Redox properties of the sulfhydrogenase from *Pyrococcus furiosus*

Alexander F. Arendsen, Peter Th.M. Veenhuizen, Wilfred R. Hagen*

Department of Biochemistry, Wageningen Agricultural University, Dreijenlaan 3, NL-6703 HA Wageningen, The Netherlands

Received 24 April 1995; revised version received 26 May 1995

Abstract The sulfhydrogenase from the extreme thermophile *Pyrococcus furiosus* has been re-investigated. The $\alpha\beta\gamma\delta$ heterotetrameric enzyme of 153.3 kDa was found to contain 17 Fe, 17 S^{2-}, and 0.74 Ni. The specific activity of the purified protein was 80 U/mg. Three EPR signals were found. A rhombic $S = 1/2$ signal ($g = 2.07, 1.93, 1.89$) was observed reminiscent in its shape and temperature dependence of spectra from [4Fe–4S]$^{(2+;1+)}$ clusters. However, in reductive titrations the spectrum appeared at the unusually high potential $E_{m,7.5} = -90$ mV. Moreover, the signal dissappeared again at $E_{m,7.5} = -328$ mV. Also, two other signals appear upon reduction: a near-axial ($g = 2.02, 1.95, 1.92$) $S = 1/2$ spectrum ($E_{m,7.5} = -303$ mV) indicative for the presence of a [2Fe–2S]$^{(2+;1+)}$ cluster, and a broad spectrum of unknown origin with effective g-values 2.25, 1.89 ($E_{m,7.5} = -310$ mV). We hypothesize that the latter signal is caused by magnetic interaction of the rhombic signal and a third cluster.

Key words: Hydrogenase; EPR; *Pyrococcus furiosus*

1. Introduction

The sulfhydrogenase of the anaerobic archaeon *Pyrococcus furiosus* was first isolated by Bryant and Adams [1]. *P. furiosus* grows near 100°C by fermentation of carbohydrates, resulting in CO_2 and H_2 as sole products. However, H_2 is only found when the organism is grown in the absence of elemental sulfur (S^0). When the culture medium contains sulfur, H_2S instead of H_2 is produced. Since H_2 inhibits growth, it was hypothesized that sulfur reduction is a means of detoxification [2]. Attempts to isolate the sulfur reductase revealed that sulfur reduction activity coincided with hydrogenase activity. Therefore, it was concluded that both H_2S and H_2 production are catalyzed by the same enzyme, and by concequence the enzyme was called sulfhydrogenase [3]. The protein is encoded by a chromosomal operon of four open-reading frames [4] for four polypeptides of 48.7, 41.8, 33.2, and 29.6 kDa [5]. Unlike other hydrogenases the *P. furiosus* enzyme catalyzed preferably H_2 production [1]. Bryant and Adams reported the enzyme to contain 31 Fe, 24 S^{2-}, and 1 Ni per 185 kDa. Despite the high metal content only two EPR signals were found. In the reduced state the enzyme exhibited resonances at $g = 2.03$, 1.93, and 1.92, which could been observed from 70 to 20 K. This signal integrated to only one spin/mol. Upon lowering the temperature a complex signal was found with fast spin-lattice relaxation rate, which accounted for another spin/mol. No Ni signals were found. The former signal was assigned to a [2Fe–2S] cluster, while the latter was proposed to arise from at least two interacting iron–sulfur clusters. In a later publication Adams reports the monitoring of a [2Fe–2S] and a [4Fe–4S] cluster in a EPR redox titration [6]. Reduction potentials of −410 mV of the [2Fe–2S] cluster and of −210 mV of the [4Fe–4S] cluster were determined. In this paper we present data on the EPR properties of *P. furiosus* sulfhydrogenase that are consistent with the presence of three, rather than two signals. We also compare differences in metal content and reduction potentials. The possibility that the rhombic signal represents a two-electron transferring cluster is discussed.

