Low-temperature magnetic-circular-dichroism spectroscopy of the iron-molybdenum cofactor and the complementary cofactor-less MoFe protein of *Klebsiella pneumoniae* nitrogenase

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The major metal clusters of the MoFe protein, Kpl, of *Klebsiella pneumoniae* nitrogenase were characterized separately by low-temperature magnetic-circular-dichroism spectroscopy. The spectra and magnetization curves of the extracted iron-molybdenum cofactor, FeMoco, and of 'P' clusters in NifB⁻-Kpl, the inactive, FeMoco-less, MoFe protein from an *nifB* mutant, were measured and compared with those of the holoprotein. (When FeMoco and NifB⁻-Kpl are combined, active Kpl is formed.) Reduced NifB⁻-Kpl had a spectrum with a weak, paramagnetic, component superimposed on a diamagnetic background. The paramagnetic component was assigned to a contaminating, e.p.r.-active, species. Thionine-oxidized NifB⁻-Kpl had a spectrum and magnetization properties very similar to those of thionine-oxidized Kpl, demonstrating that the 'P' clusters are not significantly affected by the absence of the FeMoco clusters. The spectra of reduced isolated FeMoco had similar magnetization curves but sharper features and higher intensities than those of this centre in dithionite-reduced Kpl. Furthermore, a shoulder near 580 nm in the Kpl spectrum was absent from that of FeMoco. This may be due to the loss of a ligand or to a change in symmetry of the FeMoco cluster on extraction.

Biological nitrogen fixation is mediated by the enzyme nitrogenase, which comprises an Fe protein and an MoFe protein. The latter binds N₂ during its reduction to NH₃. MoFe proteins from all sources are complex metalloproteins with *M_r* values around 220 000, containing two molybdenum atoms with about 30 iron atoms and a slightly smaller number of acid-labile sulphur atoms per molecule (Smith, 1983). Mössbauer (Smith & Lang, 1974; Zimmermann *et al.*, 1978; Huynh *et al.*, 1980; Smith *et al.*, 1980), and e.p.r. (Smith *et al.*, 1972; O'Donnell & Smith, 1978) studies of these proteins in a variety of oxidation levels have resulted in identification of three types of metal centre named 'P' clusters (16Fe), the FeMoco

centres (12–16Fe) and the 'S' atoms ($\approx 2\text{Fe}$) (Zimmermann *et al.*, 1978; Smith, 1983).

Two oxidation states of the 'P' clusters have been observed by Mössbauer spectroscopy (Zimmermann *et al.*, 1978; Smith *et al.*, 1980). In the dithionite-reduced state of the protein these clusters are diamagnetic and apparently remain so during enzyme turnover. The isomer shifts of the iron atoms in the Mössbauer spectra (Smith & Lang, 1974; Münck *et al.*, 1975), extrusion experiments (Kurtz *et al.*, 1979) and e.p.r. spectroscopy of rapid-freeze oxidation experiments (Smith *et al.*, 1983) have led to the conclusion that the reduced 'P' clusters are probably fully reduced [4Fe–4S]⁰ clusters. On oxidation with thionine, 'P' clusters become paramagnetic, possessing an odd-electron spin system with a ground-state doublet $M_s > \pm \frac{1}{2}$ such that e.p.r. transitions within the ground-state doublets are forbidden.

The isolation and characterization from mutants

Abbreviations used: Kpl and Kp2, MoFe protein and Fe protein respectively of *Klebsiella pneumoniae*; NifB⁻-Kpl, MoFe protein from *nifB* mutants of *K. pneumoniae*; FeMoco, iron-molybdenum cofactor of Kpl; m.c.d., magnetic circular dichroism.

of *Klebsiella pneumoniae* of an inactive, FeMoco-less, MoFe protein termed NifB⁻Kp1 (Hawkes & Smith, 1983) enables the 'P' clusters to be examined separately from the FeMoco centres. NifB⁻Kp1, like active wild-type Kp1, is an $\alpha_2\beta_2$ -tetramer, but is far less stable than Kp1, deteriorating rapidly at room temperature or on mild oxidation. Typical preparations contained 0.4–0.9 Mo and 9.0 ± 0.9 Fe g-atoms per mol, and when activated by FeMoco had a rather low specific activity. The molybdenum in these preparations was not associated with the e.p.r. signal normally observed from FeMoco. All preparations exhibited a weak e.p.r. signal, $g_{av.} = 1.95$, probably not associated with activatable protein.

