X-ray-absorption-spectroscopic evidence for a novel iron cluster in hydrogenase II from *Clostridium pasteurianum*

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Hydrogenase II from *Clostridium pasteurianum* contains three different iron–sulphur clusters. Two are $[4\text{Fe–4S}]^{(2+,1+)}$ clusters, whereas the other, which is thought to be the site of interaction with H_2 and is known as the ‘H cluster’, is of unknown structure and possesses unusual spectroscopic properties. Analysis of the iron e.x.a.f.s. spectra shows that the H cluster contains iron co-ordinated mostly to sulphur and possesses 2.8 Å (1 Å = 0.1 nm) Fe–Fe separations when oxidized and 3.3 Å Fe–Fe separations when reduced with H_2 . The data suggest that the reduced H cluster represents a new structural type of iron–sulphur cluster.

INTRODUCTION

The anaerobic nitrogen-fixing bacterium *Clostridium pasteurianum* possesses two different hydrogenases which belong to a small group of hydrogenases that contain only iron and no other metal (e.g. Adams *et al.*, 1986). The enzymes are among the most active hydrogenases known, with catalytic-centre activities approaching 10^6 s^{-1} (Adams *et al.*, 1986). One of the enzymes, hydrogenase I, is active in catalysing both H_2 oxidation and H_2 evolution, whereas the other, hydrogenase II, preferentially catalyses H_2 oxidation (Adams & Mortenson, 1984a). Both hydrogenases possess two different types of iron cluster, known as ‘F’ and ‘H’ clusters. The former are of the ferredoxin $[4\text{Fe–4S}]^{(2+,1+)}$ type (Adams *et al.*, 1986), whereas the latter, which are thought to be the site of hydrogen interaction, are of unknown structure, and may contain six iron atoms (M. W. W. Adams, E. Eccleston & J. B. Howard, unpublished work). Hydrogenase I possesses ~ 20 Fe atoms per molecule as four F clusters and one H cluster, whereas hydrogenase II possesses 14 Fe atoms as two different F clusters (denoted ‘F’ and ‘F’’) and one H cluster (M. W. W. Adams, E. Eccleston & J. B. Howard, unpublished work).

The structures of the H clusters are of interest not only in connection with the mechanism of the enzyme, but also in that they appear to be spectroscopically unique in bio-inorganic chemistry (Adams *et al.*, 1986; Macor *et al.*, 1987; Rusnak *et al.*, 1987; Telser *et al.*, 1986, 1987). E.x.a.f.s. spectroscopy is capable of providing structural information on polycrystalline samples that is unavailable from other techniques. We present herein an e.x.a.f.s. study of *C. pasteurianum* hydrogenase II, and show that the reduced H cluster is of a structural type previously unknown in biology.

MATERIALS AND METHODS

Sample preparation

Hydrogenase II from *C. pasteurianum*, purified as described previously (Adams & Mortenson, 1984b), was used at a final concentration of 55 mg/ml in 50 mM-Tris buffer, pH 8.0, with 50% (v/v) ethylene glycol glassing agent. Enzyme activity was determined by H_2 oxidation

under standard assay conditions (Adams & Mortenson, 1984b). Samples of the reduced enzyme were prepared under H_2 in the same buffer containing sodium dithionite (1 mM). The oxidized sample was prepared anaerobically under argon by adding thionine to slight excess, until the blue colour of the dye persisted. Samples were transferred anaerobically to Perspex (Lucite) e.x.a.f.s. cells faced with a 6 µm-thick polypropylene window, rapidly frozen, and stored in liquid N_2 . Sample integrity was assayed after data collection, after thawing under H_2 , and both samples were found to retain greater than 95% of their original activity.

Data collection

X-ray-absorption spectra were recorded at the Stanford Synchrotron Radiation Laboratory with the storage-ring SPEAR running in dedicated mode, (30–70 mA at 3 GeV). The e.x.a.f.s. measurements were performed on beam line VI-2 using an Si(111) double-crystal monochromator, whereas the edge spectra were recorded on line IV-2 with Si(220) crystals. X-ray absorption was monitored as the X-ray fluorescence excitation spectrum using a fluorescent ion-chamber detector (Stern & Heald, 1979), with appropriate-thickness Mn filters and slit assembly. Four scans, each of 30 min duration, were averaged for each sample. During data collection the samples were held close to 4 K in an Oxford Instruments CF204 liquid-helium flow cryostat. The X-ray energy was calibrated, using a three ion-chamber geometry (e.g. Scott, 1985), with reference to the first inflection point of Fe foil, which was defined as 7111.3 eV.

