Characterization of $[4\text{Fe-4S}]^{2+}$, $[4\text{Fe-4Se}]^{2+}$ and hybrid (S, Se) clusters in *Clostridium pasteurianum* ferredoxin

A resonance Raman study

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Resonance Raman spectra of native $2[4\text{Fe-4S}]$ ferredoxin from *Clostridium pasteurianum* and of its selenium-substituted analogue $2[4\text{Fe-4Se}]$ are compared. The experimental conditions used in this study included low temperature (approx. 25 K) and the absence of a glass sample cell, thus ensuring high signal-to-noise ratios. The spectra of the $2[4\text{Fe-4S}]$ and $2[4\text{Fe-4Se}]$ ferredoxins display similar numbers of bands, but the resonance Raman patterns differ largely, except for two bands observed at 353 cm^{-1} and 365 cm^{-1} in spectra of the native ferredoxin, which are only moderately shifted upon S→Se substitution and are attributed to Fe–S(cysteine) stretching modes. The activities of the latter modes, enhanced by 457.9 nm excitation, are nearly equal in both ferredoxins. These data were used to demonstrate the presence of hybrid clusters $[4\text{Fe-(4-n)S-nSe}]$ ($n = 1, 2, 3$) in a ferredoxin the active sites of which had been reconstituted in the presence of both S and Se.

It has been shown that the inorganic sulphur atoms of the two $[4\text{Fe-4S}]$ clusters present in *Clostridium pasteurianum* ferredoxin (Cp Fd) can be selectively replaced by selenium (Meyer & Moulis, 1981). The Se-substituted Fd contains two $[4\text{Fe-4Se}]$ clusters, and its biochemical properties are nearly identical with those of the native protein (Moulis & Meyer, 1982). However, in view of the differences in electronegativity, ionic radius and atomic mass of the two chalcogenides, structural discrepancies may be expected between the $[4\text{Fe-4S}]$ and $[4\text{Fe-4Se}]$ clusters in Cp Fd. These are investigated in the present work by resonance Raman (RR) spectroscopy, a useful probe for chromophores in proteins (Spiro & Gaber, 1977). In recent years RR spectroscopy of iron–sulphur proteins has yielded to significant progress, allowing the identification and structural studies of various clusters present in several ferredoxins (Spiro *et al.*, 1982; Lutz *et al.*, 1983).

The reactivities of S and Se in the reconstitution of Cp Fd have been demonstrated to be nearly equal (Moulis & Meyer, 1982), but the exact nature of the clusters present in a protein reconstituted

with a mixture of both chalcogenides has so far remained undetermined. RR spectroscopy under low-temperature conditions (20–30 K) with a set-up avoiding the use of a glass cell and of high incident powers ensured higher signal-to-noise ratios than in previous reports (Spiro *et al.*, 1982), and allowed us to establish the presence of hybrid $[4\text{Fe-(4-n)S-nSe}]$ ($n = 1, 2, 3$) clusters in a ferredoxin reconstituted in the presence of both chalcogenides.

Materials and methods

C. pasteurianum W5 (A.T.C.C. 6013) Fd was purified and total or partial Se substitutions were carried out as previously described (Moulis & Meyer, 1982). The proteins were always handled under anaerobic conditions.

For RR measurements, 5 μl droplets of sample (30–100 mg of protein/ml in 0.2–0.5 M-potassium phosphate buffer, pH 7.5) were rapidly frozen on a microscope cover-slip placed over boiling liquid N_2 . The samples were immediately introduced into a cryostat flushed with cold He gas. Temperatures were measured with accuracies close to 2 K by a calibrated resistor placed in the vicinity of the sample and were kept constant within 2 K during

Abbreviations used: Fd, ferredoxin; Cp Fd, *Clostridium pasteurianum* ferredoxin; RR, resonance Raman.

the recording of spectra. Several RR spectra of Cp Fd have been recorded in the 15–32 K range, and they were all identical. Therefore valid comparisons could be made between RR spectra recorded at various temperatures within this range.

It is well known that buffer solutions undergo pH shifts upon freezing (Williams-Smith *et al.*, 1977), namely approx. -1.0 to -1.5 pH unit in the case of potassium phosphate. These pH shifts were, however, shown to be greatly decreased or even suppressed in the presence of 40 mg of bovine serum albumin/ml (Williams-Smith *et al.*, 1977), and our experiments were carried out on samples containing 30–100 mg of protein/ml. Nevertheless, in order to check a possible effect of pH changes on the present results, we have recorded RR spectra of native Cp Fd at pH 6.0 (in phosphate) and at pH 9.0 (in borate). These spectra were identical with the one shown in Fig. 1(a) and obtained at pH 7.5.

