Biochem. J. (1982) **207**, 333–339
Printed in Great Britain

Electron-spin-resonance studies of the NADH-dependent nitrite reductase from *Escherichia coli* K12

Richard CAMMACK
Department of Plant Sciences, King's College London, 68 Half Moon Lane, London SE24 9JF, U.K.

and Ronald H. JACKSON,* Athel CORNISH-BOWDEN and Jeffrey A. COLE
Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

(*Received 12 May 1982/Accepted 4 August 1982*)

The NADH-dependent nitrite reductase of *Escherichia coli*, which contains sirohaem, flavin, non-haem iron and labile sulphide, was examined by low-temperature e.s.r. spectroscopy. The enzyme, stored in the presence of nitrite and ascorbate, gave the spectrum of a nitrosyl derivative, with hyperfine splitting due to the nitrosyl nitrogen. On removal of these reagents, a series of signals centred around $g = 6$ was observed, typical of high-spin ferric haem. Cyanide converted this into a low-spin form. On reduction of the enzyme with NADH, an axial spectrum at $g = 1.92$, 2.01 was observed. The temperature-dependence of this signal is indicative of a [2Fe–2S] iron–sulphur cluster. The midpoint potential of this cluster was estimated to be -230 ± 15 mV by two independent methods. Reduction of the enzyme with dithionite yielded further signals, which are at present unidentified, at $g = 2.1$–2.28. No signals were observed that could be assigned to a [4Fe–4S] cluster, such as is found in other sulphite reductases and nitrite reductases that contain sirohaem.

Escherichia coli K12 contains three soluble enzymes that can catalyse the reduction of nitrite. The subject of the present study is an NADH-dependent enzyme known as nitrite reductase (NADH:nitrite oxidoreductase, EC 1.6.6.4) (Coleman *et al.*, 1978; Jackson *et al.*, 1981*a*). In addition, cytochrome c_{552} can catalyse the reduction of nitrite by $FADH_2$ (Fujita & Sato, 1966; Liu *et al.*, 1981), and the NADPH-dependent sulphite reductase can catalyse the reduction of nitrite as effectively as that of sulphite (Kemp *et al.*, 1963).

The NADH-dependent nitrite reductase has been purified from a chlorate-resistant mutant of *E. coli* in which its synthesis is partially constitutive. The protein appears to be a dimer, and is remarkable for having a diverse range of prosthetic groups within each 88kDa subunit. Analysis indicated that each molecule of subunit contains one FAD group and approximately five Fe and four acid-labile S atoms (Jackson *et al.*, 1981*a*). The optical absorption spectrum shows features characteristic of sirohaem, a type of haem found in other nitrite reductases and in sulphite reductases (Siegel, 1978). The isolated enzyme rapidly loses its activity in solution, but its stability is improved by isolating and storing it in a complex medium containing NO_2^-, EDTA, ascorbate, FAD and 50% (v/v) glycerol (Jackson *et al.*, 1981*a*).

A kinetic mechanism of the NADH-dependent reductase has been proposed from studies of the reduction of NO_2^-, hydroxylamine and various single-electron acceptors by NADH (Jackson *et al.*, 1981*b*, 1982). We here present e.s.r. spectra of the enzyme that provide further information about the sirohaem and iron–sulphur clusters.

Experimental

Nitrite reductase was isolated from *E. coli* strain OR75Ch15 as described by Jackson *et al.* (1981*a*). The final isolation medium contained 50% (v/v) glycerol, 50mM-Tris/HCl, pH8, 5mM-EDTA, 5mM-ascorbate, 1mM-$NaNO_2$ and 10μM-FAD, and the enzyme was stored at −15°C. When the enzyme was required free of these reagents, it was reduced with 5mM-NADH, allowed to bind to a column (4cm × 0.5cm) of DEAE-cellulose, washed with 0.1M-NaCl + 20mM-Tris/HCl, and then eluted with 0.4M-NaCl + 20mM-Tris/HCl, pH8.0. The e.s.r. spectra of two different preparations of the enzyme

* Present address: Molecular Biology Division, Veterans' Administration Hospital, University of California, San Francisco, CA 94121, U.S.A.

0306-3275/82/110333-07$01.50/1

were essentially identical, although after removal of nitrite the haem signals showed a progressive decline in amplitude.

