

## A thermostable hybrid cluster protein from *Pyrococcus furiosus*: effects of the loss of a three helix bundle subdomain

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**Abstract** *Pyrococcus furiosus* hybrid cluster protein (HCP) was expressed in *Escherichia coli*, purified, and characterized. This is the first archaeal and thermostable HCP to be isolated. Compared with the protein sequences of previously characterized HCPs from mesophiles, the protein sequence of *P. furiosus* HCP exhibits a deletion of approximately 13 kDa as a single amino acid stretch just after the N-terminal cysteine motif, characteristic for class-III HCPs from (hyper)thermophilic archaea and bacteria. The protein was expressed as a thermostable, soluble homodimeric protein. Hydroxylamine reductase activity of *P. furiosus* HCP showed a  $K_m$  value of 0.40 mM and a  $k_{cat}$  value of  $3.8 \text{ s}^{-1}$  at 70 °C and pH 9.0. Electron paramagnetic resonance spectroscopy showed evidence for the presence of a spin-admixed,  $S = 3/2$   $[4\text{Fe-4S}]^+$  cubane cluster and of the hybrid cluster. The cubane cluster of *P. furiosus* HCP is presumably coordinated by a CXXC-X<sub>7</sub>-C-X<sub>5</sub>-C motif close to the N-terminus, which is similar to the CXXC-X<sub>8</sub>-C-X<sub>5</sub>-C motif of the *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* HCPs. Amino acid sequence alignment and homology modeling of *P. furiosus* HCP reveal that the deletion results in a loss of one of the two three-helix bundles of domain 1. Clearly the loss of one of the three-helix bundles

of domain 1 does not diminish the hydroxylamine reduction activity and the incorporation of the iron-sulfur clusters.

**Keywords** Electron paramagnetic resonance · Hybrid cluster protein · Hydroxylamine reductase · Iron-sulfur cluster · *Pyrococcus furiosus*

### Introduction

The hybrid cluster protein (HCP), also known as prismane protein or fuscodoxin in earlier literature, contains two different iron-sulfur clusters [1, 2]. The first cluster, coordinated by four cysteines near the N-terminus of the protein, is either a regular cubane-type  $[4\text{Fe-4S}]$  cluster or a  $[2\text{Fe-2S}]$  cluster. The second cluster is the unique hybrid  $[4\text{Fe-2O-2S}]$  cluster and has been named so because of the fact that it is a hybrid between an iron-sulfur and an iron-oxo cluster. The cubane cluster presumably functions in electron transfer. The hybrid cluster lies buried at the center of the protein and the distance to the cubane cluster is 11–12 Å. The hybrid cluster has an asymmetric open structure that undergoes a gross structural rearrangement upon reduction, suggesting it is the site of catalytic activity [2, 3].

HCPs are widely distributed among (facultative) anaerobic microorganisms, including bacteria, archaea, and unicellular eukaryotes. HCPs from *Desulfovibrio vulgaris*, *Desulfovibrio desulfuricans*, and *Escherichia coli* have been isolated and characterized. Differences in the predicted primary structures allow differentiation of three classes of HCPs [4–6]. Class-I HCPs have a distinct N-terminal iron-sulfur cluster binding motif: C-X<sub>2</sub>-C-X<sub>7-8</sub>-C-X<sub>5</sub>-C. Class-I *hcp* genes have so far been found in strict anaerobic bacteria and primitive eukaryotes (e.g.,

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*D. vulgaris*, *D. desulfuricans*, *Clostridium acetobutylicum*, *Giardia*, *Trichomonas vaginalis*, *Entamoeba*). Class-II HCPs have a longer spacing between the second and the third cysteine of the N-terminal iron–sulfur cluster binding motif: C–X<sub>2</sub>–C–X<sub>11</sub>–C–X<sub>6</sub>–C. Class-II *hcp* genes have been found in Gram-negative bacteria (e.g., *E. coli*, *Morganella morganii*, *Thiobacillus ferrooxidans*). In the class-II HCP from *E. coli* the N-terminal iron–sulfur cluster binding motif coordinates a [2Fe–2S] cluster. Class-II *hcp* genes are found in a dicistronic operon with an *hcr* gene encoding an iron–sulfur flavoprotein which is an NADH-dependent HCP reductase [5]. Class-III proteins have the same N-terminal iron–sulfur cluster binding motif as the class-I proteins, but they have a deletion of approximately 100 amino acid residues downstream of this motif. Class-III proteins are found in (hyper) thermophilic bacteria and archaea (e.g., *Pyrococcus furiosus*, *Ignococcus hospitalis*, *Moorella thermoacetica*, *Methanobacterium thermoautotrophicum*, *Thermatoga maritima*).