RR spectra were obtained as described previously (Lutz *et al.*, 1983), by using the 457.9 nm emission from an argon laser (Spectra Physics 170.05) and a Jobin-Yvon HG2S-UV Raman spectrometer. Typical spectral resolution was 6 cm^{-1} at 300 cm^{-1} . The signal-to-noise ratios were improved by summation of four to nine spectra in a multichannel analyser (Tracor-Northern 1710). The intensities of the Raman lines were checked after each scan, these usually remaining constant over at least five scans. Whenever a decrease in intensity was observed, a new scattering site was used. In addition, control u.v.-visible-absorption spectra run on laser-irradiated samples after thawing and diluting gave no indication of active-site degradation.

Results and discussion

Oxidized Cp Fd at 30 K and 457.9 nm excitation yields an RR spectrum (Fig. 1a) dominated by a strong 340 cm^{-1} band. Ten RR lines are resolved in the Fe–S stretching region, i.e. $200\text{--}400\text{ cm}^{-1}$, four of which had not been previously detected (Johnson *et al.*, 1982; Spiro *et al.*, 1982). This larger number of bands is not due to any temperature effect, as the same number of bands is observed at room temperature (Lutz *et al.*, 1983). Neither can this higher number be due to any equivalence of the two $[\text{4Fe-4S}]$ clusters present in Cp Fd, since the same numbers of bands were observed in the spectrum of *Bacillus stearothermophilus* Fd, which contains a single $[\text{4Fe-4S}]$ cluster (Mullinger *et al.*, 1975; Lutz *et al.*, 1983). Hence these clusters must be significantly distorted from the idealized T_d symmetry (six Raman active Fe–S stretching modes).

Fig. 1. RR spectra of $[\text{4Fe-4S}]$ and $[\text{4Fe-4Se}]$ ferredoxins from *C. pasteurianum*

The excitation wavelength was 457.9 nm, the scanning speed was $50\text{ cm}^{-1}/\text{min}$, the time constant was 1.8 s, and the slitwidth was $500\text{ }\mu\text{m}$. Other conditions are described in the Materials and methods section. (a) $[\text{4Fe-4S}]$ Fd at 30 K, four scans. (b) $[\text{4Fe-4Se}]$ Fd at 25 K, six scans.

A low-temperature RR spectrum of oxidized Se-substituted Cp Fd is shown in Fig. 1(b). The strongest lines are clustered in three groups at approx. 220, 285 and 375 cm^{-1} , thus constituting a spectrum quite different from that of native Cp Fd. This must be due to the very large mass difference between S and Se, which probably results in distinct mode patterns for the two types of clusters. The Se-substituted Cp Fd used here had been reconstituted with natural-abundance selenium: as the latter contains significant proportions of five isotopes (^{76}Se – ^{82}Se), some line-broadening is observed. Nevertheless, 11 frequencies are detected in the $150\text{--}400\text{ cm}^{-1}$ range (Fig. 1b), i.e. nearly the same number as in the spectrum of native Cp Fd between 200 and 400 cm^{-1} . A further comparison of the spectra in Figs. 1(a) and 1(b)

suggests that the 353 cm^{-1} and 365 cm^{-1} bands of the former undergo the smallest frequency shifts, to 360 cm^{-1} and 374 cm^{-1} respectively, upon S $\rightarrow$ Se substitution. Both of these bands have been assigned to $\nu[\text{Fe-S(cysteine)}]$ stretching modes (J.-M. Moulis, J. Meyer & M. Lutz, unpublished work). A single band had been previously resolved in this region at 356 cm^{-1} , and was insensitive to $^{32}\text{S}\rightarrow^{34}\text{S}$ isotopic substitution on core sulphur atoms (Johnson *et al.*, 1982; Spiro *et al.*, 1982). Therefore the frequencies of the Fe-S(cysteine) stretching modes do not appear to be greatly affected by the nature of the core chalcogenide atoms, and the quite different RR patterns observed for the native and the Se-substituted Fd can be attributed to the modes of the inorganic [4Fe-4X] (X = S, Se) cores.

It has previously been shown that, when the active sites of Cp Fd are reconstituted in the presence of iron, sulphide and selenide, the relative amounts of the two chalcogenides present in the protein are equal to those initially present in the reaction medium. The shape of the electronic spectra of such ferredoxins leaves little doubt that they contain cubane clusters (Moulis & Meyer, 1982). Two possibilities may be considered for the composition of the active sites present in such hybrid ferredoxins: they may be either only [4Fe-4S] and [4Fe-4Se] clusters, or they may include hybrid clusters containing both chalcogenides in the same cubane structure. An answer to this question is readily provided by RR spectroscopy, since it detects almost all the molecular vibrations of the Fe-X (X = S, Se) chromophores and therefore reveals marked differences between the spectra of the native and the Se-substituted Fd (Fig. 1).