The FeMoco centres contain molybdenum, iron and acid-labile sulphur in the approximate proportions 1:6–8:4–9 in a cluster that is detectable by e.p.r. spectroscopy in the dithionite-reduced protein with g values near 4.3, 3.6 and 2.01. This cluster is extractable from the protein by treatment with *N*-methylformamide to yield a material of uncertain structure (Shah & Brill, 1977). The cofactor thus extracted will activate inactive MoFe protein (e.g. NifB⁻Kp1) obtainable from mutant bacteria that are unable to synthesize FeMoco. The e.p.r. and Mössbauer spectra of FeMoco in *N*-methylformamide are similar to those of the centre in intact protein, and indicate that the e.p.r. signal integrates to one electron spin per Mo atom and that six Fe atoms are associated with each spin (Rawlings *et al.*, 1978).

The other iron atoms, called 'S', in the protein represent only about two iron atoms in all, and apparently remain diamagnetic through oxidation-state changes of the protein (Zimmerman *et al.*, 1978).

We have undertaken a characterization of the 'P' and FeMoco clusters in Kp1 by low-temperature m.c.d. spectroscopy (Johnson *et al.*, 1981a). The m.c.d. spectra of paramagnetic species are temperature-dependent, increasing in intensity as the temperature is lowered, whereas the m.c.d. of diamagnetic species are temperature-independent and invariably weaker in magnitude than those of paramagnetic centres (Johnson *et al.*, 1981b). We used this property to distinguish the m.c.d. spectra of the 'P' and FeMoco clusters since, in the dithionite-reduced state, only the FeMoco centres are paramagnetic whereas in the thionine-oxidized state only the 'P' clusters are paramagnetic (Smith *et al.*, 1980). Therefore the m.c.d. spectra at liquid-He temperature of dithionite-reduced Kp1 are dominated by the contribution from the FeMoco cluster in its e.p.r.-active oxidation state. The m.c.d. spectra of thionine-oxidized Kp1 at low temperatures revealed another paramagnet with rather similar optical properties, and this was

assigned to the oxidized 'P' clusters (Johnson *et al.*, 1981a).

A variation of the m.c.d. intensity with magnetic field and temperature can be expressed as a magnetization curve, the form of which will depend on the magnetic properties of the ground electronic state, such as spin, zero-field splitting parameters and effective g -values (Thomson & Johnson, 1980). The m.c.d. magnetization properties of the reduced FeMoco and oxidized 'P' clusters of Kp1 are quite distinctive, providing an additional criterion for assigning the m.c.d. spectrum to a cluster of a given type.

In the present paper we report the m.c.d. properties of separated FeMoco and 'P' clusters and compare them with those observed with the holoprotein. These studies confirm our original identification of the m.c.d.-spectral properties of these clusters in the wild-type protein. The data also provide further useful characterization of the centres and give an indication of their interactions in the protein.

Materials and methods

Materials

Unless otherwise stated all chemicals were from BDH Chemicals (Poole, Dorset, U.K.) and biochemicals from Sigma Chemical Co. (Poole, Dorset, U.K.).

Growth of organisms, purification of proteins and isolation of FeMoco

Klebsiella pneumoniae M5a1 (wild-type) was grown, and the nitrogenase proteins, Kp1 and Kp2, were purified from it, by the methods of Eady *et al.* (1972) as modified by Smith *et al.* (1976). The mutant strain *K. pneumoniae* 1718 was grown, and the FeMoco-deficient NifB⁻Kp1 purified from it as described by Hawkes & Smith (1983). FeMoco was extracted into *N*-methylformamide (Aldrich Chemical Co., Gillingham, Dorset, U.K.) from purified Kp1 that had been precipitated by dimethylsulphoxide and the precipitate washed with *NN*-dimethylformamide (Smith, 1980).

Assay procedures

Kp1 was assayed for acetylene-reduction activity with an approx. 20-fold molar excess of Kp2 as described by Eady *et al.* (1972). NifB⁻Kp1 was incubated with an excess of FeMoco (Hawkes & Smith, 1983), and the active Kp1 thus formed was assayed as above. Similarly, FeMoco was incubated with an excess of a crude extract of cells of an *nifB* mutant of *K. pneumoniae* followed by assay of the active Kp1 thus formed.