2. Materials and methods

2.1. Growth of organism

P. furiosus (DSM 3638) was grown on potato starch (5 g/l) in a stirred (150 rpm) 200 liter fermentor (lp 300, Bioengineering, Wald, Switzerland) at 90°C, in a medium described in [7]. NaCl p.a. was replaced by table salt. The medium also contained yeast extract (1 g/l) and cysteine (0.5 g/l). Trace elements were Na_2WO_4, 10 μM; $Fe(NH_4)SO_2$, 25 μM; $NiCl_2$, 2.1 μM; H_3BO_3, 0.2 μM; zinc acetate, 1 μM; $CuSO_4$, 0.04 μM; $MnCl_2 \cdot 4H_2O$, 1 μM; $CoSO_4$, 1.5 μM; $Na_2MoO_4 \cdot 2H_2O$, 0.03 μM. Vitamins used were biotin, 2 μM; folic acid, 2 μM; nicotinamide, 5 μM; thiamin HCl, 5 μM; riboflavin, 5 μM; pyridoxine HCl, 10 μM; cyanocobalamine, 5 μM; *p*-aminobenzoic acid, 5 μM; lipoic acid, 5 μM; and pantothenic acid, 5 μM. No elemental sulfur (S°) was present. The medium was continuously flushed with nitrogen to remove evolved hydrogen gas. Growth was followed by measuring the absorbance at 660 nm. Cells were frozen and stored at −20°C until use. Potato starch and table salt were obtained from the local grocery.

2.2. Isolation procedure

Sulfhydrogenase was purified according to a modification of the procedure of Bryant and Adams [1]. Ethanol was emitted from the buffers. Because of a higher capacity a Sephacryl PG 200 molecular sieve (2.5 × 100 cm) was used instead of a Superose 6 column. As the first purification step an ammonium sulfate precipitation was performed. Ammonium sulfate was added to the extract to 55% saturation. After a 10 min spin at 5,000 × *g* hydrogenase activity was located in the pellet. The pellet was resuspended in standard buffer and dialyzed against standard buffer. As an extra purification step a 50 ml Phenyl Sepharose hydrophobic interaction column was used. Fractions containing hydrogenase activity eluting from the hydroxyapatite column were pooled and adjusted to the appropriate ionic strenght of 0.25 M by adding crystals of $(NH_4)_2SO_4$. A 1 liter linear gradient of 0.25–0 M $(NH_4)_2SO_4$ in standard buffer was applied. Hydrogenase eluted at 0 M $(NH_4)_2SO_4$.

2.3. Activity measurements, analytical chemistry

Hydrogenase activity was measured in the hydrogen production assay according to Bryant and Adams [1] using a gas chromatograph (model 3400, Varian).

Protein was measured using the microbiuret method [8]. Iron was determined colorimetrically as the ferene complex [9]. Nickel was determined by atomic absorption spectroscopy on a Hitachi 180–80 Polarized Zeeman Atomic Absorption Spectrophotometer equipped with a pyrocuvette in the laboratory of Dr. S.P.J. Albracht (The University of Amsterdam).

SDS polyacrylamide electrophoresis was performed with a midget system (Pharmacia) holding 8-× 5-× 0.75-cm gels, according to the method of Laemmli [10]. The composition (mass/volume) of the stacking gel was 4% acrylamide and 0.1% bisacrylamide; the running gel was

*Corresponding author. Fax (31) (8370) 84 801.

SSDI 0014-5793(95)00622-2

17.5% acrylamide and 0.07% bisacrylamide. The native molecular mass of the protein was determined with a Superose 6 HR 10/30 column (Pharmacia) equilibrated with standard buffer + 0.15 M NaCl using a flow rate of 0.5 ml/min. Glucose oxidase (152 kDa), lactate dehydrogenase (109 kDa), transferrin (74 kDa), BSA (67 kDa), and ovalbumine (43 kDa) were used as the markers for the calibration. The void volume was determined with Dextran blue.

2.4. Spectroscopy

EPR measurements and mediated redox titrations were as in [11]. In titration experiments protein concentration was typically 4 μM in 100 mM HEPES pH 7.5, and the following EPR signals were monitored: the g = 1.89 peak of the broad signal at the wings; the g = 1.89 peak of the rhombic S = 1/2 signal; the g = 1.92 peak of the near-axial S = 1/2 signal. Titrations were done at 23°C.