A RR spectrum of a 1:1 mixture of native and of Se-substituted Cp Fd is shown in Fig. 2(a). Some of the features of both the [4Fe-4S] (340 cm^{-1} and 256 cm^{-1}) and the [4Fe-4Se] (221 cm^{-1} and 286 cm^{-1}) clusters can be distinguished. The spectrum of Fig. 2(a) can be readily simulated, as shown in Fig. 2(b), by adding the spectra of the native and the Se-substituted Fd as follows. The former spectrum (Fig. 1a) is normalized to the intensity of the 256 cm^{-1} band of the mixture spectrum (Fig. 2a), and the spectrum of Se-substituted Fd is normalized to the 221 cm^{-1} band of the mixture spectrum. These two bands have been chosen because they each are characteristic of one Fd alone. The experimental (Fig. 2a) and the simulated (Fig. 2b) spectra are expectedly similar and constitute the reference to which a spectrum of a hybrid (S, Se) Fd must be compared. It is worth noticing that a spectrum very similar to the one of Fig. 2(b) is obtained (not shown) by adding the two spectra of Fig. 1 after equalizing the 353 cm^{-1} band of Fig. 1(a) and the 360 cm^{-1} band of Fig.

Fig. 2. RR spectra of a 1:1 mixture of 2[4Fe-4S] Cp Fd and 2[4Fe-4Se] Cp Fd and of a hybrid (S, Se) Fd (a) Equimolar mixture of 2[4Fe-4S] Fd and 2[4Fe-4Se] Fd at 32 K, six scans. (b) Sum of the spectra of Figs. 1(a) and 1(b) calibrated as described in the text. (c) Hybrid (S, Se) Fd reconstituted in the presence of equimolar amounts of S and Se; spectrum was at 28 K, nine scans. Other conditions were as indicated in Fig. 1 legend.

1(b). Therefore similar RR activities at 457.9 nm excitation can be inferred for the Fe-S(cysteine) stretching modes of both ferredoxins, probably due to the low sensitivity of the (cysteine)S $\rightarrow$ Fe charge-transfer transitions to core chalcogenide substitution.

Fig. 2(c) shows the RR spectrum of Cp Fd reconstituted in the presence of equal amounts of sulphide and selenide, and which consequently contains equal amounts of both chalcogenides (Moulis & Meyer, 1982). It displays a strong band peaking at 365 cm^{-1} that is obviously asymmetric, and the bandwidth of which suggests the involvement of several components. At lower frequency, numerous bands are clustered in the $200\text{--}300\text{ cm}^{-1}$ region, but, unlike in the spectra of Figs. 2(a) and 2(b), none of the characteristic lines of the native nor of the Se-substituted Fd are observed. The

comparison of Figs. 2(a) and 2(c) therefore shows that, if the hybrid (S, Se) Fd may contain [4Fe-4S] and [4Fe-4Se] centres, these must be in relatively small amounts, compared with a large proportion of hybrid [4Fe-(4-n)S-nSe] ($n = 1, 2, 3$) clusters. The latter observation points to a random distribution of the chalcogenides in reconstituted clusters, yielding approx. 5% each of [4Fe-4S] and [4Fe-4Se] active sites. Thus the 'all-or-none' reconstitution reaction demonstrated for 2[4Fe-4S] *Clostridium acidi-urici* Fd (Hong & Rabinowitz, 1970) and confirmed for 2[4Fe-4Se] Cp Fd (Moulis & Meyer, 1982) does not discriminate between the two chalcogenides in mixed (S, Se) reconstitutions. In view of the differences in the general chemical reactivities of sulphide and selenide, their identical reactivity in the reconstitution of [4Fe-4X] clusters in Cp Fd suggests that in the latter reaction the incorporation of X^{2-} ($X = S, Se$) is not the limiting step. Detailed studies on the synthesis of iron-sulphur clusters (Christou *et al.*, 1977; Hagen *et al.*, 1981) have shown that the reaction proceeds in two main steps, the first being the binding of iron and the second being the reaction with sulphide, which ends up in the formation of the [4Fe-4S] core. If such a reaction scheme holds true for the reconstitution of the active sites of Cp Fd, our results would suggest that the overall reaction is limited by the binding of iron rather than by the binding of chalcogenide.

The existence of hybrid (S, Se) clusters has previously been evidenced in the case of $[Fe_4X_4(SR)_4]^{n-}$ ($n = 2, 3$) and $[Fe_2X_2(SR)_4]^{2-}$ ($X = S, Se$) synthetic clusters, by using 1H n.m.r. of thiolate ligands (Reynolds & Holm, 1981) and, in the case of [2Fe-2X] ($X = S, Se$) centres in adrenodoxin, by using e.p.r. (Mukai *et al.*, 1974). However, in the case of Cp Fd, e.p.r. spectra of the reduced, native and Se-substituted Fd, although different, are too complex and overlapping to permit any conclusion to be drawn about the nature of the [4Fe-4X] ($X = S, Se$) centres in hybrid Fd (J. Gaillard, unpublished work). The same is true for Mössbauer spectra of Cp Fd (P. Auric, un-

published work). RR spectroscopy thus brings an answer to a question that, to our knowledge, cannot be easily solved by any other currently available physicochemical techniques. We therefore expect RR spectroscopy to be useful, not only for the elucidation of the vibrational behaviour of Fe-S centres, but also for the investigation of more general problems related to the structure and function of iron-sulphur proteins.

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