Biochimica et Biophysica Acta, 679 (1982) 422–427
Elsevier Biomedical Press

BBA 41056

IRON-SULPHUR CLUSTER COMPOSITION AND REDOX PROPERTIES OF TWO FERREDOXINS FROM *DESULFOVIBRIO DESULFURICANS* NORWAY STRAIN

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(Received August 13th, 1981)

Keywords: ESR Redox potential; Iron-sulfur protein; Ferredoxin; (Desulfovibrio desulfuricans)

Two ferredoxins from *Desulfovibrio desulfuricans*, Norway Strain, were investigated by EPR spectroscopy. Ferredoxin I appears to be a conventional [4Fe-4S]$^{2+;1+}$ ferredoxin, with a midpoint reduction potential of −374 mV at pH 8. Ferredoxin II when reduced, at first showed a more complex spectrum, indicating an interaction between two [4Fe-4S] clusters, and probably, has two clusters per protein subunit. Upon reductive titration ferredoxin II changed to give a spectrum in which no intercluster interaction was seen. The midpoint potentials of the native and modified ferredoxin at pH 8 were estimated to be −500 and −440 mV, respectively.

Introduction

Desulfovibrio desulfuricans, Norway strain, [1] is distinguished by containing an unusual type of dissimilatory sulphite reductase, desulphorubidin [2]. Two ferredoxins have been isolated from this organism [3,4]. Both were found to be active in the pyruvate phosphoroclastic reaction and as electron donors to sulphite reductase [4]. Ferredoxin II, the more acidic, was very unstable in oxygen.

Several species of sulphate-reducing bacterium have now been found to contain more than one type of ferredoxin. Three different proteins have been isolated from *Desulfovibrio africanus* Benghazi strain [5,6]. The two ferredoxins from *Desulfovibrio gigas* have been extensively studied. Ferredoxin I from this organism is a trimer of a subunit of molecular weight approx. 6500, while ferredoxin II is a tetramer of the same polypeptide [7]. Ferredoxin I gives an electron paramagnetic resonance spectrum in the reduced state at g 1.94, which indicates the presence of [4Fe-4S]$^{2+;1+}$ clusters [8]. Ferredoxin II gives an EPR signal in the oxidized state at g 2.01 [8] and Mössbauer spectroscopy indicates the presence of [3Fe-3S] clusters [9]. The two ferredoxins of *D. gigas* differ in midpoint reduction potential (−455 and −130 mV, respectively) and it has been suggested by Moura et al. [10] that by existing in these two conformational states the same polypeptide chain could act in two parts of the electron transport chain.

Ferredoxins and II of *D. desulfuricans* Norway are each dimers of subunit molecular weight about 6000 [4]. The amino-acid compositions of the two ferredoxins are differen ferredoxin I, the less acidic protein, contains four atoms of iron and labile sulphur per subunit. The N-terminal amino-acid sequence resembles that of other ferredoxins from *Desulfovibrio* spp. Ferredoxin II is unstable, particularly in the presence of oxygen. Its content of iron and labile sulphide has not been determined. Its N-terminal sequence shows homology with the 2[4Fe-4S] ferredoxins of *Clostridia* [4].

In this paper we present results obtained by the use of EPR spectroscopy on the iron-sulphur clusters in ferredoxins I and II of *D. desulfuricans*. Norway strain, together with an estimate of their midpoint reduction potentials.

0005-2728/82/0000–0000/$02.75

Materials and Methods

Ferredoxins I and II were extracted from *D. desulfuricans* Norway 4 (National Collection of Industrial Bacteria No. 8310) and purified as described previously [4].

Midpoint reduction potential measurements were made by adjustment of the redox potential of the protein in the presence of mediator dyes, followed by measurement of the EPR signals [11]. For measurements at higher potentials (200 to −200 mV) the mediators (all at 40 μM concentration) were: hydroquinone, methylene blue, thionine, indigodisulphonate, indigotetrasulphonate and 2-hydroxy-1,4-naphthoquinone. For measurements at lower potentials the mediators (40 μM) were: phenosafranine, anthraquinone 2,7-disulphonate, benzyl viologen, methyl viologen, diquat, triquat, tetraquat and *N,N'*-dimethyl-3-methyl-4,4'-bipyridylium. The protein solution was reduced and oxidized with small additions of 0.1 M $Na_2S_2O_4$ and 0.2 M $K_3Fe(CN)_6$, respectively, stirred in a vessel at 25°C under argon. The redox potential was measured by platinum and calomel electrodes. After equilibration at each potential for approx. 2 min a sample was transferred anaerobically with a syringe to a quartz EPR tube and frozen for EPR measurements. The signal amplitudes from samples poised at different potentials were fitted to curves calculated from the Nernst equation, assuming that the iron-sulphur clusters are one-electron carriers.