HCP shares some sequence and structural homology with the CO dehydrogenase protein family (CO dehydrogenase and the bifunctional CO dehydrogenase/acetyl-CoA synthase) [7], which is concentrated in a striking structural similarity between the protein environment of the hybrid [Fe<sub>4</sub>O<sub>2</sub>S<sub>2</sub>] cluster of HCP and that of the [NiFe<sub>4</sub>S<sub>4–5</sub>] cluster of CO dehydrogenase. Previously it was found that a change in catalytic properties takes place upon substitution of key residues that bind to the CO dehydrogenase [NiFe<sub>4</sub>S<sub>4–5</sub>] cluster. This substitution converts CO dehydrogenase from an enzyme that catalyzes CO oxidation to one that has a hydroxylamine reductase activity [7]. It was also found that insertion of iron in the vacant position of nickel-deficient CO dehydrogenase leads to a similar hydroxylamine reductase activity. The similarity of the hybrid cluster with the iron-substituted NiFeS active-site cluster of *Rhodospirillum rubrum* CO dehydrogenase suggested that HCP may be able to reduce hydroxylamine as well. Indeed it was found that *E. coli* HCP can reduce hydroxylamine to ammonia; however, *K<sub>m</sub>* for hydroxylamine is in the millimolar range [8]. Therefore it is not likely that hydroxylamine is the physiological substrate of HCP. Concentrations of hydroxylamine in the millimolar range are toxic to *E. coli* cells, despite the presence of HCP in this organism [9].

The *hcp* gene has been found to be under the control of transcription factors that are related to anaerobicity and protection against reactive nitrogen species: Fnr, OxyR, NarL, and NsrR [10–12]. The transcription of *hcp* appears to be coregulated with the NADH-dependent periplasmic nitrite reductase Nir in *E. coli* [10]. It has been shown for *E. coli* and *M. morganii* that HCP expression is induced when they are grown anaerobically in the presence of nitrate or nitrite [4]. Conflicting results have been reported on the phenotype of the *hcp* knockout with respect to the

sensitivity towards hydrogen peroxide: it makes *C. perfringens* less sensitive and *E. coli* more sensitive. The *hcp* knockout of *Rhodobacter capsulatus* has lost the ability to grow on hydroxylamine as the only nitrogen source [9]. All these regulatory data suggest that HCP is somehow involved in the metabolism (or detoxification) of reactive nitrogen species under anaerobic conditions.

Recently HCP was found to catalyze the reduction of hydrogen peroxide with ascorbate [11]. For *D. vulgaris* and *D. desulfuricans* HCP, the reactivity is of the same order of magnitude as hydroxylamine reduction activity; however, the catalytic efficiency of this reaction is very low.

In contrast to the extensive characterization of class-I and to a lesser extent of class-II HCPs, there are only gene sequence data for the class-III HCPs. Therefore, it is not known what effect the large deletion of approximately 13 kDa may have on the iron–sulfur clusters and on the catalytic properties of the protein. DNA microarray analysis proved that the class-III *hcp* gene from *P. furiosus* is transcribed in vivo. An upregulation of *hcp* transcription was measured when *P. furiosus* cells were grown on peptides compared with starch [13]. The expression level of *hcp* in *P. furiosus* is insufficient for purification from the wild-type organism. In this report we describe the recombinant expression, purification, and characterization of the class-III HCP from the hyperthermophile *P. furiosus*.

## Materials and methods

### Cultivation

*Pyrococcus furiosus* strain DSM 3638 was cultivated anaerobically at 90 °C on a complex medium with starch as the carbon source [14] or on a complex peptide medium supplemented with elemental sulfur [15]. After cultivation, the cell pellet was used for genomic DNA extraction. Genomic DNA was isolated using phenol–chloroform extraction.

### Cloning

The *hcp* gene was amplified using the following primers: PFhcp1Forward 5'-CACCATGGAAATGGCCATACG-3' (*Nco*I site underlined) and PFhcp1Reverse 5'-GTCCGTC GACTCAATCAAGTTCTTC-3' (*Sal*I site underlined). The PCR reaction was performed with Pfx polymerase (Invitrogen) or Taq polymerase (GE Healthcare). The primer combination PFhcp1Forward/PFhcp1Reverse results in a product of 1,392 bp. PCR products were used for TOPO TA cloning (Invitrogen) according to the manufacturer's instructions. Isolated plasmids were digested with their corresponding restriction enzymes (Roche) and cloned into

the pET24d expression vector. These plasmids were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene) and sequenced for confirmation.

#### Reverse transcriptase PCR

Total *P. furiosus* RNA was isolated from cells grown overnight at 95 °C on complex peptide medium supplemented with elemental sulfur [15], using the RNeasy mini kit (Qiagen), according to the manufacturers' instructions. The reverse transcription and PCR were carried out sequentially with the OneStep reverse transcriptase PCR (RT-PCR) kit (Qiagen) using a T1 Thermocycler (Biometra) and using PFhcp1Forward and PFhcp1Reverse primers for the production of complementary DNA. Reverse transcription was performed for 30 min at 50 °C. PCR was performed according to the following protocol: 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C followed by 10 min at 72 °C. A control PCR was performed using the same PCR protocol.