E.s.r. spectra were recorded with an E4 spectrometer (Varian Associates, Walton-on-Thames, Surrey KT12 2UF, U.K.) and an ESR9 liquid-helium-flow cryostat (Oxford Instruments, Oxford OX2 0DX, U.K.). Third-harmonic spectra were obtained with a subharmonic generator (Telmore Instruments, Brynmawr, Gwent NP3 4WU, Wales, U.K.). Spectra were recorded digitally and subtracted on a DL4000 signal-processing system (Data Laboratories, Mitcham CR4 4HR, Surrey, U.K.); *g*-values were calibrated relative to the 1,1-diphenyl-2-picrylhydrazyl radical and methaemoglobin standards. Double integration was carried out digitally with correction for baseline, Cu-EDTA being used as a standard. The enzyme preparation used for estimation of spin concentrations was estimated to be 90% pure as judged by polyacrylamide-gel electrophoresis. The protein concentration was 6.2 mg/ml, as determined by the microtannin turbidometric method of Mejbaum-Katzenellenbogen & Dobryszycka (1959).

Redox-potential measurements were made either by reduction with $Na_2S_2O_4$ as described by Cammack *et al.* (1976) in an apparatus similar to that of Dutton (1971), or by electrochemical reduction in apparatus modified from that of Ke *et al.* (1977) (R. Cammack & S. K. Chamorovsky, unpublished work). The mediators used, all at 20 μM, were: 2-hydroxy-1,4-naphthoquinone, Phenosafranine, Safranine T, indigotetrasulphonate, indigosulphonate, diquat, Methyl Viologen, anthraquinone-2,7-disulphonate and anthraquinone-2-sulphonate.

$Na^{15}NO_2$ was from the British Oxygen Company Ltd., London SW19 3UF, U.K.

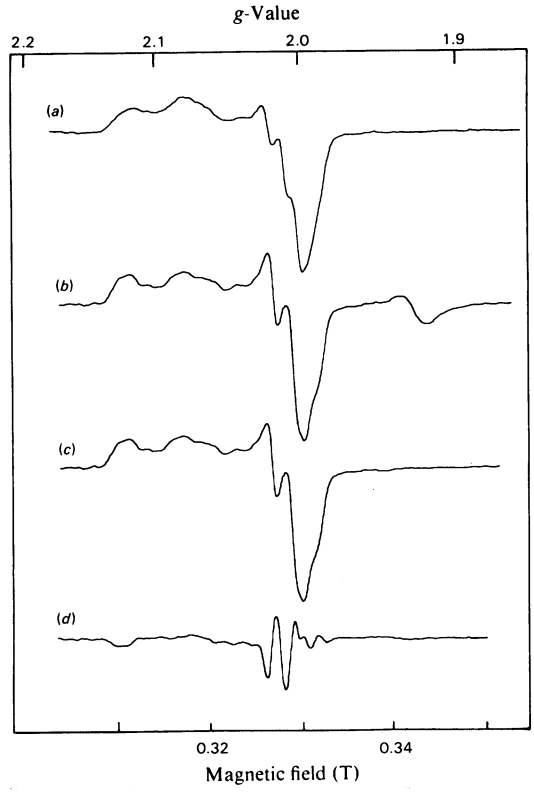

Fig. 1. *E.s.r. spectra of nitrite reductase, showing signals assigned to nitrosyl haem*

(*a*) Original preparation, containing ascorbate and $Na^{14}NO_2$; (*b*) with ascorbate and $Na^{15}NO_2$; (*c*) as (*b*), with iron–sulphur signal (cf. Fig. 5) subtracted; (*d*) difference between (*a*) and (*c*). Spectra were recorded at 65 K with the following instrument settings: microwave power 20 mW, frequency 9.24 GHz; modulation amplitude 0.5 mT, frequency 100 kHz.

Results

E.s.r. signals from nitrosyl sirohaem

The enzyme-stabilizing buffer contained 5 mM-ascorbate and 1 mM-nitrite, a mixture which would generate nitric oxide, and as a result the enzyme was in the nitrosyl form. The e.s.r. spectrum (Fig. 1) is typical of a low-spin nitrosyl-Fe compound with $g_x = 2.12$ and $g_y, g_z = 2.01$; it is similar to the spectra of nitrosyl haemoproteins such as nitrosyl cytochrome *c* (Yonetani *et al.*, 1972) and of model nitrosyl haem compounds [see Yoshimura *et al.* (1979) and references therein].