EPR spectra were recorded on a Varian E4 spectrometer with an Oxford Instruments ESR9 liquid helium flow cryostat. *g* values were measured from the principal derivative features of the spectra and referred to diphenylpicrylhydrazyl as standard, to an accuracy of ±0.002. Integrations were carried out digitally, with correction for baseline.

Results

EPR spectra of the proteins

The protein as prepared gave an extremely weak signal at *g* 2.01 (Fig. 1a). This type of spectrum is frequently seen in proteins with $[4Fe\text{-}4S]^{2+;1+}$ clusters. It has been attributed to a small amount of the 'super-oxidized' $[4Fe\text{-}4S]^{3+}$ form [12] but recent spectroscopic evidence suggests it may instead be a

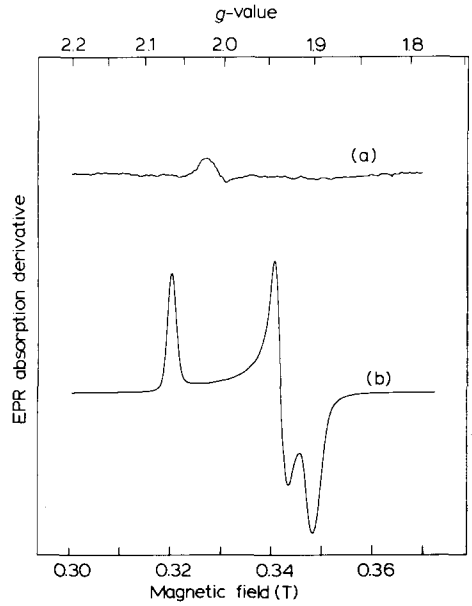

Fig. 1. EPR spectra of ferredoxin I (a) oxidized (b) reduced with 5 mM $Na_2S_2O_4$ (final concentration) for 1 min. Conditions of measurement: temperature, 24 K, microwave power, 20 mW, frequency, 9.26 GHz modulation amplitude, 0.5 mT, frequency 100 kHz. The gain setting of (a) was amplified 10-fold compared with (b).

[3Fe-3S] cluster [13]. On reduction with dithionite, the protein gave an intense signal with *g* values 1.902, 1.937, 2.068. (Fig. 1b). The signal was readily detected in the temperature range 10–40 K. These results are typical of a ferredoxin with separate [4Fe-4S] clusters [14], or *D. gigas* ferredoxin I, which contains three clusters, separated on different subunits [8].

In the oxidized state ferredoxin II gave a signal centred around *g* 2.01 with a derivative maximum at *g* 2.022 (Fig. 2a) similar in shape to the spectrum of ferredoxin of *D. gigas*. The spectrum was observed over the temperature range 6 K to 18 K. Broadening of the spectrum at high field was observed at the lower temperatures, as was observed with oxidized ferredoxin I of *D. gigas* [8]. Unlike *D. gigas* ferredoxin II, Norway ferredoxin II had only a relatively small oxidized-protein signal, corresponding to approx. 5% of the signal from the reduced protein, estimated by comparison of double integrals of the recorded spectra.

The spectrum of dithionite-reduced ferredoxin II

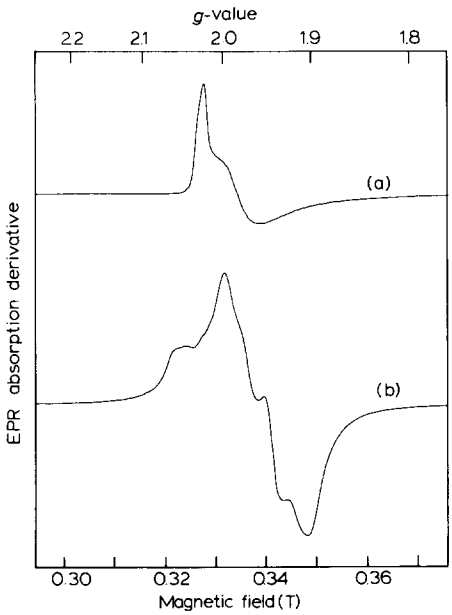

Fig. 2. EPR spectra of ferredoxin II (a) oxidized, (b) reduced with $Na_2S_2O_4$. Conditions of measurement were as for Fig. 1, except that the temperature was 9.5 K in (a) and 16 K in (b).