#### Overexpression and isolation of *P. furiosus* HCP

*Escherichia coli* strains were grown on terrific broth medium supplemented with kanamycin (25 µg/mL) and chloramphenicol (50 µg/mL) under a nitrogen atmosphere at 37 °C. Iron(III)citrate (0.01 g/L) was added prior to induction to facilitate the biosynthesis of iron–sulfur clusters. At the mid-exponential growth phase (optical density 0.4–0.6), the culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside and cooled to 28 °C. After 18 h of induction, the cells were harvested, yielding typically 3.5 g/L cells (wet weight). The cells were washed in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), pH 8.0 containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and approximately 5,000 U DNase. The cells were lysed using a cell disrupter (constant system). The cell extract was centrifuged for 1 h at 14,000g. The supernatant was concentrated using a Centriprep centrifugal filtration device with a 30-kDa cutoff (Millipore). Subsequently, the concentrated cell-free extract was passed through a 0.2-µm filter (Millipore).

All high performance liquid chromatography purification steps were performed using a Merck L-6210 Intelligent pump connected to a Waters 991 photodiode-array detector. All buffers used for purification were passed through a 0.2-µm filter and degassed. The pH of all the buffers was adjusted at room temperature.

The cell-free extract was loaded onto a Poros HQ anion-exchange column (Applied Biosystems) at a flow rate of 2 mL/min. This column was equilibrated with 50 mM Tris–HCl, pH 8.0. A gradient of 0–1.0 M NaCl in the same

buffer was applied. Brown HCP containing fractions eluted between 125 and 330 mM NaCl and were pooled. After concentration with a Centricon centrifugal filter with a 30-kDa cutoff (Millipore), the sample was run through a Superdex 200 gel-filtration column (GE Healthcare) at a flow rate of 1 mL/min. This column had been previously equilibrated with 10 mM potassium phosphate/150 mM NaCl, pH 7.0 buffer. Fractions that had the highest absorbance at 390 nm were pooled and concentrated with a Centricon filter (30-kDa cutoff). The yield of pure *P. furiosus* HCP was 8 mg/L culture.

The protein purity was estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Gel electrophoresis was carried out with a PhastSystem (GE Healthcare) according to the manufacturer's instructions. Phastgradient gels (10–15%) and SDS buffer strips were used. A sample buffer (10 mM Tris–HCl pH 8.0, 2 mM EDTA, 10% β-mercaptoethanol, 0.025% bromophenol blue) was mixed 1:1 with the sample and boiled for 5 min.

The native molecular weight was determined by native PAGE. Native PAGE was performed using a PhastSystem (GE Healthcare) using a 8–25% gradient PAGE gel with native buffer strips and native high molecular weight markers (all from GE Healthcare) according to the manufacturer's protocol.

#### Hydroxylamine reductase activity assay

The assay mixture contained 100 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid pH 9.0, 10 µM EDTA, 10 mM methyl viologen and was prepared under anaerobic conditions. For each measurement 1 mL assay mixture was used. Small aliquots (less than 1 µL) of sodium dithionite (100 mM) were added to reduce the methyl viologen until it had an absorbance of 1 at 600 nm, which corresponds to a concentration of 0.103 mM reduced methyl viologen. UV–vis absorbance was measured using a fiber-optic spectrophotometer (Avantes). Unless stated otherwise, 10 mM hydroxylamine was added as the substrate to the anaerobic assay mixture. The reaction was started by adding HCP. The decrease of absorbance at 600 nm was measured over several minutes. One unit of HCP activity was defined as 1 µmol hydroxylamine reduced per minute. The enzyme activity values were not corrected for the amount of apoprotein.

Ammonia, the product of hydroxylamine reduction, was measured enzymatically using glutamate dehydrogenase (Sigma-Aldrich) as follows. The assay reaction mixture contained 100 mM Tris–HCl pH 7.3, 15 mM α-ketoglutarate, 0.25 mM EDTA, 0.1 mM NADH. To the 1,350 µL assay mix, 100 µL HCP sample and 50 µL 100 U/mL glutamate dehydrogenase were added. The decrease in

absorption was measured over several minutes at 340 nm using the fiber-optic spectrophotometer.

#### Analytical procedures and spectroscopic methods

The iron content was determined in a colorimetric assay as the ferene complex [16]. For protein determination the bicinchoninic acid assay kit (Uptima) was used.

The N-terminal sequence of the recombinant HCP was determined after blotting onto a poly(vinylidene difluoride) membrane in a gas-phase sequenator at the Sylvius laboratories (Department of Medical Biochemistry, Leiden University, The Netherlands).