Protein determinations were by the biuret method (Gornall *et al.*, 1949) or Folin-Ciocalteu

method (Lowry *et al.*, 1951) standardized against bovine serum albumin. Colorimetric Fe (Doeg & Ziegler, 1962) and Mo (Clarke & Axley, 1955) analyses were carried out on acid-digested samples of protein or FeMoco. The *N*-methylformamide was distilled from the FeMoco samples *in vacuo* before acid digestion.

Preparation of samples for m.c.d.

NifB⁻ Kp1. This protein is unstable at temperatures above 8°C, and so all operations were at below 5°C. Concentrated (>10 mg/ml) samples of Na₂S₂O₄-reduced and thionine-oxidized protein were prepared by redissolving centrifuged pellets of 33% (w/v)-(NH₄)₂SO₄-precipitated protein in appropriate volumes of N₂-sparged 40% (v/v) glycerol in aqueous 40 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] / NaOH buffer, pH 7.5, containing either 2 mM-Na₂S₂O₄ or 0.4 mM-thionine. The oxidized protein is very unstable (Hawkes & Smith, 1983) but lost less than 10% of its activity when incubated at 5°C with the dye for 5 min before being frozen in liquid N₂ for storage. The m.c.d. cell was filled as rapidly as possible (approx. 1 min) after the sample had been thawed anaerobically. The final concentration of oxidized thionine remaining in the samples was estimated to be less than 0.1 mM from its absorbance at 600 nm. Activity and protein determinations were carried out on the samples as prepared above.

FeMoco. The FeMoco samples were prepared in *N*-methylformamide/*NN*-dimethylformamide (1:1, v/v) by evaporating an *N*-methylformamide extract almost to dryness, measuring the residual volume and then adding the appropriate volumes of the two solvents containing 2% (v/v) water, 5 mM-Na₂HPO₄, pH 8, and 2 mM-Na₂S₂O₄. The solution was then centrifuged, and the supernatant was assayed for activity and Mo content before being frozen in liquid N₂ for storage. O₂ was rigorously excluded from all of the above operations.

M.c.d. spectra were measured with a JASCO J500D spectropolarimeter. The magnetic field was generated by a split-coil superconducting magnet (SpectroMag-4; Oxford Instrument Co., Oxford, U.K.) capable of generating a field of 5 T. Field calibration was carried out with a Hall probe. Samples were immersed in boiling liquid He for the temperature range 1.5–4.2 K. Above this temperature the samples were in a stream of He gas whose temperature was held steady by a temperature controller (type DTC-2; Oxford Instrument Co.) with a cryogenic linear temperature sensor device. Temperature was measured with a carbon-glass resistor (2000 Ω nominal resistance at room temperature; Lake Shore Cryotronics, Eden, NY,

U.S.A.) calibrated by Cryogenic Calibrations (Pitchcott, Bucks., U.K.). The intensities of the m.c.d. spectra are expressed in units of Δε, where Δε = ε_L - ε_R, being the difference between the molar absorption coefficients for left and right circularly polarized light respectively. The Δε values are not normalized per unit field. The magnetic fields at which spectra were recorded are given in the appropriate Figure captions.

Samples were held in anaerobic cells consisting of a pair of silica windows separated by an annular rubber gasket. The gasket was pierced by syringe needles to allow degassing of the internal cell volume and the subsequent filling with sample. The efficiency of these cells in excluding O₂ was tested with solutions of dithionite monitored optically at 308 nm for reaction with O₂.

All sample handling was carried out in a glove-box (Faircrest Engineering, Croydon, Surrey, U.K.) with a gas-recirculation system that decreased the O₂ concentrations to below 1 p.p.m.

Results

NifB⁻ Kp1

Samples of Na₂S₂O₄-reduced and thionine-oxidized NifB⁻ Kp1 protein were examined by low-temperature m.c.d. spectroscopy. No e.p.r. signals at *g* = 4.3, 3.7 and 2.01 were observed in the Na₂S₂O₄-reduced samples, confirming the absence of the FeMoco centre. All the reduced preparations showed a weak e.p.r. signal with *g* values of 2.034, 1.928 and 1.896, similar to that of a reduced ferredoxin containing a single [4Fe-4S]¹⁺ core cluster (Hawkes & Smith, 1983).

