tris · HCl molarity and applied to the column. Elution was performed using exclusively 0.5 M Tris · HCl. The ferredoxin separated into four bands. The first contained FdI′ and was collected in a 150 ml fraction. The second, and the strongest, contained FdI in a 250 ml volume. The third and fourth band were very minor and were not further studied. The yield was 55 mg of FdI and 21 mg of FdI′.

Step V. The same procedure was used for the fraction containing FdII. The

main band (collected in 210 ml) comprised mostly FdII, whereas FdI was present in a small amount and migrated in front of FdII. A final purification was obtained by adsorbing FdII on a calcinated alumina column (20×100 mm) and eleuted with 0.01 M potassium phosphate buffer.

The yield ratios of FdI, FdII', and FdII vary from one preparation to another.

Analytical procedures

The molecular weight was estimated by molecular filtration on a Sephadex column [18] and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [19]. Non-heme (ferrous) iron was determined by the *o*-phenanthroline method [20, 21]. Protein samples were hydrolyzed according to the method of Moore and Stein [22]. 22 h of hydrolysis in 6 M HCl at 105–110 °C were employed. Amino acid analyses were carried out on a Beckman Multichrom amino acid analyzer. The values for threonine, serine or tyrosine were corrected for decomposition during hydrolysis. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone respectively [23].

Spectral measurements were performed on a Cary 14 spectrophotometer with temperature-controlled cells.

Enzyme assay

A manometric assay was utilized to determine the biological activity of the three forms of ferredoxin from *D. gigas* utilizing the reduction of sulfite by hydrogen. The main compartment of each manometric vessel contained 150 μmol phosphate buffer (pH 7.0), the carrier, pure hydrogenase and the reductase preparation in a final volume of 3.0 ml. 4 μmol of freshly prepared sodium sulfite was added from a side arm after incubation of the flask for 30 min under hydrogen at 37 °C. The center well contained 0.1 ml 10 M NaOH. Enzyme activity was calculated from the initial rate of hydrogen utilization. The sulfite reductase-containing extract, devoid of electron carriers, was prepared from *D. gigas* by ammonium sulfate fractionationas already reported [24]. Pure hydrogenase prepared from *D. gigas* by the method of Bell [25] and exhibiting a specific activity of 30 μmol hydrogen consumed/min per mg protein was added in all cases to the system to insure an excess of this activity.

RESULTS

Chemical composition and molecular weight determination

The molecular weight was determined by molecular filtration on a G-50 Sephadex column (870×20 mm) equilibrated with 0.05 M Tris · HCl buffer (pH 7.6). The molecular weights obtained for FdII were 18 000 for FdI and FdI', and 24 000 for FdII. When the tree forms are previously incubated with 0.1 % sodium dodecyl sulfate an identical mobility in 0.1 % dodecyl sulfate polyacrylamide gel electrophoresis is found. These mobilities are identical to that of *Desulfovibrio desulfuricans* (Norway 4) ferredoxin which is isolated as a monomeric unit and has a molecular weight of 6400 as determined by gel filtration (Bruschi, M., Hatchikian, C. E., Golovleva, L. A. and Le Gall, J., unpublished).

As reported in Table I, the amino acid compositions of the three forms are identical. After iron and labile sulfur determination they were shown to contain four iron and four sulfur per minimal molecular weight of 6456.