The derivative peak at $g = 2.06$ cannot be fitted by simulation of the spectrum of a single species, and therefore represents a second paramagnetic species underlying the more prominent spectrum. A similar additional component has been seen in several nitrosyl haem compounds and was termed 'absorption II' by Yoshimura *et al.* (1979). Morse & Chan (1980) interpreted it as evidence for a different conformation of the Fe-NO in the haem, rather than a different chemical species.

The total integrated intensity of the nitrosyl signals was estimated at 0.35 unpaired electron per protein molecule. Possible explanations are that not all of the enzyme was converted into the nitrosyl form, or some of the enzyme had lost its sirohaem, or both.

The *g*-value nearest to 2 is conventionally assigned as g_z, and is split by hyperfine interaction into three lines separated by 1.67 mT. This can be seen more clearly in the third-harmonic spectrum (Fig. 2*a*). Confirmation that this was due to the ^{14}N nucleus (nuclear spin, $I = 1$) of the nitrosyl group was obtained by substituting with ^{15}N. A sample of the enzyme was treated with approx. 10 mM-

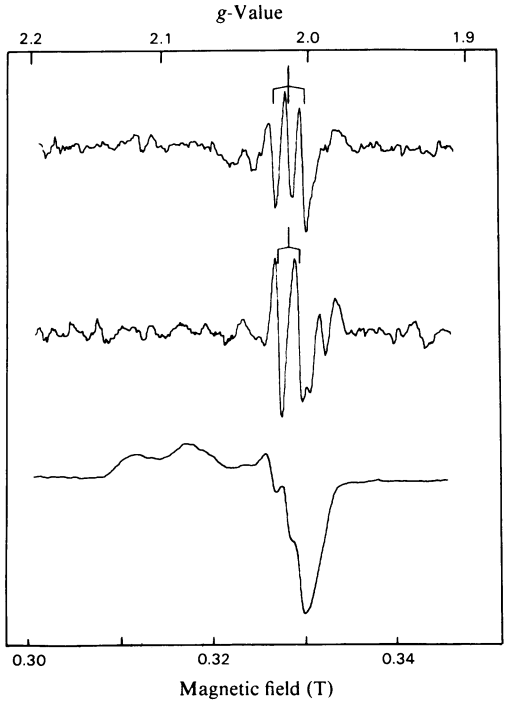

Fig. 2. *Third-harmonic e.s.r. spectra of* (*a*) [^{14}N]*nitrosyl nitrite reductase,* (*b*) [^{15}N]*nitrosyl nitrite reductase and* (*c*) *first-harmonic spectrum of* [^{14}N]*nitrosyl nitrite reductase for comparison*

Spectra were recorded at 65 K as for Fig. 1, except that for third-harmonic spectra the modulation frequency was 33.3 kHz and amplitude 1.5 mT. The ^{14}N hyperfine splitting of 1.67 mT and the ^{15}N hyperfine splitting of 2.35 mT are shown by 'stick' spectra.

$Na_2S_2O_4$ so that enzyme cycling would reduce the ^{14}NO and $^{14}NO_2^-$ in the sample to NH_3, then excess $Na^{15}NO_2$ was added. In the resulting spectrum (Figs. 1*b* and 2*b*), g_z is split into two lines separated by 2.35 mT, as expected for the nuclear spin ($I = \frac{1}{2}$) of ^{15}N. The spectrum (Fig. 1*b*) also shows features at $g = 2.01$ and 1.92 due to partial reduction of the iron–sulphur clusters. In Fig. 1(*c*) these have been subtracted out and Fig. 1(*d*) shows the $^{14}N - ^{15}N$ difference spectrum. In this difference spectrum and the third-harmonic spectra (Fig. 2), there is no evidence for a resolved hyperfine splitting at the other *g*-value positions, as was observed in *Cucurbita pepo* (vegetable marrow) nitrite reductase (Fry *et al.*, 1980; Cammack & Fry, 1980).