EPR data were recorded using a Bruker ER200D EPR spectrometer with a National Instruments interface and data acquisition and analysis software written in LabView/FORTRAN95. The liquid-helium cooling was as previously described [17]. The microwave frequency was measured with an HP5350B frequency counter. The modulation frequency was always 100 kHz. The power-saturation behavior of the EPR signals with amplitude  $A$  was fitted to the equation  $A = 1/[1 + (P/P_{1/2})^{0.5}]$ , in which  $P_{1/2}$  represents the power at 50% saturation of the EPR signal. The simulations of the EPR signals in Figs. 2a and 3 were based on  $g$ -strained  $S = 1/2$  systems according to [18]. The simulation of the signal in Fig. 2b is based on an  $E/D$ -strained  $S = 9/2$  system [19].

#### Homology modeling of *P. furiosus* HCP

A homology model of *P. furiosus* HCP was generated using the 3D-JIGSAW comparative modeling server using *D. vulgaris* HCP (Protein Data Bank entry 1gnt) as a template [20]. The 3D-JIGSAW program generated proper alignment of the N-terminal iron–sulfur cluster binding motif, unlike other protein modeling servers such as SWISS-MODEL. Figures were prepared using PyMOL (DeLano Scientific).

## Results

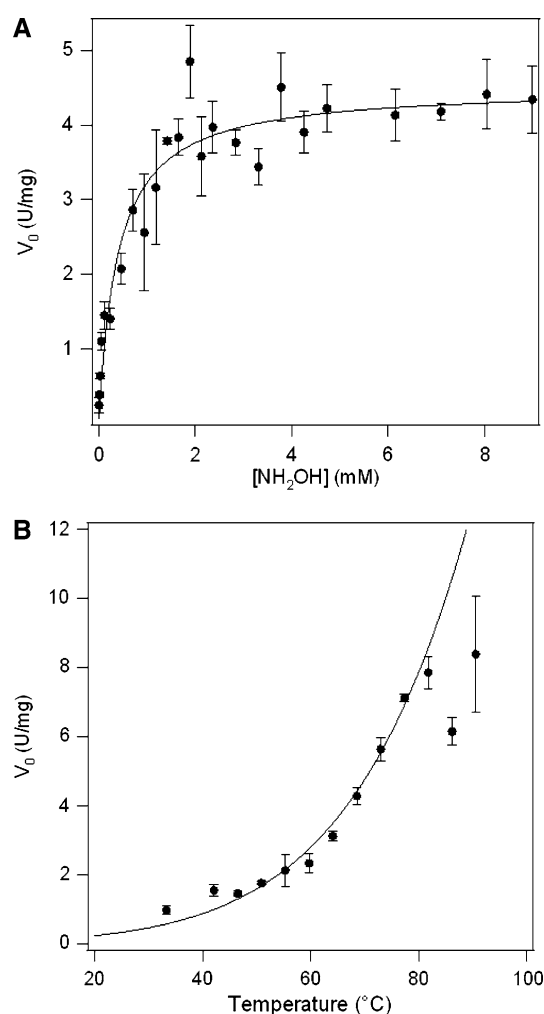
#### Expression and purification of *P. furiosus* HCP

Class-I and class-II HCPs from different species have been isolated and characterized before [1, 5, 9, 21]. Here we describe the first characterization of a class-III HCP. Owing to the low expression levels of HCP in *P. furiosus*, the *hcp* gene was overexpressed in *E. coli* using a pET expression system.

Expression plasmids were assembled in which the *hcp* gene was placed downstream of the T7lac promoter. The purified protein showed a single band at 52 kDa on an

SDS-PAGE gel (not shown). This is in good agreement with the expected molecular mass of 51.3 kDa, based on the amino acid sequence. The identity of the purified protein was confirmed by sequencing of 20 amino acids at the N-terminus. The purified HCP had a specific hydroxylamine reductase activity of 3.70 U/mg at 70 °C.

Iron determination of recombinant *P. furiosus* HCP resulted in 2.7 Fe/molecule. In the case of full occupation of a hybrid cluster and a [4Fe–4S] cluster 8 Fe/molecule can be expected. The observation of a brown color of the protein indicates the presence of iron–sulfur clusters. EPR spectroscopy was used to probe the nature of the iron–sulfur clusters in *P. furiosus* HCP.