TABLE I

AMINO ACID COMPOSITION OF THE THREE FORMS OF *D. GIGAS* FERREDOXIN

	Ferredoxin I	Ferredoxin I′	Ferredoxin II	Ferredoxin *D. gigas*[2]
Lysine	1	1	1	1
Histidine	0	0	0	0
Arginine	1	1	1	1
Tryptophan	n.d.	n.d.	n.d.	0
Aspartic acid	11	11	11	11
Threonine	0	0	0	0
Serine	3	3	3	3
Glutamic acid	9	9	9	9
Proline	4	4	4	4
Glycine	1	1	1	1
Alanine	6	6	6	6
Cystine (half)[a]	6	6	6	6
Valine	5	5	5	5
Methionine[a]	2	2	2	2
Isoleucine	5	5	5	5
Leucine	1	1	1	1
Tyrosine	0	0	0	0
Phenylalanine	1	1	1	1
Total	56	56	56	56
Molecular weight	6456[b] 18000[c]	6456[b] 18000[c]	6456[b] 24000[c]	6456[b] 6000[c]
Labile sulfur[d]	4	4	4	4
Iron[d]	4	4	4	4

[a] Calculated after performic acid oxidation.
[b] Minimum molecular weight.
[c] Molecular weight estimated by gel filtration.
[d] Per 6000 molecular weight.
n.d., not determined.

Electronic spectra data

Fig. 1 shows the spectra of oxidized and reduced FdI, FdI′ and FdII respectively. The spectra are typical of four-iron four-sulfur proteins. Upon reduction with dithionite the absorbances in the visible region are greatly decreased. Table II shows the absorption bands and molar extinction coefficients. These coefficients were calculated using protein concentrations determined by amino acid analysis and were based on the minimal molecular weight of 6456. Because glutamic and aspartic resitues are stable they were used as a basis for calculations.

The weak absorption in the ultraviolet aromatic region is in agreement with the low content in aromatic residues as determined by the amino acid analysis and makes it a good purity control index.

It should be noticed that absorption bands of the oxidized form of FdII and FdI′ are shifted to higher wavelengths when compared to those of FdI. Also important is the difference in the A_{405}/A_{300} ratio for FdI and the A_{415}/A_{305} ratios for FdII and FdI′. In the visible region a flat shoulder is observed for FdI in contrast with a

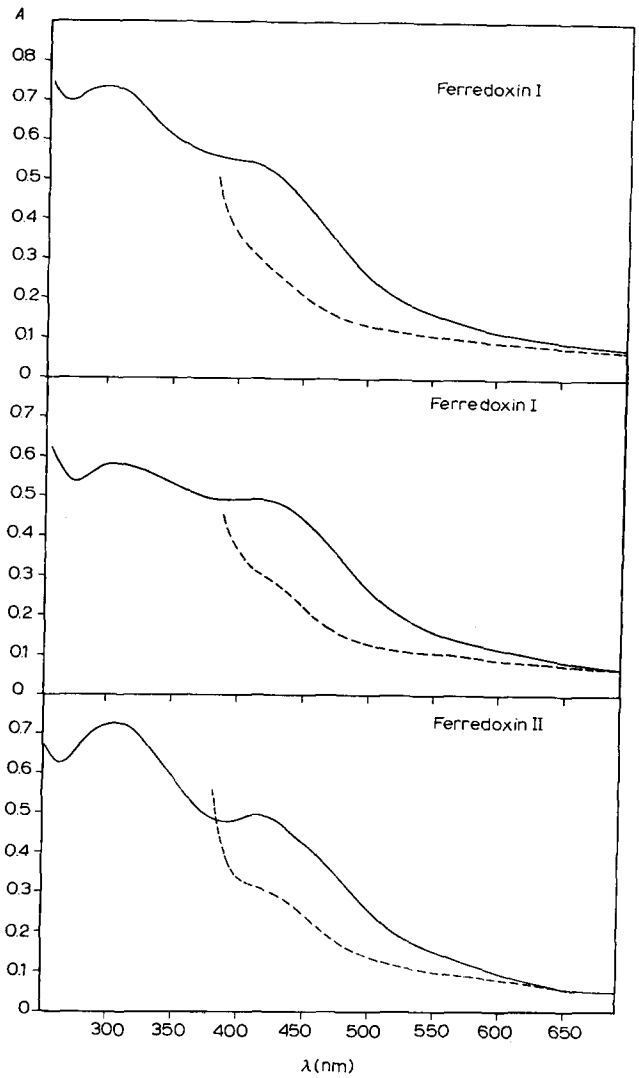

Fig. 1. Ultraviolet and visible absorption spectra of *D. gigas* FdI, FdI′ and FdII (pH 7.6). ———, oxidized protein; ----, dithionite-reduced protein.