Signals from oxidized sirohaem

The nitrite-free enzyme was obtained by treating a sample with excess NADH and removing excess reagents on a DEAE-cellulose column as described in the Experimental section. The oxidized protein then gave at low temperatures the e.s.r. spectrum presented in Fig. 3. The sharp peak at $g = 4.3$ is probably due to adventitious Fe(III); it appears intense because of its relatively narrow linewidth. The features centred around $g = 6$ are probably all due to various high-spin haem species. High-spin Fe(III) in an axial ligand field, such as a symmetrical haem, will give a spectrum at $g_x = g_y = 6$, $g_z = 2$ (Feher, 1970). The feature at $g = 2$ is often relatively inconspicuous and in the present case we did not detect it. Rhombic distortion in the haem *x*–*y* plane will cause g_x and g_y to separate on each side of $g = 6$ (Peisach *et al.*, 1971; Palmer, 1979).

The signals around $g = 6$ in Fig. 3 can therefore be interpreted in terms of three principal haem species with different degrees of rhombic distortion. The derivative peak at $g = 6$ is due to relatively undistorted haem and may be due to denatured haemoprotein. We have observed a similar broad peak in the spectrum of partly inactivated preparations of the sirohaem-containing nitrite reductase of *Cucurbita pepo* (I. V. Fry & R. Cammack, unpublished work). Native sirohaem-containing proteins typically show a large rhombic distortion (Murphy *et al.*, 1973). The other signals may thus be interpreted as a major species with *g*-values of 7.11 and 4.87 and minor species with *g*-values of 6.7 and 5.3. One or both of these may represent the native protein. Heterogeneity has also been observed in the e.s.r. spectrum of the sirohaem-containing sulphite reductase of *Desulfovibrio gigas*. This showed a mixture of signals around $g = 6$, which were tentatively interpreted as due to different protein conformations (Murphy *et al.*, 1973; Hall *et al.*, 1979).

On addition of cyanide, the iron was converted into the low-spin form, as would be expected for this strong ligand. All the signals around $g = 6$ disappeared. Only one feature of this spectrum was resolved, at $g = 2.32$ (Fig. 4). The shape of this region of the spectrum indicates axial symmetry with a third *g*-value to higher field, which presumably was too broad to be detectable. Spectra similar to this, with $g_\perp \simeq 2.3$, $g_\parallel \simeq 1.95$, have been observed in low-spin complexes of sirohaem model compounds (Stolzenberg *et al.*, 1981).

Signals from iron–sulphur clusters

Samples of the nitrite reductase reduced with excess NADH, $Na_2S_2O_4$ or reduced Methyl Viologen yielded an e.s.r. signal with apparent axial symmetry at $g_x = g_y = 1.92$, $g_z = 2.01$ (Fig. 5). The spectrum was detectable at temperatures up to 110 K. At 20 K the signal was strongly saturated at a microwave power of 10 mW. This behaviour indicates a reduced [2Fe–2S] cluster; the spectra of all

