The iron–sulfur centers of the pyruvate:ferredoxin oxidoreductase from *Methanosarcina barkeri* (Fusaro)

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Abstract The iron–sulfur clusters of a pyruvate:ferredoxin oxidoreductase isolated from a methanogenic archaeon, *Methanosarcina barkeri* (Fusaro), have been unambiguously identified for the first time. In agreement with the estimated iron and sulfur contents (Bock and Schönheit, *Eur. J. Biochem.*, 237 (1996) 35–44), the enzyme is shown to contain three $[4Fe–4S]^{2+/1+}$ clusters, which in the reduced state give a complex EPR spectrum resulting from three distinct centres, magnetically interacting. The catalytic cycle of the enzyme was studied by visible and EPR spectroscopies. A thiamine diphosphate based radical is also an intermediate in the *M. barkeri* enzyme catalytic cycle. However, under anaerobic conditions, the enzyme or *Clostridium pasteurianum* ferredoxin iron–sulfur clusters are reduced only in the presence of both substrates, pyruvate and coenzyme A.

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Key words: Pyruvate:ferredoxin oxidoreductase; EPR; Methanogens; Iron–sulfur; Thiamin diphosphate

1. Introduction

Pyruvate:ferredoxin oxidoreductases catalyze either the oxidative decarboxylation of pyruvate to acetyl CoA or the reductive carboxylation of acetyl-CoA, using ferredoxin as an electron donor or acceptor. The enzyme has been found in all the three life domains: bacteria, archaea and eukarya. In particular, it has been found in all archaea tested for its presence, both aerobic and anaerobic. Until recently, methanogenic archaea were assumed to be exceptional, apparently using the deazaflavin coenzyme F_{420} rather than ferredoxin as the electron carrier, suggesting the presence of pyruvate:F420 oxidoreductases. However, upon purification of pyruvate oxidoreductase from the methanogenic *Methanosarcina barkeri* (Fusaro) the enzyme could be clearly defined as pyruvate:ferredoxin oxidoreductase [1].

All pyruvate:ferredoxin oxidoreductases contain thiamin diphosphate (TPP) and $[4Fe–4S]^{2+/1+}$ centres. However, the number of iron–sulfur centres per enzyme has been estimated in most cases by colorimetric determinations of the iron and sulfide contents, showing variable results (see e.g. [1]). An exact quantification and characterisation of the Fe/S centres by EPR spectroscopy has been made only in a few cases [2–5]. Apparently, there is not a correlation between the number of centres and either the enzyme subunit composition or the enzyme source. We report on this article the quantification and characterisation of the iron sulfur centres of the *M. barkeri* enzyme. The catalytic mechanism and electron transfer to the ferredoxin were also probed. Although it has been commonly assumed that the oxidation of pyruvate, by the superfamily of pyruvate:ferredoxin oxidoreductases, occurs by a generally common mechanism, based on the formation of a hydroxyethyl thiamine radical intermediate [2,3], unequivocal experimental evidence is still lacking. In particular, it should be stressed that for the enzymes isolated from *Clostridia*, such a radical has not been observed [4,5].

2. Materials and methods

The enzyme was prepared as previously described [1]. All experiments were done under argon atmosphere, in buffers containing 5–10 mM dithioerythrol. *Clostridium pasteurianum* ferredoxin was prepared as in [6]. EPR experiments were performed as in [7]. Myoglobin-azide [8] was used as a standard for EPR quantification. Spectra obtained under non-saturating conditions (24 μW) were used for double integration, and corrections for the *g*-value anisotropy were performed using the Aasa and Vanngard correction factor [9]. Visible spectra were recorded on a Beckman DU-9 spectrophotometer.

3. Results and discussion

As isolated, under anaerobic conditions, the enzyme exhibits a low-intensity radical signal, with unresolved hyperfine structure and an overall linewidth of ~1.5 mT, similar to those observed in other PORs [10,11] (Fig. 1AFig. 2A), which has been assigned to a hydroxyalkyl thiamine diphosphate