is shown in Fig. 2b. It differs from the simple spectrum of reduced ferredoxin I in having additional absorption around g 2 and 1.92, and broader outer lines. These features are characteristic of a system of [4Fe-4S] clusters that are close enough to interact magnetically, as in the 2[4Fe-4S] ferredoxins of *Clostridium* spp. and *Veillonella alcalescens* [15]. The lineshapes of such spectra are critically dependent on the detailed structure of the protein [14]. In support of this assignment, a half-field ($\Delta m_s = 2$) transition was detected at g 3.96 (not shown). No signal of this type was detected in reduced ferredoxin I. The spectrum of reduced ferredoxin II was readily detected at low temperatures (under 8 K) at which the spectrum of ferredoxin I was strongly saturated. This reflects a faster electron spin relaxation rate which again is typical of reduced 2[4Fe-4S] proteins [16].

On treatment of ferredoxin II with excess dithionite for several minutes the lineshape of the spectrum changed to one in which there was no evidence for spin-spin interaction between clusters. This was investigated further during redox titrations (see below).

Spectra of the proteins in 80% dimethylsulphoxide

A guide to the type of cluster in proteins can be obtained by measuring the spectrum of the reduced form in 80% dimethylsulphoxide. This treatment is thought to remove the constraints of the protein and effects of other groups which can cause the clusters to give atypical spectra in the native state. [2Fe-2S] and [4Fe-4S] clusters have different characteristic spectra under these conditions [17], the [2Fe-2S] clusters being detectable up to considerably higher temperatures. [3Fe-3S] clusters are unstable under these conditions, and may isomerize to produce a small proportion of [4Fe-4S] or [2Fe-2S] clusters (Cammack, R., unpublished observations). However, the known [3Fe-3S] clusters are detectable by EPR in the oxidized and not in the reduced state, which does not apply to ferredoxins I and II.

Upon treatment with 80% dimethylsulphoxide and reduction by dithionite both ferredoxin I and ferredoxin II gave signals characteristic of [4Fe-4S] clusters (Fig. 3) and no [2Fe-2S] cluster was detected.

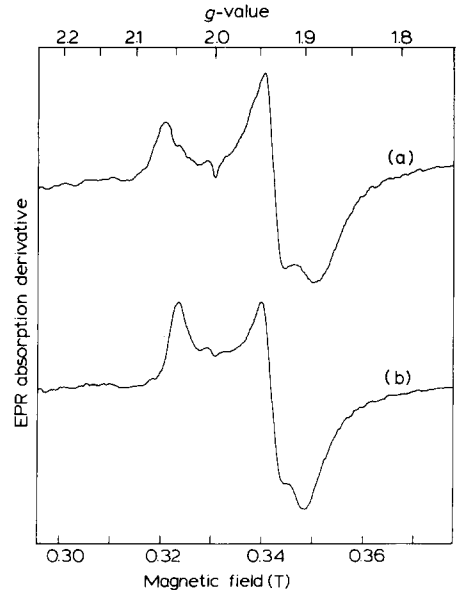

Fig. 3. Spectra of reduced ferredoxins in 80% (v/v) DMSO solution (a) ferredoxin I, (b) ferredoxin II. 30 µl samples of the proteins at pH 9.2 were purged with argon and treated anaerobically with 120 µl DMSO at 0°C for 2 min. 2 mM (final concentration) $Na_2S_2O_4$ solution was then added and the samples were frozen after a further 1 min. Conditions of measurement: Temperature, 22 K, microwave power, 20 mW; frequency, 9.26 GHz; modulation amplitude, 1 mT.