well-defined peak for FdII and FdI′. In the reduced state FdII and FdI′ show a well-defined band at 425 nm indicating that in the last case there is still a considerable contribution from the chromophore whereas the spectrum of reduced FdI does not show such a large contribution. An attempt to reduce the proteins with ascorbic acid was unsuccessful since no alteration of the spectra was observed indicating that they do not have positive redox potentials. This was recently confirmed by EPR redox titrations (J. J. G. Moura, A. V. Xavier, R. Cammack, M. Bruschi, J. Le Gall, and J. P. Gayda, unpublished results).

TABLE II

MOLAR EXTINCTION COEFFICIENTS OF THE THREE FORMS OF FERREDOXIN FROM *D. GIGAS*

Ferredoxin	λ (nm)	ε ($M^{-1} \cdot cm^{-1}$)	Ratio
I (oxidized)	300	20,600	$A_{405}/A_{300} = 0.77$
	405	16,000	
I (reduced)[a]	420	9,150	
I′ (oxidized)	305	19,400	$A_{415}/A_{305} = 0.87$
	415	16,900	
I′ (reduced)[a]	425	10,050	
II (oxidized)	305	23,100	$A_{415}/A_{305} = 0.68$
	415	15,700	
	453[b]	13,300	
II (reduced)[a]	425	9,600	

[a] Values corrected for dithionite absorption.
[b] Shoulder.

Thermal stability

Absorbance changes were observed by increasing the sample temperature. From the results indicated in Fig. 2, instability temperatures of 55, 61 and 73 °C respectively for FdI′, FdI and FdII were obtained. No appreciable spectro-

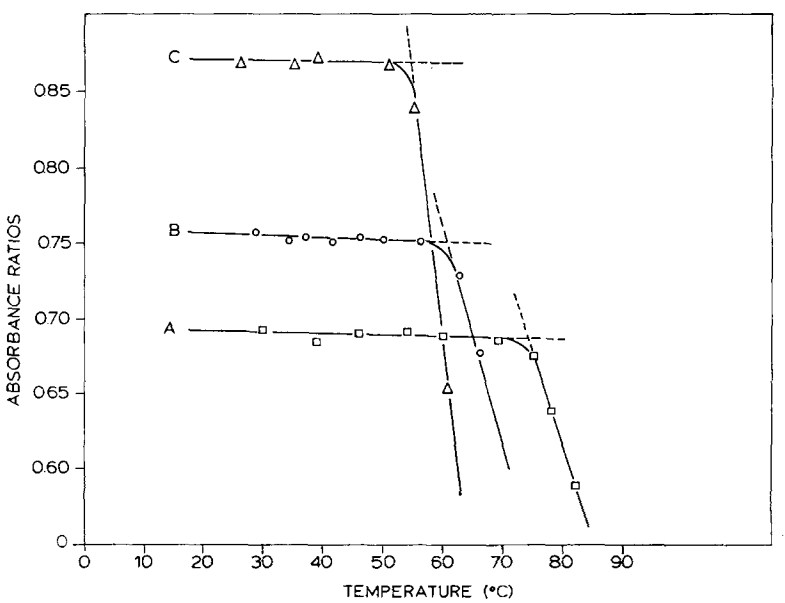

Fig. 2. Thermal stability of the three forms of *D. gigas* ferredoxin. Absorbances were observed with increasing sample temperature. A, Ferredoxin I, A_{405}/A_{300}; B, Ferredoxin I′, A_{415}/A_{305}; C, Ferredoxin II, A_{415}/A_{305}.

photometric changes are observed until the instability temperature is reached. The bleaching of the chromophore is irreversible, but the protein is stable for several hours at intermediate temperatures.