3. Results

3.1. Isolation

Our first attempts to isolate the sulfhydrogenase from *Pyrococcus furiosus* using the procedure described by Bryant and Adams [1] did not result in a pure hydrogenase sample. Instead, more then four bands were observed on SDS gel. By use of a modified procedure, i.e. by using ammonium sulfate precipitation as the first step, and a Phenyl-Sepharose hydrophobic interaction column, a preparation with a higher apparent purity on SDS gel was obtained. Clearly, four subunits can be observed of 45, 43, 31, and 28 kDa (Fig. 1). These values fit reasonably with the values of the subunits deduced from the amino acid sequence, being 48.7, 41.8, 33.2, and 29.6 kDa for the α, β, γ, and δ subunit, respectively [5]. Starting with a specific activity of ≈1.0 U/mg, an 80-fold purification was accomplished, resulting in a specific activity of 80 U/mg (see also Table 1). This is lower than the value reported by Bryant and Adams, i.e. 360 U/mg, which accounted for a 350-fold purification [1].

3.2. Analytical data

The molecular mass of the protein was previously reported to be 185 kDa [1]. However, this value was based on an assumed hexameric subunit composition of $\alpha_2\beta_2\gamma_2$. Recently, it was shown that the the enzyme consists of four different gen products (α, β, γ, and δ) [5]. The sum of the masses of the individual subunits is 153.3 kDa. To obtain the subunit composition we performed a gel-filtration experiment. On a Superose 6 column the native protein ran with an apparent molecular mass of 135 kDa (Fig. 2). This value fits reasonably with a $\alpha\beta\gamma\delta$ subunit composition. Moreover, on a SDS gel all four subunits have similar intensities (Fig. 1). We therefore conclude that *P. furiosus* sulfhydrogenase is an $\alpha\beta\gamma\delta$ heterotetramer of 153.3 kDa. Table 1 lists the iron, sulfide, and nickel contents of three different preparations. The enzyme contained 17 ± 2 Fe, 17 ± 2 S^{2-}, and 0.74 ± 0.21 Ni atoms per 153.3 kDa.

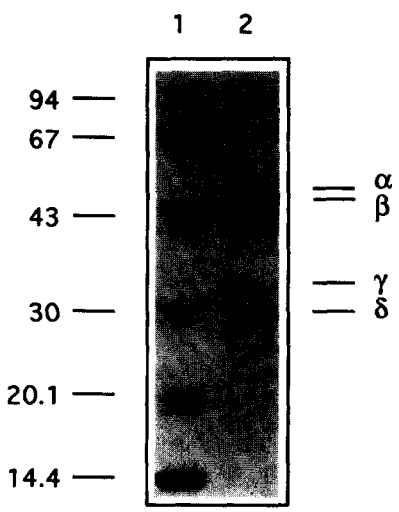

Fig. 1. SDS-polyacrylamide gel with purified *P. furiosus* sulfhydrogenase. Lane 1, molecular marker mixture; lane 2, purified sulfhydrogenase.

Table 1
Physico-chemical data on *P. furiosus* sulfhydrogenase

	Previous reports		This paper
Ni	0.98 ± 0.05	[1]	0.74 ± 0.21
Fe	31 ± 3	[1]	17 ± 2
S^{2-}	24 ± 4	[1]	17 ± 2
Spec. activity (U/mg)	360	[1]	80
E_m [2Fe–2S] (mV)	−410	[6]	−303
E_m [4Fe–4S] (mV)	nr		−90
E_m 'broad signal' (mV)	−210	[6]	−310

nr, not reported.

3.3. EPR spectroscopy

In the reduced state *P. furiosus* sulfhydrogenase exhibits two EPR signals. A near-axial (g = 2.02, 1.95, 1.92) S = 1/2 spectrum (Fig. 3C) indicates the presence a [2Fe–2S] cluster as already proposed by Bryant and Adams [1]. Moreover, a broad spectrum of unknown origin with effective g-values 2.25, 1.89 was detected (Fig. 3E). This signal showed fast relaxation properties since no saturation was observed using microwave powers up to 200 mW. Double integration of the total spectrum in the reduced state integrated to 1.8 spin/mol; the putative [2Fe–2S] cluster alone comprised 0.7 spin/mol. In addition to the two signals detected in the reduced state we discovered a third signal, which is observed when the protein is partially reduced. The shape and temperature dependence of this rhombic S = 1/2 signal (g = 2.07, 1.93. 1.89) are reminiscent of a $[4Fe\text{–}4S]^{(2+;1+)}$ cluster (Fig. 3A). Integration of this spectrum yielded ≈ 0.45 spin/mol. In the oxidized state the enzyme was EPR silent; no [3Fe–4S] signals could be found, nor did we detect any Ni resonances. No high-spin resonances were detected at low field at any stage of the reduction.