Fig. 1 shows the low-temperature m.c.d. spec-

Fig. 1. Low-temperature m.c.d. spectra of reduced NifB⁻ Kp1

The protein concentration was 50 μM, in 50 mM-Tris/HCl buffer, pH 7.8, containing 40% (v/v) glycerol. The path-length was 2.69 mm. The magnetic field was 5.1 T. Temperature: —, 1.55 K; ---, 4.22 K; ·····, 20 K.

trum of the dithionite-reduced $\text{NifB}^- \text{Kp1}$ at a series of temperatures between 1.5 and 20 K. The solution contained glycerol at a concentration of 40% (v/v) in order to obtain good-quality optical glasses. The e.p.r. spectrum of the sample (not shown) integrated to 0.035 electron/molecule of $\text{NifB}^- \text{Kp1}$ of M_r 218 000, and the overall intensity of the m.c.d. spectrum was very low, with $\Delta\epsilon$ values per molecule of protein at a given field and temperature about one-tenth those of the oxidized protein (Fig. 2). The signal (Fig. 1) was temperature-dependent, as comparison of the 1.5 K and 20 K spectra clearly shows. However, the temperature-dependent component is superimposed on a diamagnetic signal with $\Delta\epsilon_{550} < 20 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 5 T. The $\Delta\epsilon$ values of diamagnetic $[\text{4Fe-4S}]^{2+}$ clusters are typically $5\text{--}6 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at magnetic fields of 5 T (Johnson *et al.*, 1981b). This is the value obtained for reduced HiPIP (high-potential

iron-sulphur protein) and oxidized ferredoxin from *Clostridium pasteurianum*, i.e. $[\text{4Fe-4S}]^{2+}$ -oxidation-level protein. Hence a protein containing between 2 and 4 such diamagnetic $[\text{4Fe-4S}]$ clusters per molecule should not have a $\Delta\epsilon$ value larger than about $20 \text{ M}^{-1} \cdot \text{cm}^{-1}$, in accord with our spectrum at 20 K. The m.c.d. of the major paramagnetic component between 450 and 800 nm has a form rather similar to that of a $[\text{4Fe-4S}]^{1+}$ ferredoxin, as in reduced *Clostridium* ferredoxin (Johnson *et al.*, 1981b). Hence it seems likely that this component is associated with the weak e.p.r. signals previously reported (Hawkes & Smith, 1983). The sharp negative peak at 420 nm and the positive peak at 403 nm clearly also belong to a paramagnetic species, and are probably haem transitions from low concentrations of a contaminating protein. The m.c.d. of haems include a sharp peak and trough in the Soret region.

Fig. 2. (a) Low-temperature m.c.d. spectra of thionine-oxidized $\text{NifB}^- \text{Kp1}$ and (b) comparison of the low-temperature m.c.d. spectra of thionine-oxidized $\text{NifB}^- \text{Kp1}$ and wild-type Kp1 protein

(a) The protein concentration was $54 \mu\text{M}$, in 50 mM-Tris/HCl buffer, pH 7.8, containing 40% (v/v) glycerol. The solution contained thionine. The path-length was 2.36 mm. The magnetic field was 5.1 T. Temperature: —, 1.54 K; ----, 2.07 K. (b) The magnetic field was 5.1 T in both cases and the temperature was 1.54 K for thionine-oxidized $\text{NifB}^- \text{Kp1}$ (—) and 1.53 K for wild-type Kp1 (----). The sample conditions for the sample were as given in (a) above for $\text{NifB}^- \text{Kp1}$ and as given in the legend to Fig. 3 of Johnson *et al.* (1981a) for Kp1 .

The thionine-oxidized form of NifB⁻Kp1 was prepared as described in the Materials and methods section. No attempt was made to remove oxidizing dye because of the instability of the protein. Thionine does not, however, contribute to the low-temperature m.c.d. spectrum between 300 and 750nm even at concentrations much greater than those used in the protein samples.