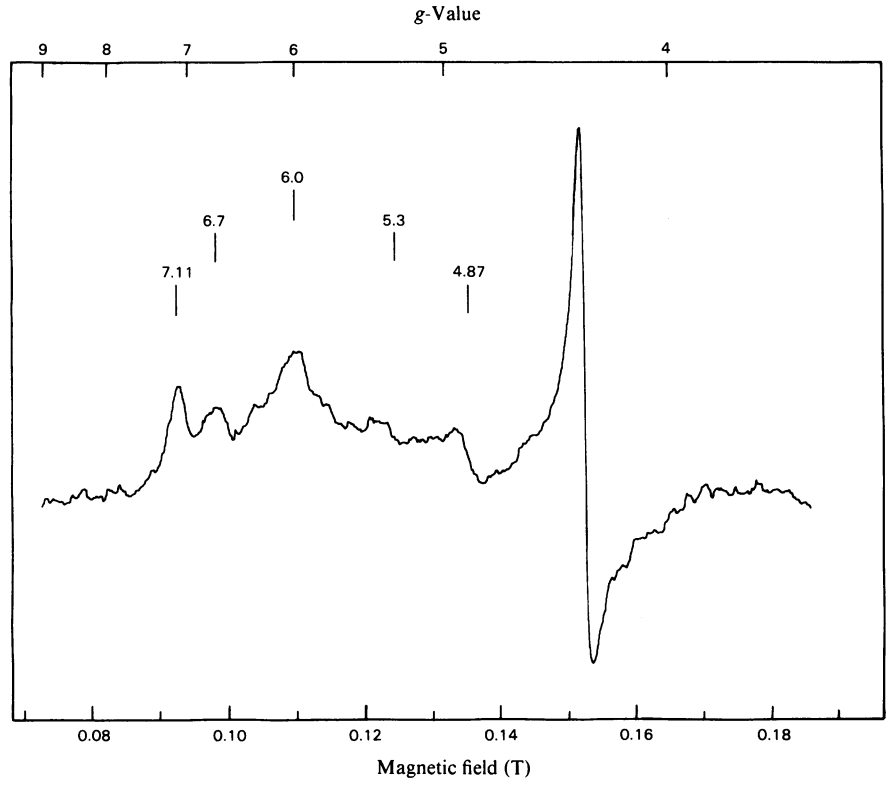

Fig. 3. *Low-field spectrum of substrate-free oxidized enzyme*
The spectrum was recorded at 9 K with the following instrument settings: microwave power 20 mW, frequency 9.24 GHz, modulation amplitude 1 mT, frequency 100 kHz.

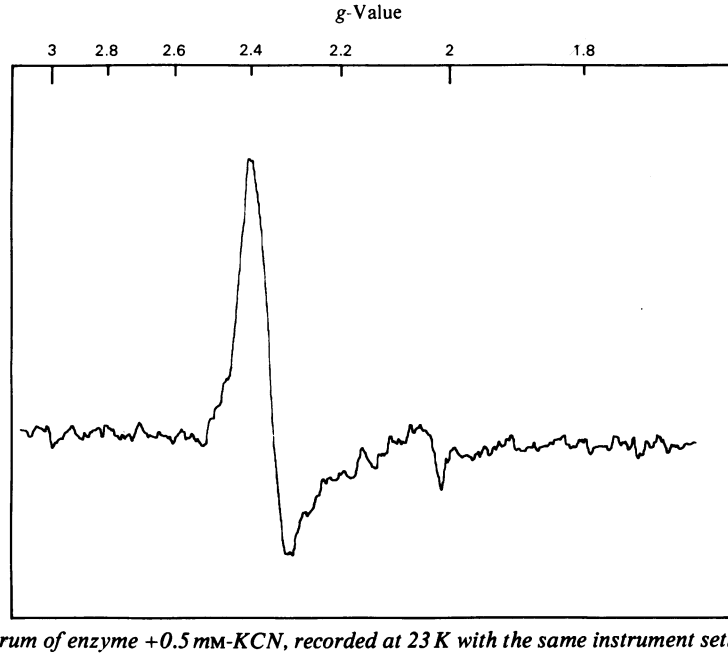

Fig. 4. *Spectrum of enzyme +0.5 mM-KCN, recorded at 23 K with the same instrument settings as in Fig. 3*

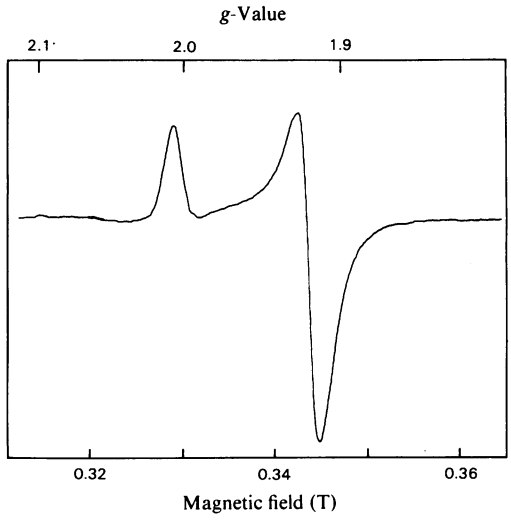

Fig. 5. *Spectrum of enzyme reduced with approx. 10mM-NADH recorded at 65K with the same instrument settings as for Fig. 1*