Data analysis

The e.x.a.f.s. oscillations $\chi(k)$ were extracted from raw data using standard methods (Scott, 1985) and were quantitatively analysed by curve fitting to the following approximate expression:

$$\chi(k) = \sum_b \frac{N_b S_b A_b(k, R)}{k R_{ab}^2} e^{-2\sigma_{ab}^2 k^2} \sin[2k R_{ab} + \alpha_{ab}(k, R)] \quad (1)$$

Where N_b is the number of b-type atoms at a distance R_{ab} from the absorber atom a, k is the photoelectron wave-number and σ_{ab} is the root-mean-square deviation of R_{ab}

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(σ being the Debye–Waller factor). $A_b(k, R)$ and $\alpha_{ab}(k, R)$ are the amplitude and phase-shift functions respectively. The curve-wave theoretical amplitude and phase-shifts functions of McKale *et al.* (1988) were used with $1/R_{ab}$ interpolation, and with the amplitudes reduced by an empirical linear correction factor, S_b , to allow for amplitude reductions not accounted for by theory, such as the so-called ‘shake-up’ and ‘shake-off’ losses. Values for S_b were obtained by fitting the e.x.a.f.s. of model compounds $[\text{Fe}_4\text{S}_4(\text{SC}_6\text{H}_5)_4]$ for Fe–Fe, $S_b = 0.28$; $\text{Fe}[\text{S}_2\text{CN}(\text{n-C}_4\text{H}_9)_2]_3$ for Fe–S, $S_b = 0.37$, and $\text{Fe}(\text{CH}_3\text{COCH}_2\text{CO-CH}_3)_3$ for Fe–O interactions, $S_b = 0.48$].

RESULTS

Edge spectra

Fig. 1 shows the iron X-ray absorption K-edge region for oxidized and reduced *C. pasteurianum* hydrogenase II. The pronounced peak centred near 7112.2 eV is due to a $1s \rightarrow 3d$ bound-state transition. This transition, which is formally dipole-forbidden, gains intensity with tetrahedral geometries (such as those in Fe–S clusters) due to metal $3d$ – $4p$ mixing (Shulman *et al.*, 1976). Higher-energy features within the edge are also visible as shoulders close to 7119 and 7127 eV. These can also be described as bound-state transitions to excited states (e.g. $1s \rightarrow 4p$; cf. Shulman *et al.*, 1976), but quantitative interpretation of this part of the edge is highly complex, and beyond the scope of this work.

The overall shapes of the iron edge spectra bear very strong resemblance to the spectra of other biological Fe–S systems and models thereof (Shulman *et al.*, 1976; Cramer, 1977). This indicates that the bulk of the iron in *C. pasteurianum* hydrogenase II is tetrahedrally coordinated to sulphur. The edge spectrum of the reduced enzyme has only subtle differences from that of the oxidized enzyme. The position of the edge did not shift appreciably on reduction; although such a shift is anticipated, it is expected to be too small to be detected (Cramer, 1977). In agreement with this, we have found in separate experiments that the iron K-edge spectrum of *C.*

Fig. 1. Iron K-edge X-ray-absorption spectra of oxidized (—) and reduced (---) *C. pasteurianum* hydrogenase II

pasteurianum eight-iron ferredoxin changes very little upon reduction.

E.x.a.f.s. spectra

Fig. 2. shows the e.x.a.f.s. spectra, together with the e.x.a.f.s. Fourier transforms, of *C. pasteurianum* hydrogenase II in oxidized and reduced forms. The oxidized protein shows two major peaks in the e.x.a.f.s. Fourier transform, at $R + \Delta = 1.8$ and $2.4 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$), which correspond to Fe–S and Fe–Fe interactions respectively. E.x.a.f.s. curve-fitting analysis indicates an Fe–S distance of $2.27 \text{ \AA}$ and an Fe–Fe distance of $2.76 \text{ \AA}$ (Fig. 2 and Table 1). The Fe–Fe distance is typical of that of $[2\text{Fe-2S}]$, $[4\text{Fe-4S}]$ and $[3\text{Fe-4S}]$ clusters reported in the literature (Antonio *et al.*, 1982a; George & George, 1988).