The m.c.d. spectra shown in Fig. 2 are at temperatures of 1.54 and 2.07 K. Two different batches of thionine-oxidized NifB⁻Kp1 have been examined and the forms of the m.c.d. spectra are identical. $\Delta\epsilon$ values, based on protein determinations, agreed to within 5%. The form of the spectrum is very similar to that of thionine-oxidized wild-type Kp1, which is shown in Fig. 2(b). There is a slight shift of the long-wavelength

band from 800nm in oxidized Kp1 to about 795 nm in NifB⁻Kp1. The striking new feature of the NifB⁻Kp1 m.c.d. spectrum is the presence of a sharp trough of 420nm. We assign this band to the presence of the haem impurity, which was also detected in the m.c.d. spectrum of the dithionite-reduced NifB⁻Kp1 protein. Presumably the haem remains in a reduced state under our conditions for thionine oxidation.

M.c.d. magnetization curves for the peaks of NifB⁻Kp1 protein at 800, 515 and 380nm are given in Fig. 3. The curves are very distinctive, with a steep initial slope at low magnetic field, and show magnetic saturation at 1.5 K at the relatively low magnetic field of approx. 3 T. These properties have also been observed for the thionine-oxidized 'P' clusters in wild-type Kp1 (Johnson *et al.*,

Fig. 3. M.c.d. magnetization curves plotted at three different wavelengths from the m.c.d. spectra of thionine-oxidized NifB⁻Kp1

The conditions for the sample were as given in the legend to Fig. 2(a). Wavelength: (a) 800nm; (b) 515nm; (c) 380nm. Temperature: $\circ$, 1.54 K; $\square$, 2.07 K; $\triangle$, 4.22 K. Magnetic-field values were between 0 and 5.1 T. Points indicated $\bullet$ have been normalized to 100% of magnetic saturation for all three curves.

1981a). The intercept values of approx. 0.127 lie in close agreement with those of Kp1. These curves prove unambiguously that the three major features of the m.c.d. spectrum belong to the same paramagnet, and furthermore they reveal a paramagnet with an electronic doublet as a ground state because the signal magnetizes completely (Thomson & Johnson, 1980). The effective g -factors of

this doublet are very large, since the signals magnetize readily. These properties are similar for thionine-oxidized NifB⁻ Kp1 and wild-type Kp1, showing the essential similarity of the ground-state electronic structure of the clusters in the two proteins. We determined the ground-state spin, S , as $\frac{5}{2}$ or $\frac{7}{2}$ for Kp1 (Johnson *et al.*, 1981a). No information has previously been available about

Fig. 4. (a) Low-temperature m.c.d. spectra of reduced *Klebsiella pneumoniae* FeMoco and (b) comparison of low-temperature m.c.d. spectra of reduced Kp1 at 1.57 K and FeMoco at 1.53 K.

The FeMoco concentration was 46 μM in molybdenum. The path-length was 2.32 mm. The magnetic field was 5 T. The specific activity was 276 units/ng-atom of Mo. The solvent was *NN*-dimethylformamide/*N*-methylformamide (1:1, v/v). Temperature: —, 1.53 K; ----, 4.22 K; ·····, 23.5 K. (b) The sample conditions for FeMoco were as given in (a) above. The spectrum of reduced Kp1 (—) is taken from Johnson *et al.* (1981a). The conditions for this latter were as follows: concentration 62 μM in protein, 50 mM-Tris/HCl buffer, pH 7.4, containing 10 mM-MgCl₂ and 50% (v/v) ethanediol, path-length 3.36 mm; specific activity 1700 units/mg of Kp1; estimated Mo content 1.35 ± 0.13 ng-atom/mol of Kp1; magnetic field 5 T.

the electronic properties of the iron-sulphur clusters in NifB⁻ Kp1 because they are e.p.r. silent and yield optical spectra lacking in useful detail.

The $\Delta\epsilon$ values (expressed per mol of protein) of thionine-oxidized NifB⁻ Kp1 and Kp1 at comparable fields and temperatures are in the same range, although the values of the former are consistently lower by 20–30%. The wild-type Kp1 had a specific activity of 1500 units/mg (Johnson *et al.*, 1981a). The specific activity of Kp1 preparations can be related to their FeMoco content (Hawkes *et al.*, 1984), with fully active protein containing two FeMoco clusters/molecule having a specific activity of 2520 ± 250 units/mg. If we accept the 'all-or-none' hypothesis in which each protein molecule contains all, exactly half or none of its maximum metal complement (for a discussion see Smith, 1983), then the protein's specific activity will also be related to its 'P'-cluster content. Thus the Kp1 used for the m.c.d. spectra would be expected to contain approx. 9.5 g-atoms of Fe as 'P' clusters per molecule. Our NifB⁻ Kp1 preparations contained 9 ± 0.9 Fe atoms per molecule. These data, coupled with the similarity in intensities of the m.c.d. spectra, are striking evidence that the same number of 'P' clusters with the same absorption coefficients were present in the wild-type and mutant protein samples used, despite the relatively low specific activity (approx. 370 units/mg) of NifB⁻ Kp1 after activation by FeMoco.