**Fig. 1** Kinetic properties of *Pyrococcus furiosus* hybrid cluster protein (HCP). **a** Hydroxylamine reduction activity of *P. furiosus* HCP at pH 9.0 and 70 °C. The line is a least-squares fit to a Michaelis–Menten curve with  $K_m = 0.40 \pm 0.08$  mM and  $V_{max} = 4.5 \pm 0.2$  U/mg. **b** Temperature dependence of hydroxylamine reduction activity of *P. furiosus* HCP. The solid line represents a fit of the data points below 80 °C to the Eyring equation with  $\Delta H^\ddagger = 48 \pm 3$  kJ/mol and  $\Delta S^\ddagger = -93 \pm 10$  J/K mol

**Table 1** Comparison of the kinetic parameters of hydroxylamine and hydrogen peroxide reduction activities of hybrid cluster protein (HCP) from different organisms

Organism	Class	Substrate	$k_{\text{cat}}^a$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ (M <sup>-1</sup> s <sup>-1</sup> )	Reference
<i>Desulfovibrio vulgaris</i>	I	NH <sub>2</sub> OH	3.1 <sup>b</sup>	NR	NR	[3]
		H <sub>2</sub> O <sub>2</sub>	0.04	0.3	$0.1 \times 10^3$	[11]
<i>Rhodobacter capsulatus</i>	II	NH <sub>2</sub> OH	1.9	1	$1.9 \times 10^3$	[9]
<i>Escherichia coli</i>	II	NH <sub>2</sub> OH	396	2.5	$1.59 \times 10^5$	[8]
		H <sub>2</sub> O <sub>2</sub>	0.17	0.3	$0.6 \times 10^3$	[11]
<i>Pyrococcus furiosus</i>	III	NH <sub>2</sub> OH	3.8	0.40	$9.5 \times 10^3$	This work

NR not reported

<sup>a</sup> The temperatures at which the *D. vulgaris*, *R. capsulatus*, and *E. coli* HCP activities assays were performed were not reported. The activities of the *P. furiosus* enzyme were measured at 70 °C

<sup>b</sup> Measured at pH 7.0 but reported to be unchanged at pH 8.8

Native PAGE showed that *P. furiosus* HCP has a native molecular mass of 102 kDa. This is consistent with the predicted molecular mass of 102.6 kDa for the HCP homodimer. Previously characterized HCPs have been found to be monomeric [1].

#### *Pyrococcus furiosus* HCP exhibits hydroxylamine reductase activity

HCP catalyzes the two-electron reduction of hydroxylamine to form ammonia and water [7, 8]. Figure 1a shows the rate of hydroxylamine reduction by the recombinant HCP from *P. furiosus* versus the concentration of hydroxylamine at pH 9.0 at 70 °C. The enzyme exhibits Michaelis–Menten kinetics with  $K_m$  of  $0.40 \pm 0.08$  mM and  $V_{\text{max}}$  of  $4.5 \pm 0.2$  U/mg ( $k_{\text{cat}} = 3.8/\text{s}$ ). The catalytic efficiency  $k_{\text{cat}}/K_m = 9.5 \times 10^3$  M<sup>-1</sup> s<sup>-1</sup> shows that the enzyme turnover rate is far from the diffusion-limited value of  $10^8$ – $10^9$  M<sup>-1</sup> s<sup>-1</sup>; however, the value is comparable to the values of HCPs from other organisms (Table 1). The low catalytic efficiency of all HCPs for hydroxylamine and for hydrogen peroxide suggests that these reactivities may represent minor activities, leaving the major physiological substrate unknown.

*Pyrococcus furiosus* is a hyperthermophilic organism so it can be expected that its HCP is relatively thermostable. Figure 1b shows that *P. furiosus* HCP indeed exhibits thermostable hydroxylamine reduction activity. The formation of the product, ammonia, was confirmed enzymatically using glutamate dehydrogenase. With RT-PCR, we were able to qualitatively confirm the transcription of HCP in *P. furiosus* grown on casein and inorganic sulfur (not shown). Additional control PCRs with total RNA as a template showed no bands, confirming that the RNA preparation was not contaminated with (genomic) DNA. These results confirm previous

DNA microarray analysis showing that the *hcp* gene is transcribed in vivo [13].