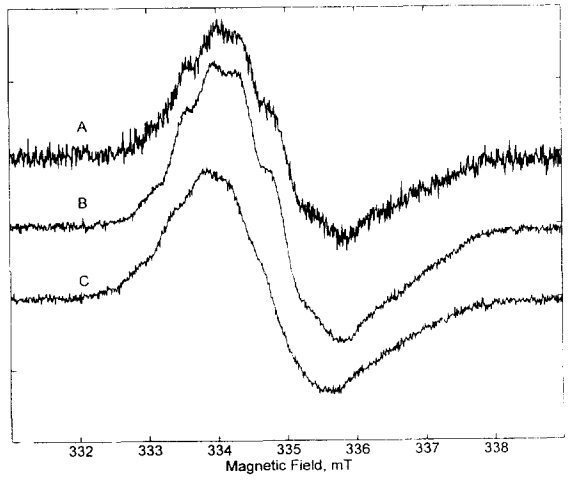

Fig. 1. EPR spectra at 134 K of *M. barkeri* POR: (A) native; (B) incubated with 10 mM sodium pyruvate; (C) incubated with 10 mM sodium pyruvate labelled at C-2 with ^{13}C. Microwave power: 2.4 mW; modulation amplitude: 0.1 mT

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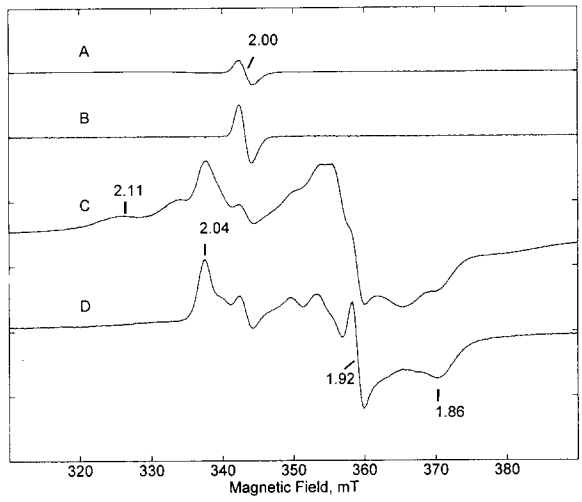
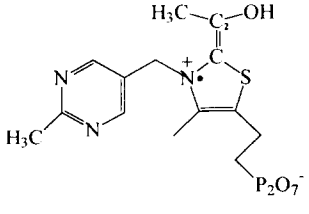


Fig. 2. EPR spectra of *M. barkeri* POR: (A) native; (B) incubated with 10 mM sodium pyruvate; (C,D) upon addition of 0.1 mM CoA. Temperature: (A–C) 8 K; (D) 25 K. Microwave power: 2.4 mW; modulation amplitude: 1 mT.

based radical. In some preparations, a very low-intensity rhombic signal is observed at low temperatures, with *g*-values at ~2.03, 1.96 and 1.88, typical of reduced $[4Fe\text{–}4S]^{2+/1+}$ centres (not shown). Upon incubation of the enzyme with 10 mM sodium pyruvate, the intensity of this signal is unaltered, while the intensity of the radical species increases by about 100 times, but there are no changes in its lineshape (Fig. 1BFig. 2B). Spectra recorded at low-temperature do not show new EPR resonances, indicating that the enzyme iron–sulfur centres remain in the same redox state (Fig. 2B). It has been suggested that this may be due to the presence of oxygen [2,3]. Using redox mediators, the solution redox potential was determined to be stable at ~−200 mV, which seem to exclude the presence of oxygen. Pyruvate labelled with ^{13}C at C-2 was also used as a substrate. Apart from a very slight loose of resolution in the central part of the spectrum (Fig. 1C), there are no differences on the radical EPR spectra obtained with natural abundance or ^{13}C-labelled pyruvate, as was observed on the enzymes from *Pyrococcus furiosus* and *Thermotoga maritima* [12]. This clearly shows that the spin density at C-2 is negligible, in contrast to previous formulations of the radical structure [12,13]. Although the actual radical structure has not yet been demonstrated, either in enzymatic systems or using thiazolium salts, the former proposal [14] in which the electron density is clearly distributed on the pentadienylic group, is compatible with the negligible effect observed upon labelling pyruvate at C-2:



After addition of CoA the EPR spectra change drastically (Fig. 2C,D). A complex spectrum appears, with an average *g*-value at 1.94. Lines are observed from *g* ~2.11 to ~1.81, with main features at *g* ~2.11, 2.06, 2.04, 1.93, 1.92, 1.88, 1.86 and 1.81. These features are accounted by a minimum of three distinct centres. Even at the maximum attainable microwave power (246 mW) and at 4.6 K no other resonances are observed in the entire magnetic field range, indicating the absence of $S > 1/2$ spin systems. Change of the microwave power, at 4.6 K or 15 K do not result in a simplification of the spectra and above 40 K the spectrum broadens beyond detection. At ca. 20 K the spectrum is slightly simplified and a main set of resonances at $g = 2.04$, 1.92 and 1.86 is detected (Fig. 2D). Both the EPR *g*-values and the relaxation behaviour observed are typical of reduced $[4Fe\text{–}4S]^{2+/1+}$ centres, in an $S = 1/2$ ground state, magnetically interacting. By double integration of the spectrum at 15 K, a value of 2.7 spin/mol was obtained. This value shows that the enzyme contains three 4Fe centres, as expected from the previously published iron quantitation [1]. Also, it indicates that in the presence of both substrates an almost complete reduction of all the Fe centres is achieved. Upon addition of excess sodium dithionite there are no changes of either the EPR line-shape or of the EPR signal intensity. The minor difference from the expected value of 3 spin/mole may result from several factors. It is possible that not all the enzyme centres are fully reduced; in fact, as observed for other PORs, the reduction potentials of these centres are extremely low. At pH 7.0 the reduction potentials estimated by an EPR monitored redox titration (data not shown) are well below −350 mV, and hence at this point it was not possible to determine the reduction potentials by chemical reduction with sodium dithionite, at this pH value. Furthermore, due to the magnetic interaction between the reduced Fe centres, the total spectral intensity may be lower than that expected for magnetically isolated centres.

The temperature behaviour of the spectrum suggests that two of the clusters have a stronger magnetic interaction between them, while the third cluster, which yields an essentially isolated species at higher temperature, has a weaker interaction with the other centre(s). Although no three-dimensional structure has yet been reported for this enzyme, these observations may be tentatively explained on the basis of the available amino acid sequences for the four subunit enzymes, as previously advanced for the *Clostridium pasteurianum* homodimeric enzyme [5]. The N-terminal amino acid sequences of the four subunits of *M. barkeri* POR [1] show a considerable homology with the corresponding subunits of the PORs from *T. maritima* and *P. furiosus* [15], as well as with the amino

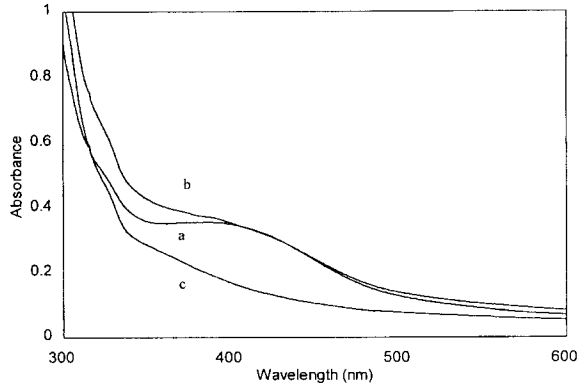


Fig. 3. Visible spectra of *M. barkeri* POR: (A) native; (B) incubated with 10 mM sodium pyruvate; (C) upon addition of 0.1 mM CoA.

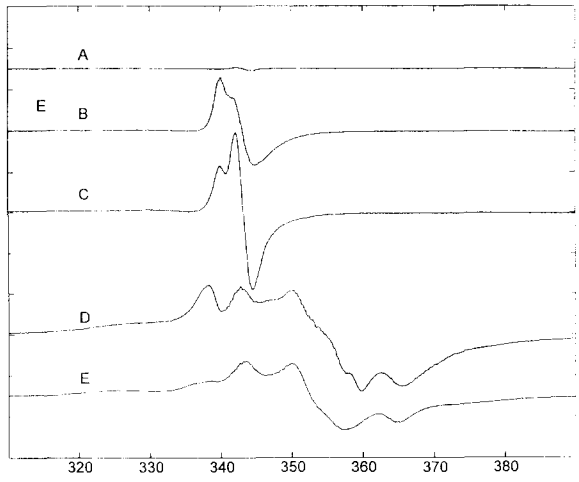


Fig. 4. EPR spectra of *M. barkeri* POR mixed with *C. pasteurianum* ferredoxin. (A) *M. barkeri* POR native; (B) *M. barkeri* plus *C. p.* ferredoxin (0.3 mM each); (C) after incubation with 10 mM pyruvate; (D) C after addition of 0.1 mM CoA; (E) *Cp* Fd reduced with excess sodium dithionite. Temperature: 8 K; other experimental conditions as in Fig. 2.