FeMoco

FeMoco was extracted from wild-type Kp1 by the procedures described in the Materials and methods section. The cofactor was diluted to 50% (v/v) with degassed *NN*-dimethylformamide. We found that *NN*-dimethylformamide/*N*-methylformamide (1:1, v/v) forms excellent-quality optical glasses, and we therefore employed this medium for the low-temperature m.c.d. studies. Na₂S₂O₄ was always present in excess in the samples, although its solubility in the solvent mixture is low. On addition of *NN*-dimethylformamide to a sample the characteristic e.p.r. signals, $g = 4.6, 3.3$ and 2.0 , were lost unless extra dithionite was added.

The m.c.d. spectra of FeMoco at several temperatures between 1.5 K and 23.5 K are shown in Fig. 4(a). $\Delta\epsilon$ values are based on molybdenum determinations. A comparison between the forms of the m.c.d. spectra of FeMoco as extracted and protein-bound in wild-type Kp1 is given in Fig. 4(b). Note that the $\Delta\epsilon$ scale is expressed per g-atom of molybdenum in the case of FeMoco and per mol of protein (M_r 218000) in the case of reduced Kp1. The Mo content of the Kp1 sample can be calculated as 1.35 g-atom of Mo/mol from the known relationship between molybdenum content

and activity (Hawkes *et al.*, 1984), and should be accurate to $\pm 10\%$. All of the transitions observed with the protein are present in the FeMoco spectra, except for a shoulder at approx. 580 nm, which is much less distinct. However, the relative intensities of the 800 nm and 520 nm bands differ and the $\Delta\epsilon$ per g-atom Mo of the protein spectrum is lower than that of the extracted FeMoco.

The m.c.d. magnetization curves of the FeMoco are presented in Fig. 5. These curves we call a complex nested set with curves run at different temperatures having different field-dependencies (Thomson & Johnson, 1980). Qualitatively similar curves were observed with the holoprotein (Johnson *et al.*, 1981a), but the curve measured at 1.5 K saturates more completely than does the curve for the holoprotein. Hence the sets of magnetization curves for the extracted FeMoco and the protein are not superimposable. In the holoprotein there is a sizeable contribution to the magnetization curves from the diamagnetic contribution of the 'P' clusters. This is of course absent from the FeMoco curves. There was some slight variation in the magnetization properties of the FeMoco, depending on the preparation. E.p.r.-spectral parameters also vary slightly with FeMoco preparation. We have taken this nested set of curves as an empirical indicator of the identity of the $S = \frac{3}{2}$ electronic ground state with a predominantly axial zero-field splitting, as identified by an analysis of the e.p.r. spectrum (Münck *et al.*, 1975). The simulation of theoretical m.c.d. magnetization curves for an

Fig. 5. *M.c.d. magnetization curves for Klebsiella pneumoniae FeMoco plotted at 520 nm*

The conditions for the sample were as given in the legend to Fig. 4(a). Magnetic-field values were between 0 and 5.1 T. Temperature: ○, 1.58 K; □, 1.934 K; △, 4.22 K. ● has been normalized to 100% of magnetic saturation.

electronic ground state of this complexity has not yet been achieved. However, in support of our argument, it has been possible to simulate the form of a nested set of magnetization curves for the much simpler example of an atomic-like transition $^4S \rightarrow ^4P$ with a zero-field splitting imposed upon the 4S ground state only (E. Kamalaku, A. J. Thomson & R. Grinter, unpublished work).