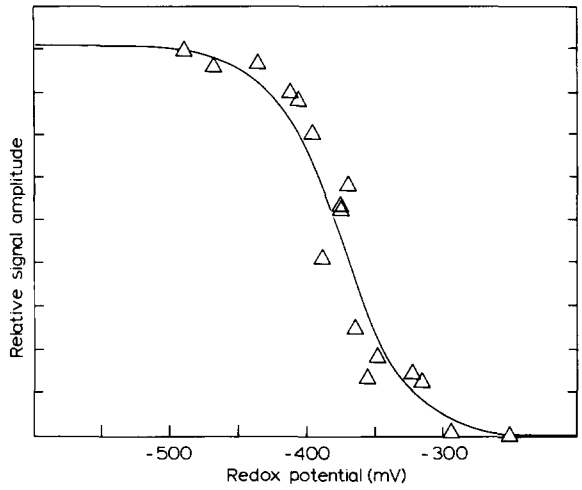

Fig. 4. Redox titration of ferredoxin I. The protein concentration was approx. 0.29 ml/ml. in 0.1 M Tris-HCl, pH 8.0. EPR spectra were recorded as for Fig. 1 and the relative amplitudes of the feature of g 1.94 plotted against the redox potential at which samples were poised before freezing.

Redox potential measurements

Redox potential titrations of ferredoxin I monitored by EPR spectroscopy showed that the ferredoxin reduced as a single species, with good reversibility (Fig. 4). The midpoint reduction potential was estimated to be -374 ± 15 mV at pH 8.0. Comparison of spectra of fully and partly reduced protein gave no evidence for more than one type of cluster, or interaction between clusters.

Upon titration of the oxidized form of ferredoxin II the signal at g 2.01 titrated with a potential approx. 115 ± 20 mV (Fig. 5a). The signal did not show complete reversibility upon reoxidation, possibly due to instability of the oxidized form.

The reduction titration of ferredoxin II to low potentials, was accompanied by a gradual and irreversible change in signal lineshape (Fig. 6). Upon first adjusting to a potential at which the protein was partially reduced (−505 mV) (Fig. 6a) a signal typical of isolated reduced [4Fe-4S] clusters was seen, and upon further reduction to −540 mV (Fig. 6b) the additional peak at g 1.996 appeared as seen in the dithionite reduced protein. This is the behaviour expected for a protein with interacting [4Fe-4S] clusters. At first only one of the clusters per molecule will be reduced on average; upon further reduction, when both clusters become reduced, a signal appears which is characteristic of the interaction between them [18]. However, as the titration was continued, the signal lineshape changed to give a new species, with g 1.898, 1.943, 2.058 (Fig. 6c). This spectrum

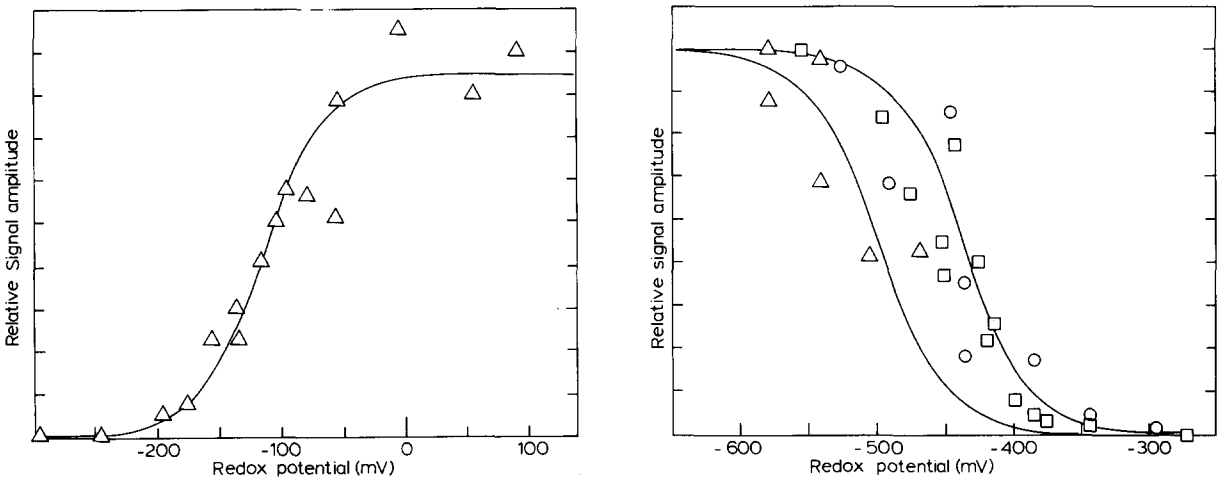

Fig. 5. a. Redox titration of ferredoxin II, oxidized form. in 0.1 M Tris-HCl, pH 8.0. The size of the signal at g 2.01 was recorded as for Fig. 2a. b. Redox titration of ferredoxin II, reduced signals. The size of the g 1.94 signals is plotted for three separate titrations: Points ○ are from a titration at pH 8.0 in which the protein had previously been titrated to high potentials, and had been in the titration vessel for 1 h. Points □ are from a titration at pH 8.0. which started at −270 mV, total duration 30 min. Points △ are from a titration at pH 8.5 in which the potential was adjusted to low potentials (−505 mV) within 3 min. The signal lineshape was changing during this titration (Fig. 6). The theoretical curves are for midpoints of −500 and −440 mV.