3.4. Redox titration

To investigate the redox properties of the *P. furiosus* sulfhydrogenase the enzyme was titrated in the presence of mediators. By adding sodium dithionite or ferricyanide the sample was stepwise reduced or oxidized, and EPR samples were drawn at redox equilibrium. Table 1 lists the reduction potentials of the different EPR signals. The rhombic S = 1/2 signal appeared upon reduction of the enzyme; a reduction potential $E_{m,7.5}$ = −90 mV was determined. Further reduction causes this signal to disappear with an apparent reduction potential $E_{m,7.5}$ = −328 mV; a bell-shaped curve is obtained (Fig. 4A). For the near-axial S = 1/2 signal a reduction potential $E_{m,7.5}$ = −303 mV was determined (Fig. 4B). The broad signals at the wings of the spectrum appeared with a reduction potential $E_{m,7.5}$ = −310 mV (Fig. 4C). Reversibility was tested by fully

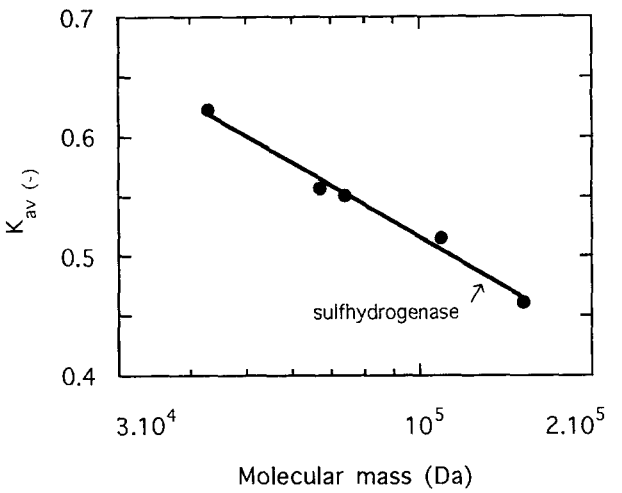

Fig. 2. Calibration of the native molecular mass of *P. furiosus* sulfhydrogenase with a Superose 6 column. Gelfiltration was performed as described in section 2. K_{av} is the ratio of the elution volume V_e minus the void volume V_0 over the total volume V_t minus the void volume V_0.

reducing the enzyme with sodium dithionite and subsequently stepwise re-oxidizing the enzyme with ferricyanide. No changes in EPR signals or reduction potentials were observed.

We have also done an aerobic isolation of *P. furiosus* sulfhydrogenase. The enzyme thus obtained was identical with respect to its activity and redox properties, as well as its EPR spectra, compared to the anaerobically isolated enzyme. Therefore, we suggest that this hydrogenase is not particularly oxygen-sensitive and there is no necessity to perform the purification under strict anaerobic conditions.

4. Discussion

4.1. Isolation and analytical data

According to the purification scheme published by Bryant and Adams it should be possible to obtain a 350-fold purification of *Pyrococcus furiosus* sulfhydrogenase in four subsequent column chromatography steps [1]. However, we were unable to reproduce their results, as SDS gel electrophoresis of our final preparation showed that the enzyme was not yet pure. Also, Mura et al. [4] found it impossible to purify the sulfhydrogenase to homogeneity using this method. By use of a modified purification scheme we were able to purify the enzyme to near homogeneity. The final preparation had a specific activity of 80 U/mg, which represents an 80-fold purification. Both the specific activity and the purification factor are lower than reported earlier [1]. Ma et al. present the isolation of a sulfur reductase of *P. furiosus* [3]. Since sulfur reductase activity and hydrogenase activity coincided during the isolation procedure, it was concluded that sulfur reductase and hydrogenase are one and the same enzyme. However, the sulfur reductase could only be purified thirty-fold instead of 350-fold for the hydrogenase. The purification factor of thirty-fold appears to be a more realistic value when compared to our results.