Discussion

In a previous paper (Johnson *et al.*, 1981a) the low-temperature m.c.d. spectra of the two major metal-containing components of Kpl were characterized in their accessible paramagnetic states. They were the FeMoco centre in its $S = \frac{3}{2}$ state and the oxidized state of the 'P' clusters. This represented the first attempt to deconvolute the optical properties of these two types of metal cluster. In the present investigation we have been able to examine the low-temperature m.c.d. spectra of these two clusters in physically separated forms. The results show that the spectra of the separated components are remarkably similar to those in the active protein. This establishes the correctness of our earlier analysis.

The NifB⁻ Kpl protein is unusual among iron-sulphur-containing proteins in containing clusters that are e.p.r.-silent in both the oxidized and the reduced states. (We take the variable-intensity e.p.r. signals at $g = 1.94$ to be either decomposition products of the active clusters or to be protein-containing $[4Fe-4S]^+$ clusters from impurities that have co-purified.) Hence there are rather few spectroscopic techniques with which to characterize it. Low-temperature m.c.d. spectroscopy is clearly very valuable for this purpose. The comparison that we have been able to make with the m.c.d. properties, both spectra and magnetization curves, of the 'P' clusters in wild-type Kpl and in NifB⁻ Kpl establishes for the first time a structural and an electronic similarity of the clusters in both proteins. Since the mutant protein is enzymically activated by addition of FeMoco, it was obvious that the former must contain the clusters in their correct state in the re-activated enzyme. However, the importance of the present observation is a demonstration that the 'P' clusters are virtually identical in the two proteins. The m.c.d. magnetization properties establish that the ground-state electronic spin is the same and that the zero-field splitting parameters of the ground state are little altered. This suggests that the 'P' clusters are not significantly altered chemically or physically by the presence of the cofactor centres, and lends credence to the suggestion that their role is to store electrons and mediate electron transfer to and from the FeMoco centres, where binding of

N₂ takes place (Hawkes *et al.*, 1984). We do not fully understand why Kpl and NifB⁻ Kpl preparations containing, as indicated in the present work by m.c.d. spectroscopy, almost identical numbers of 'P' clusters per molecule should have such different specific activities (1500 and 370 units/mg respectively). Current evidence indicates (T. R. Hawkes & B. E. Smith, unpublished work) that a large proportion of putative FeMoco-binding sites in our NifB⁻ Kpl preparations were blocked in some way, possibly by a molybdenum-containing but inactive species. This hypothesis would be consistent with the observation that when FeMoco is bound by NifB⁻ Kpl its full activity is expressed (Hawkes & Smith, 1983).

The m.c.d. spectra of isolated FeMoco are similar to those of the centre in the active protein. The m.c.d. magnetization properties are also very similar. E.p.r. and Mössbauer studies (Rawlings *et al.*, 1978) had already shown that the centre was essentially unchanged on extraction from the protein environment. The low-temperature m.c.d. spectra of iron-sulphur clusters in this paramagnetic state are relatively well-structured compared with the electronic absorption spectra. We have as yet little understanding of the assignment of spectral features, but it is likely that they involve charge-transfer transitions from the ligands, both protein-bound and sulphide ion, on to the metal core. One clear difference between the two spectra is that the spectrum of the extracted cofactor has sharper features. Thus the pronounced shoulder at 580 nm in the m.c.d. spectrum of the reduced protein-bound centre is not visible in that of the extracted cofactor. It could be either that this transition is absent because one or more crucial ligands are lacking or that the shoulder has coalesced with the major peak at 520 nm. This could occur if the geometry of the cluster were more regular in the extracted state. Similarly the two features at 720 and 800 nm in the spectrum of the protein-bound centre are slightly less well-resolved in the extracted spectrum.

The $\Delta\epsilon$ values of the m.c.d. of the extracted FeMoco are apparently higher (on a molybdenum basis) than for the centre in the protein, particularly if the background due to the diamagnetic 'P' clusters in the protein is taken into account. For the 520 nm band this might be explained in terms of the sharpening of the band in the spectrum of extracted FeMoco, i.e. the area under the band may not change. However, the 800 nm band is also more intense relative to the 520 nm band in this spectrum. M.c.d. spectroscopy clearly provides a valuable spectroscopic probe of the electronic states of FeMoco and a means of comparing the nature of this centre both within the protein environment and as extracted.

We have no evidence from any of our experiments of the presence of the minor component called 'S'. This is not surprising if it remains diamagnetic under all conditions and is only a small fraction of the total iron, and thus would be expected to make only small contributions to the spectra.

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