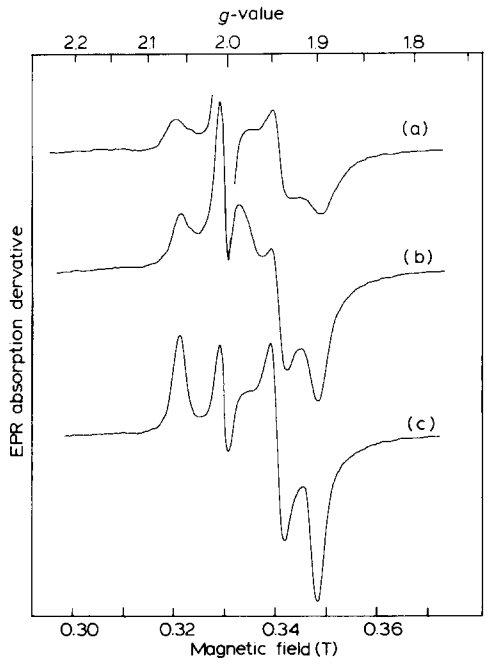

Fig. 6. Spectra of ferredoxin II samples from the redox titration vessel. Samples poised at (a) −505 mV, (b) at −540 mV, (c) at −550 mV, but taken after 1 h incubation at 25°C. The narrow features at g 2.00 are radical signals from the mediators. Conditions of measurement: temperature, 20 K; microwave power, 20 mW; modulation amplitude, 1 mT.

showed narrower linewidths than those in Fig. 6a. This spectrum is characteristic of separate [4Fe-4S] clusters.

Since this change took place while the redox potential was being held constant we interpret this unusual result as indicating that the protein was unstable under these reducing conditions. Either one of the interacting clusters was becoming denatured by the reduction treatment, or the interaction between the clusters was being destroyed. In order to estimate the redox potential of the undenatured protein it was necessary to adjust the potential of the system quickly, in which case the redox system may not have reached redox equilibrium. However, the results are consistent with low redox potential, about −500 mV for the clusters in the native protein and −440 mV for the modified form (Fig. 5b).

Discussion

The less acidic ferredoxin (Fd I) is a typical [4Fe-4S] ferredoxin constituted by two subunits of 6000 molecular weight. The N-terminal sequence is homologous to that of *D. gigas* ferredoxin, and ferredoxin I and ferredoxin II from *D. africanus* [5,19].

The more acidic ferredoxin (Fd II) is not so typical. Studies of it are hampered by instability during the purification. The sensitivity to O_2 and the influence of temperature are more severe than Probst et al. [2] described for *Desulfuramonas acetoxidans* ferredoxin. After some experiments with neutral salts as described by Masumi et al. [21] and Petering et al. [22] it was found that the presence of 0.5 M NaCl or Na_2SO_4 and a temperature of 4°C preserved the protein.

This instability was also observed in the EPR spectra of the dithionite-reduced protein. At first the protein showed a spectrum indicative of interacting [4Fe-4S] clusters. Prolonged reduction caused the appearance of a signal typical of isolated [4Fe-4S] clusters. This latter state, although relatively stable, presumably represents a degraded form of the protein. It is noteworthy that if more severe reducing conditions had been used, or the protein had been even more unstable, the EPR spectra data would not have detected the presence of the interacting clusters. This should be borne in mind when interpreting the EPR spectra of other ferredoxins with [4Fe-4S] clusters.