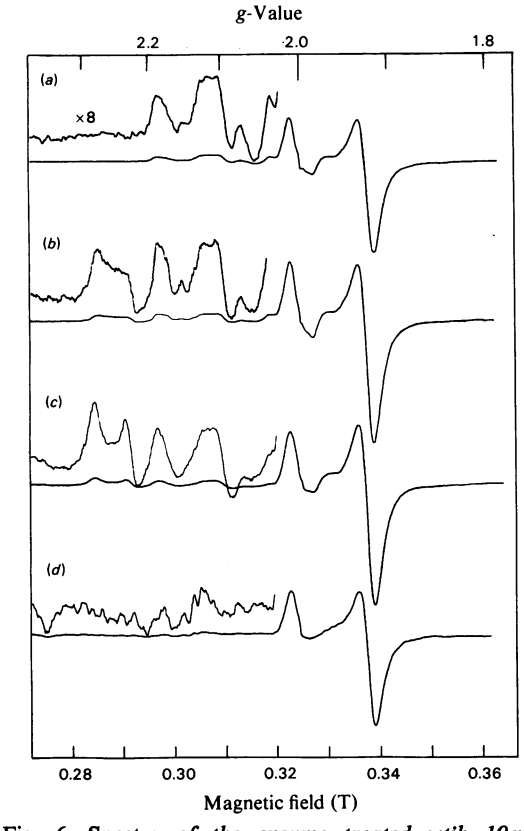

Fig. 6. *Spectra of the enzyme treated wtih 10mM-$Na_2S_2O_4$ for (a) 15s, (b) 1min, (c) 5min, and (d) 20min*
The spectra at the low-field region are expanded by a factor of 8. Conditions of measurement were as for Fig. 1.

reduced [4Fe–4S] clusters become too broad to be detected as the temperature is raised to 77K (see Rupp *et al.*, 1978).

Spectra of the reduced enzyme measured over the temperature range 4.2–77K indicated only one type of iron–sulphur cluster. The integrated intensity of the signal with g-values of 2.01 and 1.92 was estimated as 0.63 unpaired electron per protein molecule. Hence there was no evidence for more than one iron–sulphur cluster per molecule. Other sirohaem-containing enzymes have been found to contain a [4Fe–4S] cluster [see Hall *et al.* (1979) and references therein], but they are not normally detectable by e.s.r. unless they are reduced in the presence of a strong ligand to the sirohaem (e.g. Aparicio *et al.*, 1975). In the case of *E. coli* sulphite reductase, this behaviour has been attributed to strong antiferromagnetic coupling between the haem iron and the [4Fe–4S] cluster (Christner *et al.*, 1981). Thus the $[4Fe–4S]^{1+}$ state is only found in an e.s.r.-detectable state, with electron spin $S = \frac{1}{2}$ when the haem iron is in the diamagnetic low-spin Fe(II) state. To investigate the possibility of such a cluster in *E. coli* nitrite reductase, samples of the nitrite-free enzyme were treated with 0.5mM-KCN and reduced with 5mM-$Na_2S_2O_4$ and 0.2mM-Methyl Viologen. In other experiments the enzyme solution was saturated with CO gas and similarly reduced. In neither case were any additional e.s.r. signals around $g = 1.94$ observed.

The midpoint potential of the [2Fe–2S] cluster was determined by redox titration in the presence of mediator dyes and a platinum electrode, withdrawing samples and freezing for measurement of the $g = 2.01$, 1.92 signal. The enzyme was reduced either chemically with dithionite as in the apparatus of Dutton (1971) or electrochemically with a gold electrode (Ke *et al.*, 1977). The midpoint potential of the cluster was estimated to be -230 ± 15mV. The same value was obtained both by chemical reduction with dithionite and by electrochemical reduction. The iron–sulphur cluster appeared to be relatively stable. The duration of each redox titration was up to 2h, during which time the intensity of the signal with g values of 2.01 and 1.92 of the fully reduced enzyme did not diminish. However, it is likely that other components of the enzyme were damaged during this time. For example, it was not possible to obtain the midpoint potential of the sirohaem because of a rapid and irreversible loss of the e.s.r. signals around $g = 6$. The midpoint potential therefore probably refers to [2Fe–2S] clusters in the inactivated enzyme. However, it is

consistent with the observation that in the active enzyme, the iron–sulphur cluster was completely reduced by NADH in the presence of a 3-fold excess of NAD^+. Thus the midpoint potential in the active enzyme is considerably less negative than that of the NAD^+/NADH couple (−350 mV at pH 8).