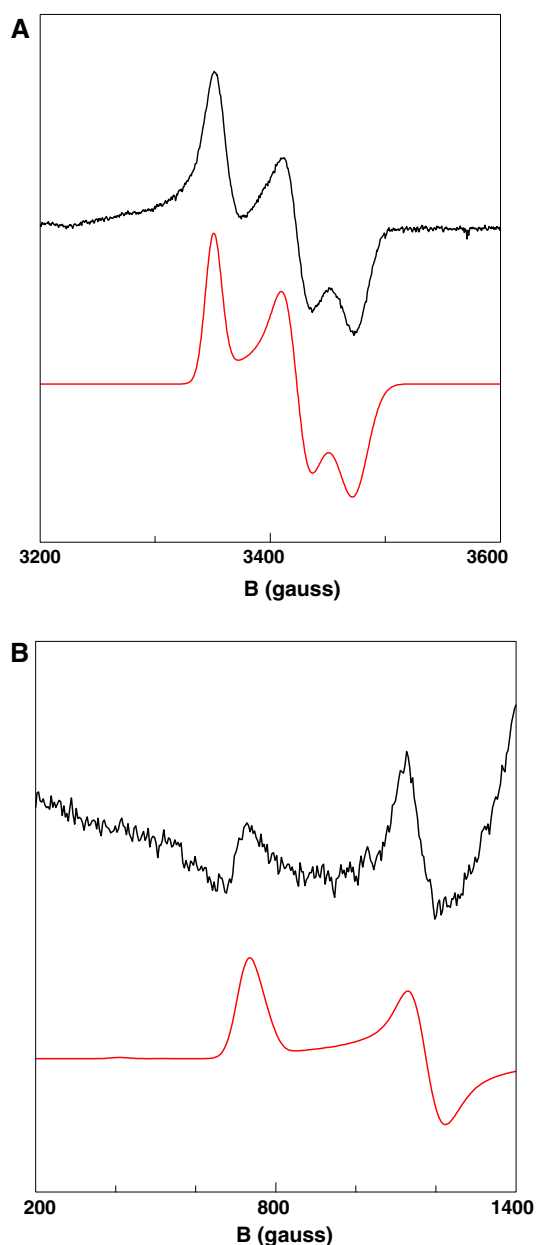
#### EPR spectroscopic characterization of the iron–sulfur clusters of *P. furiosus* HCP

Figure 2 shows EPR spectra of *P. furiosus* HCP as isolated. In the  $g = 2$  region the spectrum shows (Fig. 2a) an EPR signal which can be simulated as an  $S = 1/2$  species with  $g$  values of 2.010, 1.967, and 1.939. Power saturation of this species at 22.5 K is characterized by  $P_{1/2} = 43$  mW. This  $S = 1/2$  species is assigned to the hybrid cluster; highly similar signals have been found in *E. coli* HCP (2.01, 1.96, 1.94) [1] and recombinant *D. vulgaris* HCP [22].

The low-field signals in Fig. 2b probably originate from an excited doublet from the  $S = 9/2$  spin multiplet of a high-spin hybrid cluster as in *E. coli* HCP [5, 21]. These signals could not be simulated with  $S < 9/2$ .

Figure 3 shows the EPR spectra of dithionite-reduced HCP. Upon reduction of the sample with excess sodium dithionite, the resonances that were described above disappear. The spectra are now dominated by a complex signal centered around  $g = 2.0$  composed of signals with different temperature-dependent relaxation behavior. The signal measured at 22 K with  $g$  values of 2.013, 1.89, and 1.82 (Fig. 3, spectrum A) is attributed to the hybrid cluster. At low temperature a very broad signal is found (Fig. 3, spectrum B) which is similar to that in the EPR of reduced *D. vulgaris* and *D. desulfuricans* HCP [23, 24]. This signal has been attributed to a spin-admixed  $S = 3/2$  ground state of the  $[4\text{Fe-4S}]^+$  cluster. This is in accordance with the fact that the *P. furiosus* protein has the same N-terminal  $[4\text{Fe-4S}]$  cluster binding motif as HCP from *D. vulgaris* and *D. desulfuricans*. The loss of the three-helix bundle of domain 1 in *P. furiosus* HCP does not appear to alter the environment of the cubane cluster.

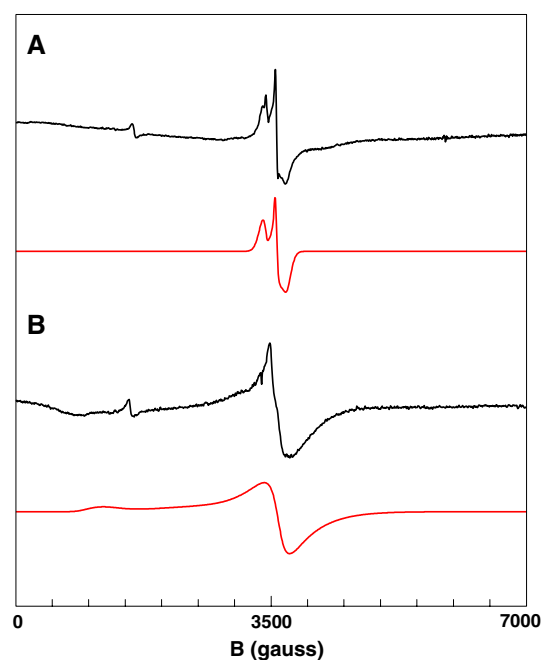




**Fig. 2** EPR spectra of as-isolated HCP. **a** Putative  $S = 1/2$  hybrid cluster signal. The EPR conditions were as follows: microwave frequency 9.427 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 12.5 gauss, temperature 22.5 K. The simulation parameters were as follows:  $g_x = 1.939$ ,  $g_y = 1.967$ ,  $g_z = 2.010$ ,  $W_x = 0.005$ ,  $W_y = 0.007$ ,  $W_z = 0.008$  (in  $g$ -value units). **b** Low-field EPR spectrum of *P. furiosus* HCP as isolated. The EPR conditions were as follows: microwave frequency 9.433 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 12.5 gauss, temperature 11.8 K. The simulation parameters were as follows:  $S = 9/2$ ,  $E/D = 0.155$ ,  $W_{\text{iso}} = 20$  gauss,  $\sigma(E/D) = 1.5\%$