Ferredoxin II is a dimeric molecule with two subunits of molecular weight approx. 6000 [4]. The extinction coefficient per subunit is 31 580 $M^{-1} \cdot cm^{-1}$, typical of 2[4Fe-4S] proteins such as those from Clostridia. If each subunit contains two clusters, the spin-spin interaction which is observed in the spectra of freshly-reduced protein (Fig. 2b) might be due to interaction between two clusters in one subunit, or between clusters in different subunits. The former possibility seems more likely because of the similarity of the spectrum to those of the clostridial ferredoxins. Moreover, the [4Fe-4S] ferredoxins with multiple subunits such as ferredoxin I from the same organism and ferredoxin I of *D. gigas* [8] do not show such spin-spin interaction.

Ferredoxin II, therefore, appears to resemble ferredoxin from *D. acetoxidans* in containing eight atoms

of iron and eight of labile sulphide per protein subunit.

Acknowledgements

We are grateful to Dr. M. Bruschi for her supervision during this projects, and to Dr. J. Le Gall and A.V. Xavier for their advice and encouragement. We also thank Mr. I.G. Paterson for technical assistance. This work was supported by grants from The U.K. Science Research Council, National Institute of Health (Grant No. GM 25879); Instituto Nacional de Investigação Científica, Junta Nacional de Investigação Científica e Tecnológica and Fundação Calouste Gulbenkian, Portugal.

References

1 Miller, J.D. and Saleh, A.M. (1964) J. Gen. Microbiol. 37, 419–423
2 Lee, J.P., Yi, C.S., Le Gall, J. and Peck, H.D. Jr. (1973) J. Bacteriol. 115, 453–455
3 Bruschi, M. Hatchikian, E.C., Golovleva, L.A. and Le Gall, J. (1977) J. Bacteriol. 129, 30–38
4 Guerlesquin, F., Bruschi, M., Bovier-Lapierre, G. and Fauque, G. (1980) Biochim. Biophys. Acta 626, 127–135
5 Hatchikian, E.C., Jones, H.E. and Bruschi, M. (1979) Biochim. Biophys. Acta 548, 471–483
6 Hatchikian, E.C. and Bruschi, M. (1981) Biochim. Biophys. Acta 634, 41–51
7 Bruschi, M., Hatchikian, E.C., Le Gall, J. Moura, J.J.G. and Xavier, A.V. (1976) Biochim. Biophys. Acta 449, 275–284
8 Cammack, R., Rao, K.K., Hall, D.O., Moura, J.J.G., Xavier, A.V. Bruschi, M, Le Gall, J., Deville, A. and Gayda, J.-P. (1977) Biochim. Biophys Acta 490, 311–321
9 Huynh, B.H., Moura, J.J.G., Moura, I., Kent, T.A., Le Gall, J., Xavier, A.V. and Munck, E. (1980) J. Biol. Chem. 255, 3232–3244
10 Moura, J.J.G., Xavier, A.V., Hatchikian, E.C. and Le Gall, J. (1978) FEBS Lett. 89, 177–179
11 Cammack, R., Barber, M.J. and Bray, R.C. (1976) Biochem. J. 157, 469–478
12 Sweeney, V.W. and Rabinowitz, J.C. (1980) Annu. Rev. Biochem. 49, 139–161
13 Thomson, A.J., Robinson, A.E., Johnson, M.K., Cammack, R., Rao, K.K. and Hall, D.O. (1981) Biochim. Biophys. Acta 637, 423–432
14 Cammack, R., Dickson, D.P.E. and Johanson, C.E. (1977) in Iron-Sulfur Proteins (Lovenberg, W., ed.), vol. 3, pp. 283–330, Academic Press, New York
15 Mathews, R., Charlton, S. Sands, R.H. and Palmer, G. (1974) J. Biol. Chem. 249, 4326–4328
16 Rupp, H., Rao, K.K., Hall, D.O. and Cammack, R. (1978) Biochim. Biophys. Acta 537, 255–269
17 Cammack, R. (1975) Biochem. Soc. Trans. 3, 482–488
18 Orme-Johnson, W.H. and Beinert, H. (1969) Biochem. Biophys. Res. Commun. 36, 337–344
19 Bruschi, M. (1979) Biochem. Biophys. Res. Commun. 91, 623–628
20 Probst, I., Moura, J.J.G., Moura, I., Bruschi, M., Le Gall, J. (1978) Biochim. Biophys. Acta 502, 38–44
21 Masumi, H., Nahamura, S., Koga, K. and Yoshizumi, H. (1979) Biochem. Biophys. Res. Commun. 87, 1095–1101
22 Petering, D., Fee, J.A. and Palmer, G. (1971) J. Biol. Chem. 246, 643–653