Enigmatic is also the difference in metal content. We find 17 Fe and 17 S^{2-}, which is almost twofold lower than reported previously, namely 31 and 24, respectively [1]. However, the numbers of Bryant and Adams [1] were based on an assumed molecular mass of 185 kDa. With the exact molecular masses of the subunits currently known [4], and with a proposed total molecular mass of 153.3 kDa, we can now correct these numbers to 27 Fe and 21 S^{2-}. These values are still higher than ours. We find 0.74 Ni, which is close to the previously published value. Unfortunately, no data on metal content have been presented for the sulfur reductase [3].

4.2. EPR spectroscopy and redox properties

We have found two EPR signals in *P. furiosus* sulfhydroge-

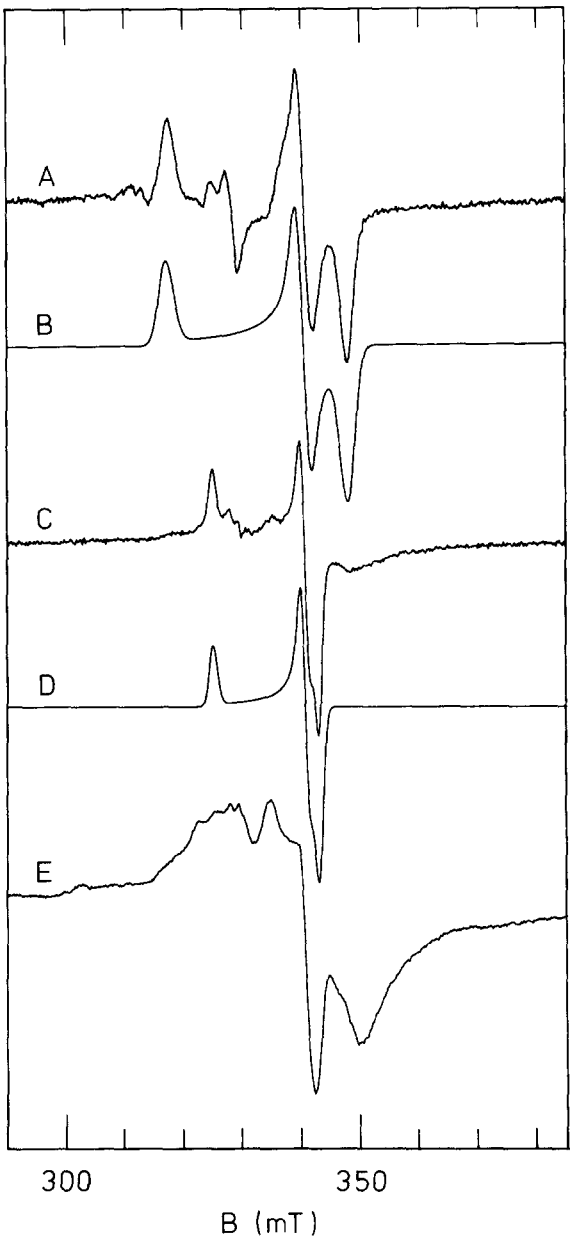

Fig. 3. EPR spectrum of purified *P. furiosus* sulfhydrogenase as a function of redox potential and microwave power. Trace A, spectrum of enzyme poised at a redox potential of −146 mV; trace B, simulation of A (see Table 2 for parameters); trace C, fully reduced enzyme; trace D, simulation of C; trace E, fully reduced enzyme at high microwave power. EPR conditions: microwave frequency, 9182 ± 1 MHz; microwave power, 5 mW or (trace E) 200 mW; modulation frequency, 100 kHz; modulation amplitude, 0.8 mT; temperature, 20 K (trace A) or 17 K.