Physiological activity

The results of the coupling activity of the three forms of ferredoxin between hydrogenase and sulfite reduction are reported on Table III. As compared with the endogenous activity of the control containing reductase preparation and hydrogenase without electron carrier the systems containing FdI, FdI′ and FdII exhibited a significant stimulation of sulfite reduction. It may be seen from these data that all

TABLE III

REDUCTION OF SULFITE BY HYDROGEN IN THE PRESENCE OF FERREDOXIN I, I′ AND II WITH *D. GIGAS* ENZYMATIC EXTRACT

Sulfite reductase activity was determined as reported in Experimental Procedure. The control without added electron carrier contained: reductase preparation (17.2 mg protein) and pure hydrogenase (400 μg protein). 100 nmol of each of the electron carriers (methyl viologen, FdI, FdI′ and FdII) were added to the enzymatic preparation.

	μmol H_2 consumed	
	After 15 min	After 30 min
Control (without added carrier)	0.20	0.42
+methyl viologen	5.60	—
+ferredoxin I	1.42	2.94
+ferredoxin I′	1.21	2.72
+ferredoxin II	1.43	3.30

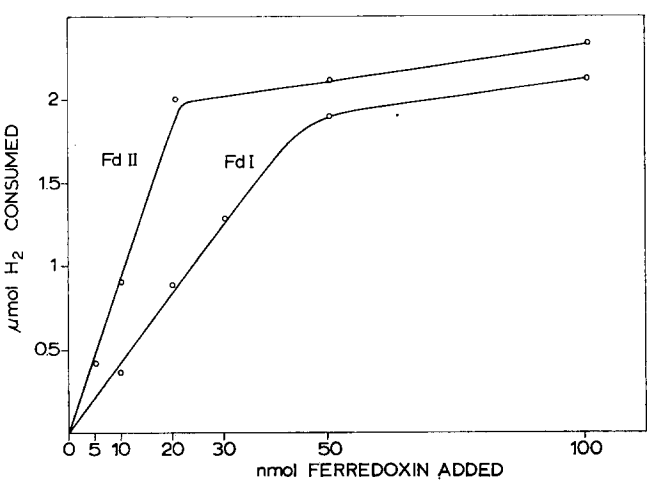

Fig. 3. Effectiveness of *D. gigas* ferredoxin I and II in the sulfite reduction reaction. Sulfite reductase activity was determined as reported in Experimental Procedure. The reaction mixture contained: reductase preparation (17.2 mg protein) and pure hydrogenase (400 μg protein). The values of hydrogen uptake indicated in the figure for each concentration of ferredoxin represent the activity after 24 min of reaction and after subtraction of the endogenous activity.

the ferredoxins function as carriers in the reduction of sulfite with hydrogen in the presence of *D. gigas* reductase preparation and that the slight differences of activity between the three forms of ferredoxin found at the saturation level is not significant (see Table III). However for this activity FdII is more effective than FdI at an electron carrier concentration lower than the saturation concentration (Fig. 3).

DISCUSSION

The reported results show that three forms of ferredoxin can be isolated from *D. gigas*. Although the presence of two ferredoxins have been already reported [11–14] in other organisms, this is the first time that different forms of ferredoxins derived from the same polypeptide chain are described.

Important differences are observed between the spectra of the three forms of *D. gigas* ferredoxin. The ultraviolet visible ratios (see Table II) present marked differences (0.77, 0.87 and 0.68 for FdI, FdI′ and FdII, respectively) showing that the chromophore contribution to the visible region is not the same for the three forms. Thus it may be expected that they have their clusters in different oxidation states.