The E.x.a.f.s. spectrum of reduced hydrogenase II, illustrated in Fig. 2, is remarkably different from that of the oxidized protein. The Fourier transform shows three major peaks, two of which are attributable to Fe–S and Fe–Fe interactions with distances similar to those of the oxidized protein ($2.30 \text{ \AA}$ and $2.76 \text{ \AA}$ respectively). The additional major transform peak at $R + \Delta = 2.9 \text{ \AA}$ fits well to an Fe–Fe interaction at $3.3 \text{ \AA}$ (although a rather large E_0 shift of -11 eV was required in the fit). The large amplitude of this interaction (Table 1) indicates that a significant fraction of the iron in the reduced H cluster is separated by $3.3 \text{ \AA}$. The shoulder in the e.x.a.f.s. Fourier transform of the reduced enzyme (Fig. 2) at $R + \Delta = 3.4 \text{ \AA}$ suggests that there may be additional small contributions to the e.x.a.f.s. from interactions longer than $3.3 \text{ \AA}$, but data with a more extended k -range are required for an unambiguous interpretation.

The apparent absence of long-distance Fe–Fe interactions in the e.x.a.f.s. of the oxidized sample does not conclusively preclude their presence in this sample. For example, it is possible that the e.x.a.f.s. from two different Fe–Fe distances might cancel to some extent. Thus

Table 1. Curve-fitting analysis of *C. pasteurianum* hydrogenase II Fe e.x.a.f.s. data

The Table lists the average number (N) per Fe and distance (R) of each scatterer detected, together with the Debye–Waller factor (σ), and the shift in threshold energy (ΔE_0) required for the fit. The data and best fits are illustrated in Fig. 2. Values for R are considered accurate to $\pm 0.02 \text{ \AA}$ and values for N and σ to $\pm 30\%$, although it is important to note that N and σ are highly correlated. The k -range dictates a resolution (the smallest discernible difference in R for two similar interactions) of approx. $0.13 \text{ \AA}$. (Note that the amplitude profiles of the interactions assigned as Fe–Fe cannot be reproduced by significantly lighter atoms, such as carbon or nitrogen.)

	N	$R \text{ (\AA)}$	$\sigma \text{ (\AA)}$	$\Delta E_0 \text{ (eV)}$
Oxidized				
Fe–S	4.1	2.27	0.044	–0.1
Fe–Fe	3.5	2.76	0.062	0.9
Reduced				
Fe–S	4.0	2.28	0.043	–1.1
Fe–Fe	2.3	2.76	0.038	3.6
	1.1	3.30	0.032	–10.6

Fig. 2. Iron e.x.a.f.s. (a) and Fourier transforms (b) of oxidized (curves i) and reduced (curves ii) *C. pasteurianum* hydrogenase II. The broken lines indicate the best fits (see Table 1 for parameters).

calculations show that e.x.a.f.s. from Fe-Fe separations of 3.29 Å and 3.47 Å would effectively cancel, decreasing the contributions of both. Such cancellations, however, are very sensitive to the distances concerned, and we view their possibility as rather unlikely. Another possibility, again rather unlikely, is that the Debye-Waller factor of the 3.3 Å interaction is so great in the oxidized form that the interaction is not seen. In any case, the differences between the oxidized and reduced forms of the H cluster shown in Fig. 2 indicate that there are substantial changes in the structure upon reduction.

No Fe-(O, N) interactions (note that e.x.a.f.s. cannot, in general, discriminate scatters of similar atomic mass) can be distinguished in the e.x.a.f.s. of either the oxidized or the reduced protein, and inclusion of such scatters did not improve the fit. Similarly the inclusion of outer-shell sulphur, carbon or nitrogen atoms did not improve the fit. Nevertheless, bearing in mind that the 14 Fe complement of the enzyme possesses a total of approx. 56 ligands, the presence of a number of Fe-(O/N) ligands cannot be excluded, especially with the relatively weak e.x.a.f.s. amplitudes of such interactions. Whereas ENDOR (electron nuclear double resonance) experiments on the oxidized H cluster detected no ^{14}N couplings (Telser *et al.*, 1986), recent electron-spin-echo experiments have found at least three different such couplings (H. Thomann, M. Bernardino & M. W. W. Adams, unpublished work), indicating the possibility of Fe-N co-ordination in the H cluster.

DISCUSSION

The structures of [4Fe-4S] clusters differ little between oxidized and reduced forms (Antonio *et al.*, 1982a), so