Table 2
Simulation parameters of EPR signals from *P. furiosus* sulfhydrogenase

	z-value	*y*-value	*x*-value
'Rhombic Signal'			
g-value:	2.068	1.927	1.886
Line width:	0.0098	0.0064	0.0080
'Axial Signal'			
g-value:	2.0185	1.926	1.913
Line width:	0.0050	0.0040	0.0045

The simulations assume $S = 1/2$ and are based on 100×50 molecular orientations. The line shape is assumed to be a symmetrical Gaussian in frequency space; the line width is described as a *g*-strain tensor collinear with the *g*-tensor [14].

nase in the reduced state. As previously proposed the near-axial signal probably represents a $[2Fe\text{–}2S]^{(2+;1+)}$ cluster [1]. A reduction potential $E_{m,7.5} = -303$ mV was determined for the putative [2Fe–2S] cluster from *P. furiosus* hydrogenase. The broad signals at the wings of the spectrum ($g = 2.25$; 1.89) appeared with a reduction potential $E_{m,7.5} = -310$ mV, which is almost identical with the observed reduction potential of the putative [2Fe–2S] cluster. We also detected a rhombic $S = 1/2$ signal in the partially reduced enzyme, which possibly arises from a $[4Fe\text{–}4S]^{(2+;1+)}$ cluster. Its reduction potential is −90 mV. Upon further reduction the signal disappeared again at $E_{m,7.5} = -328$ mV, hence a bell-shaped titration curve is obtained (Fig. 3A). Adams reported to have monitored a [2Fe–2S] cluster in a titration experiment by following the $g = 2.03$ peak. A reduction potential of −410 mV was determined [6]. Although the rhombic signal has not been observed before [1], Adams reported to have followed also a putative [4Fe–4S] cluster by monitoring the amplitude of the $g = 1.88$ signal; a reduction potential of −210 mV was determined [6]. The latter resonance is presumably identical to our 'broad signal'. There is a large disparity between the reduction potentials determined in our experiments and the potentials reported by Adams. For the [2Fe–2S] cluster we find a reduction potential which is 100 mV more positive. The [4Fe–4S] cluster reported by Adams presumably represents the broad signals for which we determined a reduction potential that is 100 mV more negative (Table 1). Our calculations were done with data from three independent titrations, we therefore propose that the data presented by Adams are not accurate.

The bell-shape of the titration curve of the rhombic signal could imply that the corresponding cluster is capable of transferring two electrons. This is unprecedented for this type of cluster. So far [4Fe–4S] clusters have only been shown to switch between either the 2+ and the 1+ state, or between the 2+ and 3+ state. Iron–sulfur clusters that can take up more than one electron are thus far limited to a few examples, e.g. the P-cluster in nitrogenase [12], and the prismane protein [13]. In the periplasmic Fe-only hydrogenase from *Desulfovibrio vulgaris* (Hildenborough) a rhombic signal ($g = 2.06$) is observed when the enzyme is partially reduced. Upon further reduction this signal disappears again, giving rise to a bell-shaped titration curve. When the enzyme is re-oxidated this signal does not re-appear. It has been hypothesized that the appearance of this signal is caused by reductive activation [11]. To exclude the possibility that the rhombic signal in *P. furiosus* sulfhydrogenase is due to reductive activation, reversibility was checked by re-oxidation after reduction. The bell shape of the curve was reproduced and is therefore not a reflection of some form of activation. However, the cluster that gives rise to the rhombic signal could be coupled to a third spin system that is not observed. In this case, reduction of this third system could lead to a transition of the rhombic signal into the broad signal, i.e. the broad signal is the resultant of a magnetic interaction between the cluster of the rhombic signal and a third cluster that has become EPR detectable upon reduction with $E_{m,7.5} = -310$ mV. Because the resulting interaction spectrum has rapid spin-lattice relaxation properties the unseen third spin system is presumably also fast relaxing. The axial signal has slow relaxation properties and the shape does not point to any interaction, therefore, it is unlikely that the coupling is caused by this spin system. The high iron and sulfide content (17 Fe, 17 S^{2-}) alllows