A spectral characteristic of *D. gigas* ferredoxin lies in the fact that the peak centered around 300 nm is perfectly symmetrical with no shoulder near 280 nm. This result is only obtained in the last purification step and less refined methods lead to ferredoxin with such a shoulder or even a peak at 280 nm. The refinement of the preparation of *D. gigas* ferredoxin is only made possible because of its excellent stability in aerobic conditions. Nevertheless, the question may be posed whether in previous examples, in particular in the cases of *B. polymyxa* [11, 12] and *A. vinelandii* [14] the two ferredoxins reported are different proteins. This question is sustained by the large discrepancy in the reported amino acid compositions of *B. polymyxa* ferredoxins.

As it can be seen from Table I, the amino acid compositions of the three forms of *D. gigas* ferredoxin are identical. After iron and labile sulfur determination they were shown to contain four iron and four sulfur per minimal molecular weight of 6456. Although the three forms of ferredoxin are present in different aggregation states, treatment with sodium dodecyl sulfate induces a depolymerization into monomeric units of molecular weight of approx. 6400, a value similar to the minimal molecular weight obtained from sequence data.

One of the characteristics of *D. gigas* ferredoxin is the presence of two residues of cysteine which are not involved in the formation of the 4Fe-4S cluster. These two residues can be present as free -SH or can participate as intra- or intermolecular disulfide bridges. In this last case they would be responsible for the maintenance of the polymerisation of the molecule. Some experiments using *p*-chloromercuribenzoate titrations have shown that the cysteine residues of the different forms are not all accessible, even in the presence of denaturating conditions (8 M urea, 4 M guanidine). This difficulty of access is particularly evident for FdII implying that the degrree of polymerisation contributes to an additional protection of the cysteine residues. Attempts are being made to localize the cysteine residues that participate in intrachain disulfide bridge, by purification and identification of the peptides obtained by tryptic cleavage of the only lysine (number 30) of the molecule [2].

The aggregation states in which the three forms were isolated may explain

the distinct temperatures of thermal denaturation and the slight difference in the apparent isoelectric point (due to different exposition of charged residues) which allows a rough separation of the three forms in DEAE-cellulose. Due to lack of available data on the aggregation states of iron-sulfur proteins, it is not possible to distinguish whether the factor responsible for the differences in the environment of the cluster is due to tertiary or quaternary type interactions. The influence of these interactions in the magnetic properties of the three forms will be discussed in following papers which will deal with NMR (J. J. G. Moura, A. V. Xavier, M. Bruschi and J. Le Gall, unpublished results) and EPR R. Cammack, J. J. G. Moura, A. V. Xavier, M. Bruschi, J. Le Gall, and J. P. Gayda, unpublished results).

A dimerization of *Clostridium pasteurianum* ferredoxin has been reported [26], but in this example the aggregation is due to the loss of one of the two clusters, which cannot be the case in *D. gigas* ferredoxin. Furthermore aggregation induces a loss of activity in *C. pasteruianum* ferredoxin while for *D. gigas* ferredoxin the most effective form is that which has a higher aggregation value.

Although all the three forms are active in the reduction of sulfite by molecular hydrogen and have roughly the same coupling effect at saturating concentrations, FdI and FdII do not present the same activity at lower concentrations. This fact suggests that the aggregation and redox states have an influence on the physiological properties of the protein. Thus, proteins have a way of varying their redox properties without a change of their primary structure. Such a property, which will be discussed in more detail in following papers, may have important consequences at the level of the regulation of electron transfer by organisms or organelles like mitochondria.

It is tempting to point out that FdII, the most effective form, exists as a tetramer and that since cytochrome c_3 possesses four hemes [27], conditions are realized for a rapid electronic exchange between the two proteins.

ACKNOWLEDGEMENTS

We are indebted to Mrs N. Forget and G. Bovier-Lapierre for their skilful technical help, to Dr. M. Scandellari and R. Bourrelli (from the Laboratoire de Chimie Bactérienne) and to the Fermentation Plant of the University of Georgia for growing the bacteria.