Additional e.s.r. signals

An unusual series of signals was observed when samples of the enzyme were treated with excess $Na_2S_2O_4$ (Fig. 6). The most prominent of these were at $g = 2.18$ and 2.10, and at $g = 2.28$ and 2.23. The former were the first to appear (Fig. 6*a*). All the signals disappeared on prolonged treatment with $Na_2S_2O_4$ (Fig. 6*d*) and were not observed on reduction with reduced Methyl Viologen. The origin of these signals is not clear at present. However, dithionite, or its oxidation product, is capable of binding sirohaem. Transient e.s.r. signals in this region have been observed in spinach (*Spinacia oleracea*) nitrite reductase and *E. coli* sulphite reductase during treatment with $Na_2S_2O_4$ (Lancaster *et al.*, 1982), although they were not observed in *Cucurbita pepo* nitrite reductase (Cammack *et al.*, 1978). They were interpreted as due to unreduced low-spin haem. In *E. coli* sulphite reductase, a signal at $g = 2.52$, 2.29 and 2.07 was observed in a half-reduced state and attributed to the interaction between an oxidized [4Fe–4S] cluster and a high-spin ferrous haem (Janick & Siegel, 1979; Christner *et al.*, 1981). Both interpretations would imply that complete reduction of the enzyme by dithionite is relatively slow.

Discussion

The enzyme, as stored in the medium containing nitrite and ascorbate, gave an e.s.r. signal characteristic of nitrosyl haem. It seems likely that the effectiveness of these components of the stabilizing mixture is due to the formation of this relatively stable derivative of the sirohaem, which may thus be prevented from dissociating.

The removal of ^{14}NO by reduction with NADH or dithionite and its replacement with ^{15}NO indicates that the nitrosyl enzyme can turn over. Thus sirohaem–NO may be an intermediate in the reduction of NO_2^-, as has been suggested for the nitrite reductases of plants (Cammack *et al.*, 1978; Lancaster *et al.*, 1979). However, evidence that this species is kinetically competent is at present lacking.

E. coli nitrite reductase differs from the enzyme of higher plants in that is uses NADH rather than reduced ferredoxin as electron donor. In composition it differs in that it contains a flavin and a [2Fe–2S] cluster. This combination is commonly found in haem and iron–sulphur proteins that interact with NADH (Cammack, 1979).

The temperature-dependence and power-saturation behaviour of the e.s.r. signal imply that just one type of [2Fe–2S] cluster is present in the enzyme, which is in conflict with the analysis of five atoms of iron and four of labile sulphide per molecule (Jackson *et al.*, 1981*a*). The comparison of e.s.r. and analytical data is, however, complicated by the extreme lability of some of the prosthetic groups and the low integrated intensities of the e.s.r. spectra. All other sirohaem-containing nitrite reductases and sulphite reductases that have been examined contain [4Fe–4S] clusters (Lancaster *et al.*, 1979; Hall *et al.*, 1979; Christner *et al.*, 1981). There are thus several possibilities.

(*a*) The *E. coli* nitrite reductase contains two [2Fe–2S] clusters per haem, with identical e.s.r. spectra. This seems unlikely, as in other enzymes that contain two [2Fe–2S] clusters, such as xanthine oxidase, the two clusters are distinguishable by their e.s.r. lineshape and redox potentials.

(*b*) The enzyme contains a [2Fe–2S] cluster and also a [4Fe–4S] cluster that is for some reason undetectable by e.s.r. Such clusters occur, for example, in nitrogenase.

(*c*) The enzyme has a [2Fe–2S] cluster that replaces the [4Fe–4S] cluster in other nitrite reductases. This would imply that the analyses (Jackson *et al.*, 1981*a*) were confused by adventitious iron and sulphide.

The question as to the possible existence of a [4Fe–4S] cluster in this enzyme may only be resolved by the use of other spectroscopic techniques such as magnetic circular dichroism or Mössbauer spectroscopy.

We thank Dr. D. Patil and Dr. S. K. Chamorovsky for assistance with the redox potential measurements. This work was supported by grants from the U.K. Science Research Council.