#### HCP family structural comparison

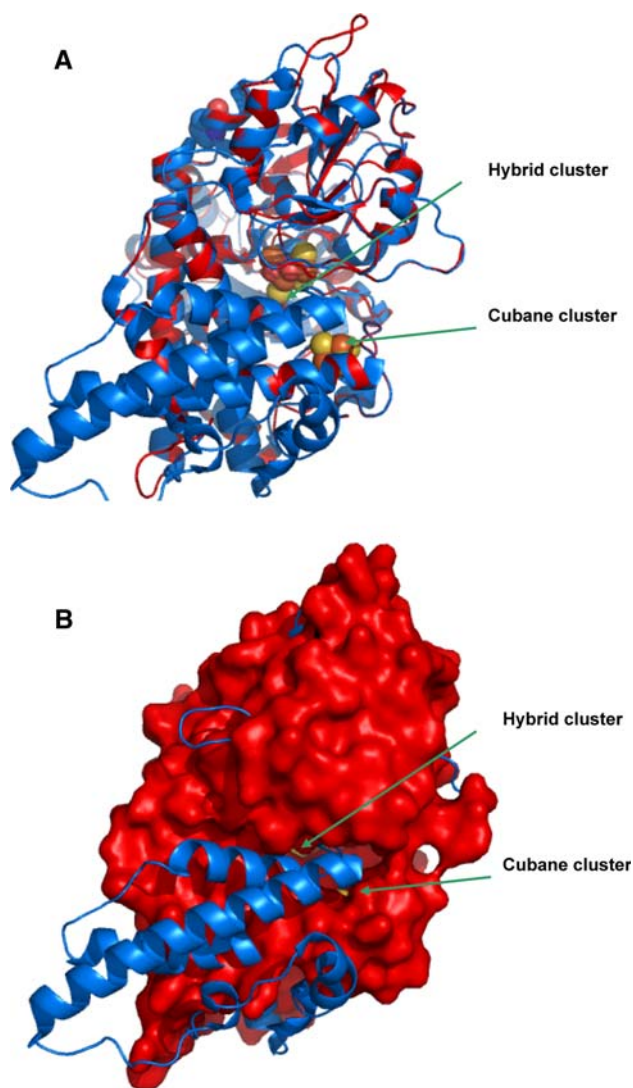
The crystal structures of *D. vulgaris* HCP and *D. desulfuricans* HCP show that the enzyme is composed of three



**Fig. 3** EPR spectra of HCP reduced with 10 mM sodium dithionite. **A** EPR spectrum at 12 K. The EPR conditions were as follows: microwave frequency 9.429 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 12.5 gauss. The simulation parameters were as follows:  $g_x = 1.820$ ,  $g_y = 1.890$ ,  $g_z = 2.013$ ,  $W_x = 0.03$ ,  $W_y = 0.018$ ,  $W_z = 0.035$ ,  $W_{xy} = 0$ ,  $W_{xz} = 0.02$ ,  $W_{yz} = 0.025$ . **B** EPR spectrum at 7.7 K. The EPR conditions were as follows: microwave frequency 9.432 GHz, microwave power 200 mW, modulation frequency 100 kHz, modulation amplitude 12.5 gauss. The simulation parameters were as follows:  $g_x = 1.63$ ,  $g_y = 1.88$ ,  $g_z = 4.8$ ,  $W_x = 0.17$ ,  $W_y = 0.09$ ,  $W_z = 1.2$ ,  $W_{xy} = 0.1$ ,  $W_{xz} = 0$ ,  $W_{yz} = 0.1$ ,  $W$  residual = 0.02 (all in  $g$ -value units)

domains [4, 24]. Domain 1 contains the cysteine motif for the cubane cluster and two three-helix bundles that exhibit structural similarity with the molecular chaperone DnaK and the proteasome activator REG $\alpha$  [4]. Domain 2 and domain 3 contain a core of a six-strand parallel twisted sheet and together provide all the ligands to the hybrid cluster. Sequence alignment of *P. furiosus* HCP with the *D. vulgaris* and *D. desulfuricans* HCPs shows that the deletion in the archaeal HCP sequences results in the loss of one of the two three-helix bundles of domain 1, while the metal binding part is conserved. By overlaying a homology model of *P. furiosus* HCP with the *D. vulgaris* HCP structure, one can clearly see the effect of the deletion on the whole structure (Fig. 4). The loss of one of the two three-helix bundles of domain 1 would increase the solvent accessibility of both the cubane and the hybrid cluster if *P. furiosus* HCP were monomeric like the other HCPs. The dimeric state may be necessary to protect the active site and maintain substrate selectivity.