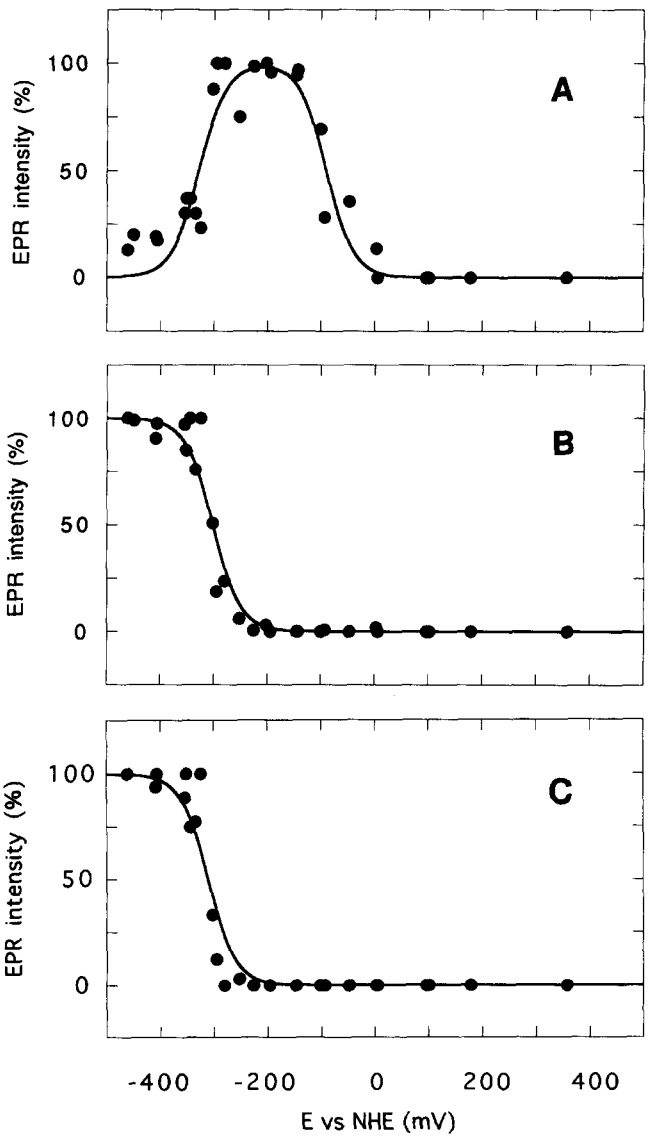

Fig. 4. Mediated redox titration of purified sulfhydrogenase from *P. furiosus*. Relative intensities of EPR signals are plotted as a function of the equilibrium redox potential in the presence of mediators (see section 2). The solid traces are least-square fits to the Nernst equation ($n = 1$) with the following E_m values: A, −328 mV, −90 mV; B, −303 mV; C, −310 mV.

for the presence of more than two clusters. Also, the four subunits have recently been shown to be homologous to the two subunits of common Ni-hydrogenases and to two of the subunits of a sulfite reductase [5] and, therefore, the $\alpha\beta\gamma\delta$ sequence carries five motifs for five putative iron–sulfur clusters. We therefore suggest that the rhombic EPR signal does not represent a two-electron transferring cluster, but that its disappearance upon reduction is due to magnetic coupling to an as yet unidentified spin system.

When we compare the results presented in this paper with previously presented data we find that there is a remarkable disparity in metal content, EPR spectra, and reduction potentials. The possibility that we have purified a different protein does not appeat to be a realistic one. The rhombic EPR signal ($g = 2.07$) has been detected only in our enzyme preparation, yet the axial signal ($g = 2.06$) and the broad signal ($g = 2.25$) have been observed in both studies; it is very unlikely that different proteins exhibit identical EPR spectra. Alternatively, the stoichiometry of the four subunits may not be constant. Because of its homology with a hydrogenase and a sulfite reductase, the *P. furiosus* sulfhydrogenase can be considered to be the sum of two functional units: a hydrogenase (α and δ subunit) and a sulfur reductase (β and γ subunit) each unit containing its own metal centers. The two units (i.e. either set of two subunits) need not necessarily be stoichiometrically expressed, for example, the expression of the subunits could be dependent on the growth conditions, and this could be reflected by the relative intensities of the different EPR signals, and, possibly, also by the metal content. However, except for some minor changes (e.g. the use of starch instead of maltose as carbon and energy source) the growth conditions used in our experiments and those of Bryant and Adams [1] are similar. Moreover, the four structural genes are organized in one transcriptional unit, and no indication was found for posttranslational processing at the amino-terminus [5]. Also, SDS gels of purified protein presented in both studies revealed the presence of four subunits. The significant discrepancies between the here presented data and the previous work remain to be resolved.

Acknowledgements: We thank W. Roseboom for help with atomic absorption spectrometry. Professor C. Veeger is acknowledged for his continuous interest and support. This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

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