References

Aparicio, P. J., Knaff, D. B. & Malkin, R. (1975) *Arch. Biochem. Biophys.* **169**, 102–107

Cammack, R. (1979) in *Metalloproteins: Structure, Function and Clinical Aspects* (Weser, U., ed.), pp. 162–184, Thieme Verlag, Stuttgart

Cammack, R. & Fry, L. V. (1980) *Biochem. Soc. Trans.* **8**, 642

Cammack, R., Barber, M. J. & Bray, R. C. (1976) *Biochem. J.* **157**, 469–478

Cammack, R., Hucklesby, D. P. & Hewitt, E. J. (1978) *Biochem. J.* **171**, 519–526

Christner, J. A., Münck, E., Janick, P. A. & Siegel, L. M. (1981) *J. Biol. Chem.* **256**, 2098–2101

Coleman, K. J., Cornish-Bowden, A. & Cole, J. A. (1978) *Biochem. J.* **175**, 483–493

Dutton, P. L. (1971) *Biochim. Biophys. Acta* **226**, 63–80

Feher, G. (1970) *Electron Paramagnetic Resonance with Applications to Selected Problems in Biology*, pp. 43–50, Gordon and Breach, New York

Fry, I. V., Cammack, R., Hucklesby, D. P. & Hewitt, E. J. (1980) *FEBS Lett.* **111**, 377–380

Fujita, T. & Sato, R. (1966) *J. Biochem. (Tokyo)* **60**, 691–700

Hall, M. H., Prince, R. G. & Cammack, R. (1979) *Biochim. Biophys. Acta* **581**, 27–33

Jackson, R. H., Cornish-Bowden, A. & Cole, J. A. (1981*a*) *Biochem. J.* **193**, 861–867

Jackson, R. H., Cole, J. A. & Cornish-Bowden, A. (1981*b*) *Biochem. J.* **199**, 171–178

Jackson, R. H., Cole, J. A. & Cornish-Bowden, A. (1982) *Biochem. J.* **203**, 505–510

Janick, P. A. & Siegel, L. M. (1979) *Abstr. Int. Congr. Biochem. 11th, Toronto* 437

Ke, B., Dolan, E., Sugahara, K., Hawkridge, F. M., Demeter, S. & Shaw, E. R. (1977) in *Photosynthetic Organelles — Structure and Function* (Miyachi, S., Katoh, S., Fujita, Y. & Shibata, K., eds.), pp. 373–382, Elsevier, Amsterdam

Kemp, J. D., Atkinson, D. E., Ehret, A. & Lazzarini, R. A. (1963) *J. Biol. Chem.* **238**, 3466–3471

Lancaster, J. R., Vega, J. M., Kamin, H., Orme-Johnson, N. R., Orme-Johnson, W. H., Krueger, R. & Siegel, L. M. (1979) *J. Biol. Chem.* **254**, 1268–1272

Lancaster, J. R., Salerno, J. C., Siegel, L. M. & Kamin, H. (1982) in *Electron Transport and Oxygen Utilization* (Ho, C., ed.), Elsevier/North-Holland, New York

Liu, M.-C., Peck, M. D., Jr., Abou-Jaoudé, A., Chippaux, M. & Le Gall, J. (1981) *FEMS Microbiol. Lett.* **10**, 333–337

Mejbaum-Katzenellenbogen, W. & Dobryszycka, W. H. (1959) *Clin. Chim. Acta* **4**, 515–522

Morse, R. H. & Chan, S. I. (1980) *J. Biol. Chem.* **255**, 7876–7882

Murphy, M. J., Siegel, L. M., Kamin, H., DerVartanian, D. V., Lee, J. P., Le Gall, J. & Peck, H. D. (1973) *Biochem. Biophys. Res. Commun.* **54**, 82–88

Palmer, G. (1979) in *The Porphyrins* (Dolphin, D., ed.), vol. 4, pp. 313–353, Academic Press, New York

Peisach, J., Blumberg, W. E., Ogawa, S., Rachmilewitz, E. A. & Oltzik, R. (1971) *J. Biol. Chem.* **246**, 3342–3355

Rupp, H., Rao, K. K. & Cammack, R. (1978) *Biochim. Biophys. Acta* **537**, 255–269

Siegel, L. M. (1978) in *Mechanisms of Oxidizing Enzymes* (Singer, T. P. & Ondarza, R. N., eds.), pp. 201–214, Elsevier, Amsterdam

Stolzenberg, A. M., Strauss, S. H. & Holm, R. H. (1981) *J. Am. Chem. Soc.* **103**, 4763–4778

Yonetani, T., Yamamoto, H., Erman, J. E., Leigh, J. S. & Reed, G. H. (1972) *J. Biol. Chem.* **247**, 2447–2455

Yoshimura, T., Ozaki, T., Shintani, Y. & Watanabe, H. (1979) *Arch. Biochem. Biophys.* **193**, 301–313