Interestingly, the three-helix bundle of HCP domain 1 is also absent in CO dehydrogenase. CO dehydrogenase can



**Fig. 4** Structural comparison of *P. furiosus* HCP and *Desulfovibrio vulgaris* HCP. **a** Overlay of the homology model of *P. furiosus* HCP (red) and the crystal structure of *D. vulgaris* HCP (Protein Data Bank entry 1GNT) (blue). The effect of the deletion is clearly visible in this picture. One three-helix bundle of domain 1 is lost in the *P. furiosus* HCP, leaving the cubane cluster more exposed to the solvent. **b** The effect of the deletion on the solvent accessibility of the hybrid cluster and the cubane cluster

occur as a monomer, a homodimer, or an  $\alpha_2\beta_2$  heterotetrameric bifunctional (CO dehydrogenase/acetyl-CoA synthase) enzyme. CO dehydrogenase has an extra 45 amino acid sequence preceding the cubane binding motif which is absent in all HCPs. The similarity between CO dehydrogenases and the class-III HCPs, in particular, has prompted researchers to predict that the class-III HCPs are dimeric unlike the other HCPs [25]. A structural topology was proposed in which the missing helix bundle is replaced by the helix bundle from the other monomer. Here we provide evidence that, at least for one of the class-III HCPs,

this is true. The nature of the dimerization interface has to be determined from X-ray crystallographic data.

## Discussion

A class-III HCP from *P. furiosus* was expressed and characterized for the first time. The optimum activity is at approximately 80 °C, confirming the thermostable nature of the protein.

EPR spectroscopy of the recombinant *P. furiosus* HCP gave signals that can be attributed to a [4Fe–4S] cluster and the hybrid cluster. The dithionite-reduced sample showed a [4Fe–4S]<sup>+</sup> cluster with a spin-admixed  $S = 3/2$  ground state as in *D. vulgaris* HCP and *D. desulfuricans* HCP. Extensive EPR and Mössbauer spectroscopic studies on other HCPs have shown that the hybrid cluster can exist in four different redox states that can be designated as 3+, 4+, 5+, and 6+ according to prismane protein nomenclature [2], where the 6+ state represents the all-ferric state of the cluster. The 6+ state is  $S = 0$ , the 5+ state is a mixture of  $S = 1/2$  and  $9/2$ , the 4+ state is a mixture of  $S = 0$  and 4, and finally the 3+ state is  $S = 1/2$ . In short, the EPR signals from the hybrid cluster are  $S = 1/2$ , for the reduced cluster (3+ state) and  $S = 1/2$  and  $9/2$  for the oxidized cluster (5+ state). We were able to observe signals for the 5+ state in as-isolated *P. furiosus* HCP and for the 3+ state in dithionite-reduced *P. furiosus* HCP.

*Pyrococcus furiosus* has a second smaller *hcp* gene in its genome. This gene encodes a 13.5-kDa protein (Pf6089) that contains four cysteines, but misses several essential residues for the coordination of the cubane and the hybrid cluster. The sequence of residues 1–85 from this “mini-HCP” is nearly identical (89% identity) to that of residues 111–168 and 200–226 of the full-length HCP. The C-terminal sequence, residues 86–123, does not resemble any known gene product, but provides two of the four cysteines. This “mini-HCP” gene is only present in *P. furiosus* and was not found in any other genome database.

In the light of the enigmatic physiological role of HCP, it is interesting that a close relative of *P. furiosus*, *P. horikoshii*, does not contain the *hcp* gene in its genome. The genomes of *P. furiosus* and *P. horikoshii* have been compared and analyzed in detail [26]: despite the high sequence identity at the 16S ribosomal RNA level, the two genomes have diverged significantly. *P. horikoshii* has lost a significant number of operons, including several involved in amino acid biosynthesis, polysaccharide degradation, and the tricarboxylic acid cycle. The physiological role of HCP in *P. furiosus* may be linked to one of these operons.

In conclusion, the deletion of one three-helix bundle of domain 1, which is common to all class-III HCPs, does not

diminish hydroxylamine reduction activity. The activity combined with the EPR spectroscopic data indicate that both a regular [4Fe–4S] cubane cluster and a hybrid cluster are present in *P. furiosus* HCP. Hydroxylamine reduction activity has now been measured for HCPs of all three classes and hydrogen peroxide reduction has been measured for HCPs from classes I and II; however, the low catalytic efficiencies for all these reactions indicate that these are minor promiscuous activities. The physiological substrate of HCP is still to be discovered.

Deletion of a 116 amino acid stretch from domain 1 may offer a site at which dimerization occurs. The deletion also appears to lead to a more compact structure of the monomer, which together with the dimeric structure could be a mechanism to increase the thermal stability of this class-III archaeal HCP.

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