acid sequences deduced from the *Methanococcus jannaschii* genome [16]. From the complete sequences of these enzymes, it was found that δ-subunit contains two cluster binding motifs Cys–XX–Cys–XX–Cys–XXX–...Cys–Pro, as observed in two $[4Fe–4S]^{2+/1+}$ ferredoxins. In these proteins, magnetic interactions between the two clusters are always observed, due to their close spatial proximity [8,17,18]. The third cluster in the multi-subunit PORs is located in the β-subunit, which besides the TTP-binding domain contains four cysteine residues, in an atypical sequence. Hence, the electron transfer pathway may be assured from the β-subunit cluster, close to the catalytic (TPP) site, to the δ-subunit centres, which as evidenced by the EPR data, are also close to the β-subunit cluster.

A parallel set of experiments monitored by Visible spectroscopy, under anaerobic conditions, was performed (Fig. 3), which fully corroborate the EPR data; as deduced from the bleaching of the absorbance at 410 nm, only in the presence of both pyruvate and CoA a reduction of the Fe centres is observed.

M. barkeri POR was shown to transfer electrons efficiently to *C. pasteurianum* ferredoxin [1]. Hence, the reduction of this protein was also probed by EPR spectroscopy. A ferredoxin sample (30 μM) prepared in deaerated buffer, containing 5 mM DTT, exhibits an almost isotropic signal, with g_{max} at 2.03, typical of oxidised [3Fe–4S] centres (Fig. 4B). Integration of this signal in relation to the EPR signal of the dithionite reduced ferredoxin indicates that in this preparation ~25% of the [4Fe–4S] centres were converted into $[3Fe–4S]^{1+/0}$ clusters, a behaviour common in bacterial ferredoxins. The ferredoxin was mixed with an almost equimolar amount of enzyme, and the EPR spectrum of the mixture was recorded (Fig. 4B). Besides the resonance from the 3Fe centre, a low intensity superimposed radical signal is observed, due to the enzyme. After incubation of the mixture with 10 mM sodium pyruvate for 10 min at room temperature, the EPR spectrum shows an increase of the intensity of the radical signal, but the intensity of the 3Fe centre resonance remains unchanged (Fig. 4C), indicating that there is no reduction of the Fd at this stage. This observation was corroborated by parallel mode EPR: no low-field resonances, due to the reduced 3Fe centre (S = 2 ground state), were observed [7]. Longer incubation with pyruvate did not change the spectrum. Upon addition of 0.1 mM CoA a complex spectrum is obtained, in the $g = 2.1–1.8$ region (Fig. 4D). This spectrum is assigned to the superposition of the signals from the reduced enzyme and ferredoxin. For comparison, the EPR spectrum of the dithionite reduced ferredoxin is also shown in Fig. 4E. As the EPR signal of the reaction mixture is not completely reproduced by adding the individual spectra of reduced enzyme and Fd, it may be that either a slightly different degree of reduction of one of the components or a change on the EPR spectra of the 4Fe centres due to protein–protein interactions occur.

4. Conclusion

The data presented above unambiguously show that the pyruvate ferredoxin oxido reductase from *M. barkeri* contains three $[4Fe–4S]^{2+/1+}$ centres, all with very low reduction potentials and with an S=1/2 ground state in the reduced form. The centres interact magnetically, indicating that they are in close proximity, as expected for at least two of the centres from the primary structures available for these enzymes. The overall EPR characteristics of the *M. barkeri* enzyme are similar to those of *Desulfovibrio africanus* POR [2], in spite of the completely different subunit composition of these enzymes: the methanogenic enzyme is of the αβγδ type, while the *Desulfovibrio* enzyme is a single subunit. Recent sequence comparisons of several pyruvate oxidoreductases have shown that the one and two subunit enzymes contain domains homologous to the four subunit enzymes [15].

Two types of experiments, using three independent sets of experiments probed the catalytic mechanism. Both in the presence and in the absence of *Cp* Fd, only after addition of both pyruvate and CoA the reduction of the Fd or the enzyme iron–sulfur centres is observed. The same results were obtained with the enzyme isolated from *Sulfolobus* strain 7 [19]. Although in the *M. barkeri* enzyme the decarboxylation of pyruvate also proceeds through a thiamine based radical, the exact nature of this radical as well as the electron transfer steps and electron acceptor(s) remain to be unequivocally demonstrated.

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