REFERENCES

1 Le Gall, J. and Dragoni, N. (1966) Biochem. Biophys. Res. Commun. 23, 145–149
2 Travis, J., Newman, J. D., Le Gall, J. and Peck, Jr., H. D. (1971) Biochem. Biophys. Res. Commun. 45, 452–458
3 Hall, D. O., Cammack, R. and Rao, K. K. (1974) in Iron in Biochemistry and Medicine, Jacobs, A. and Worwood, M., eds.), p. 279. Academic Press, London
4 Shethna, Y. I., Stombaugh, N. A. and Burris, R. H. (1971) Biochem. Biophys. Res. Commun. 42, 1108–1116
5 Johnson, P. W. and Canale-Paraola, E. (1973) Arch. Mikrobiol. 89, 341–353
6 Hall, D. O., Rao, K. K. and Mullinger, R. (1975) Biochem. Soc. Trans. 3, 472–479
7 Zubieta, J. A., Mason, R. and Postgate, J. R. (1973) Biochem. J. 133, 851–854
8 Bartsch, R. G. (1963) in Bacterial Photosynthesis, (Gest, H., San Pietro, A. and Vernon, L. P., eds.), p. 315, Antioch Press, Yellow Springs, Ohio.
9 De Klerk, H. and Kamen, M. D. (1966) Biochim. Biophys. Acta 112, 175–178

10 Tedro, S. M., Meyer, T. E. and Kamen, M. D. (1974) J. Biol. Chem. 249, 1182–1188
11 Stombaugh, N. A., Burris, R. H. and Orme-Johnson, W. H. (1973) J. Biol. Chem. 248, 7951–7956
12 Yoch, D. C. (1973) Arch. Biochem. Biophys. 158, 633–640
13 Shanmugam, K. T., Buchanan, B. B. and Arnon, D. I. (1972) Biochim. Biophys. Acta 256, 477–486
14 Yoch, D. C. and Arnon, D. I. (1972) J. Biol. Chem. 247, 4514–4520
15 Le Gall, J., Mazza, G. and Dragoni, N. (1965) Biochim. Biophys. Acta 99, 385–387
16 Dubourdieu, M. and Le Gall, J. (1970) Biochem. Biophys. Res. Commun. 38, 965–972
17 Bruschi, M., Le Gall, J., Hatchikian, C. E. and Dubourdieu, M. (1969) Bull. Soc. Franç. Physiol. Végét. 15, 381–390
18 Whitaker, J. R. (1963) Anal. Chem., 35, 1950–1953
19 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
20 Harvey, A. E., Smart, Jr., J. A. and Amis, E. S., Anal. (1955) Anal. Chem. 27, 26–35
21 Lovenberg, W., Buchanan, B. B. and Rabinowitz, J. C. (1963) J. Biol. Chem. 238, 3899–3913
22 Moore, S. and Stein, W. H. in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.), pp. 819–831, Vol. 6, Academic Press, New York
23 Hirs, C. H. W. in Methods in Enzymology (Hirs, C. H. W., ed.), Vol. XI, Academic Press, New York
24 Hatchikian, E. C., Le Gall, J., Bruschi, M. and Dubourdieu, M. (1972) Biochim. Biophys. Acta 258, 701–708
25 Bell, G. R. (1973) Studies on electron transfer systems in *Desulfovibrio:* purification and characterization of hydrogenase, catalase and superoxyde dismutase. Ph.D. thesis, p. 206, University of Georgia, Athens, U.S.A.
26 Gersonde, K., Trittelvitz, E., Schlaak, H. E. and Stabel, H. H. (1971) Eur. J. Biochem. 22, 57–65
27 Dobson, C. M., Hoyle, N. J., Geraldes, C. F., Bruschi, M., Le Gall, J., Wright, P. E. and Williams, R. J. P. (1974) Nature 249, 425–429