the long Fe-Fe interaction observed in reduced hydrogenase II is almost certainly from the H cluster, rather than the F clusters. Consistent with this, the amplitudes of the different Fe-Fe interactions are approximately what would be expected for two 4Fe and one 6Fe cluster (Table 1), with an appreciable fraction of the iron in the 6Fe cluster separated by 3.3 Å. Assuming a stoichiometry for the two F clusters of [4Fe-4S] (4 Cys), the iron, sulphide and amino acid analyses of hydrogenase II indicate a stoichiometry for the H cluster of [6Fe-3S] (4 Cys) (M. W. W. Adams, E. Eccleston & J. B. Howard, unpublished work). Such a cluster is clearly deficient in cysteine ligands, and accepting the inherent inaccuracy in acid-labile sulphide determinations, in bridging sulphide ligands as well. μ_2 cysteine-bridging ligands are possible, but ligation to other amino acids seems likely. Possibilities include methionine ligands, bridging carboxylates from amino acids such as glutamate, and nitrogenous ligands from histidine, lysine or arginine. As discussed above, recent spin-echo experiments support the hypothesis of Fe-N co-ordination to the H cluster. The Fe-Fe separation of 3.3 Å is in the range observed in proteins where two irons are bridged by two carboxylates plus an oxygen bridge (see Prince *et al.*, 1988). Such distances are also found in a variety of more extended oxygen-bridged iron clusters in non-biological complexes (Lippard, 1988). The presence of such Fe-Fe distances in hydrogenase II might argue for similar structures as components of the H cluster.

Hydrogenase II has been extensively characterized by a number of different spectroscopies (Adams *et al.*, 1986; Macor *et al.*, 1987; Rusnak *et al.*, 1987; Telser *et al.*, 1987), although some of the original interpretations may have to be re-evaluated, since they relied upon the

erroneous formulation of the H cluster as a three-iron cluster (M. W. W. Adams, E. Eccleston & J. B. Howard, unpublished work). Mossbauer studies of the H cluster of hydrogenase II indicate that it possesses only two spectroscopically distinct types of iron, in the ratio of 2:1 (Rusnak *et al.*, 1987). The H cluster possesses an $S = 0$ ground state when reduced, and an $S = \frac{1}{2}$ ground state when oxidized (Rusnak *et al.*, 1987), and has g -values and ^{57}Fe hyperfine couplings that are distinct from those of classical iron-sulphur clusters (Rusnak *et al.*, 1987; Telser *et al.*, 1987). The unique spectral properties of the H cluster are emphasized by recent resonance-Raman investigations, which showed that the H cluster gave no detectable resonance-Raman contributions at 406.7 nm or 488.0 nm excitation wavelengths (Macor *et al.*, 1987). This contrasts with classical [3Fe-4S] and [4Fe-4S] clusters, which give very pronounced resonance Raman spectra at these excitation wavelengths (Johnson *et al.*, 1983a,b).

Several low-molecular-mass six-iron clusters have been synthesized, but no such cluster has been definitely shown to exist in biology. Four different basic structural types have been reported: the [6Fe-6S] prisms of Kanatzidis *et al.* (1984, 1985), the [6Fe-8S] stellated octahedra of Cecconi *et al.* (1981), the [6Fe-6S] 'basket' clusters of Snyder & Holm (1988) and the [6Fe-9S] cluster of Christou *et al.* (1982). The latter was the first reported example of a six-iron cluster, and possesses a quite complex structure resembling two [3Fe-4S] clusters joined via one common μ_4 sulphide, plus two external μ_2 sulphides. The [6Fe-6S] basket cluster is of some interest in that it is a topological isomer of a prismane cluster, into which it can readily and reversibly be converted (Snyder & Holm, 1988). This process could be analogous to the changes in H-cluster geometry between oxidized and reduced forms. It should be noted that none of these models contain 3.3 Å Fe-Fe separations, and all contain too much sulphide to mimic our current estimate (M. W. W. Adams, E. Eccleston & J. B. Howard, unpublished work) of the stoichiometry of the H cluster. Interestingly, of the known clusters containing ≥ 6 Fe atoms, our current view of the H cluster resembles, albeit distantly, the FeMo cofactor of nitrogenase. With a stoichiometry of $\text{Fe}_{6-8}\text{S}_{9-10}\text{Mo}$, this cluster is also thought to be a site of H_2 production (and N_2 reduction), and it also shows evidence of nitrogeneous ligands (Thomann *et al.*, 1987). The presence of Fe-Fe interactions at > 3 Å has recently been suggested in the FeMo cofactor (Arber *et al.*, 1988), although it is important to note that there are significant disagreements between this and previous work (Antonio *et al.*, 1982b).

The e.x.a.f.s. data presented here show that the H cluster undergoes a substantial rearrangement upon reduction. In the oxidized form, the Fe-S and Fe-Fe distances resemble those in other iron-sulphur clusters, but, upon reduction, the cluster undergoes a significant rearrangement, and a form with a significantly longer Fe-Fe separation is revealed. The rather more expanded structure of the reduced form apparently represents a new form of Fe-S cluster. Since the fully oxidized H cluster is thought to bind H_2 and become reduced as a consequence of H_2 oxidation, the changes brought about

by oxidation and reduction should provide new insights into the catalytic mechanism of the enzyme.

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