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The hyperthermophilic bacterium, *Thermotoga maritima*, contains an unusually complex iron-hydrogenase: amino acid sequence analyses versus biochemical characterization[1]

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Abstract

The hyperthermophilic bacterium, *Thermotoga maritima*, grows up to 90°C by fermenting carbohydrates and it disposes of excess reductant by H_2 production. The H_2-evolving cytoplasmic hydrogenase of this organism was shown to consist of three different subunits of masses 73 (α), 68 (β) and 19 (γ) kDa and to contain iron as the only metal. The genes encoding the subunits were clustered in a single operon in the order *hydC* (γ), *hydB* (β), and *hydA* (α). Sequence analyses indicated that: (a) the enzyme is an Fe-S-cluster-containing flavoprotein which uses NADH as an electron donor; and (b) the catalytic Fe-S cluster resides within the α-subunit, which is equivalent to the single subunit that constitutes most mesophilic Fe-hydrogenases. The α- and β-subunits of the purified enzyme were separated by chromatography in the presence of 4 M urea. As predicted, the H_2-dependent methyl viologen reduction activity of the holoenzyme (45–70 U mg^{-1}) was retained in the α-subunit (130–160 U mg^{-1}) after subunit separation. However, the holoenzyme did not contain flavin and neither it nor the α-subunit used NAD(P)(H) or *T. maritima* ferredoxin as an electron carrier. The holoenzyme, but not the α-subunit, reduced anthraquinone-2,6-disulfonate (apparent K_m, 690 μM) with H_2. The EPR properties of the reduced holoenzyme, when compared with those of the separated and reduced subunits, indicate the presence of a catalytic 'H-cluster' and three [4Fe-4S] and one [2Fe-2S] cluster in the α-subunit, together with one [4Fe-4S] and two [2Fe-2S] clusters in the β-subunit. Sequence analyses predict that the α-subunit should contain an additional [2Fe-2S] cluster, while the β-subunit should contain one [2Fe-2S] and three [4Fe-4S] clusters. The latter cluster contents are consistent with the measured Fe contents of about 32, 20 and 14 Fe mol^{-1} for the holoenzyme and the α- and β-subunits, respectively. The *T. maritima* enzyme is the first 'complex' Fe-hydrogenase to be purified and characterized, although the reason for its complexity remains unclear.

Keywords: Hydrogenase; *Thermotoga maritima*; Hyperthermophile

Abbreviations: AQ, Anthraquinone 2,6-disulfonic acid; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; POR, pyruvate ferredoxin oxidoreductase; TPP, thiamine pyrophosphate chloride

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1. Introduction

Hydrogenases catalyze the reversible oxidation of H_2 gas and they have been purified from a diverse array of both aerobic and anaerobic microorganisms. Most of them contain nickel and Fe-S clusters, the so-called NiFe hydrogenases [1], but some contain only Fe-S clusters and these are termed the Fe-hydrogenases [2]. There is also one hydrogenase from a methanogen which does not contain any metal, although its H_2-oxidizing activity is obligately dependent on its cofactor substrate [3,4].

To date, Fe-hydrogenases have been purified only from anaerobic bacteria, and these include the enzymes from *Desulfovibrio vulgaris* [5–7], *Desulfovibrio desulfuricans* [8], *Desulfovibrio fructosovorans* [9], *Megasphaera elsdenii* [10] and two from *Clostridium pasteurianum* [11,12]. All six enzymes have molecular masses of ~60 kDa and consist of either one or, in the case of the *Desulfovibrio* enzymes, two subunits (of 46 and 11 kDa). All of these enzymes contain the so-called 'H-cluster', the proposed site of H_2 catalysis, as shown by the unique rhombic EPR signal of the oxidized enzymes, and at least two ferredoxin-type [4Fe-4S] clusters. Gene sequences have been reported for the *D. vulgaris* and *D. fructosovorans* hydrogenases and for one of the *C. pasteurianum* enzymes [9,13,14], together with those for several putative Fe-hydrogenases that have yet to be purified. These include genes in two *Clostridium acetobutylicum* strains, in two *Desulfovibrio* species (including a second Fe-hydrogenase in *D. fructosovorans*), in the anaerobic protist, *Trichomonas vaginalis*, and in the ciliate *Nyctotherus ovalis* [15–20]. Sequence comparisons reveal that both the purified and the hypothetical Fe-hydrogenases are highly similar. All of the hypothetical Fe-hydrogenases appear to consist of a single subunit, with the exception of the enzyme from *D. fructosovorans*, in which adjacent genes have been suggested to play a role in H_2 metabolism [16].

The structures of the Fe-hydrogenases from *C. pasteurianum* and *D. desulfuricans* were recently obtained from X-ray crystallography [21,22]. These provided detailed information on the nature of the catalytic H-cluster, which was shown to be a hybrid of a [4Fe-4S] cluster and a [2Fe] subcluster bridged by a single cysteinyl sulfur. The [2Fe] subcluster contained several diatomic ligands which, by analogy to the situation in the NiFe hydrogenases, were identified as CO and CN based on FTIR studies of the *D. vulgaris* enzyme [21–28]. It was, furthermore, postulated that the two Fe-atoms in the [2Fe] subcluster are bridged by a 1,3-propanedithiol (PDT) molecule instead of two inorganic sulfide atoms [22]. The crystallographic analysis also provided insight into the likely electron transfer pathways in the hydrogenases of *C. pasteurianum* and *D. desulfuricans*. The latter enzyme has two [4Fe-4S] centers which are thought to form a pathway for electron flow from the surface of the protein to the active site H-cluster. The clostridial enzyme contains additional clusters, one of the [4Fe-4S] and one of the [2Fe-2S] type, which extend the putative electron transfer pathway providing two possible sites where electrons can enter the enzyme from physiological electron donor(s) [21].

Herein we focus on the Fe-hydrogenase from *Thermotoga maritima*. This is a strictly anaerobic organism which grows up to 90°C. It represents one of the two most slowly evolving branches within the bacterial domain; therefore, evolutionary information might be obtained by comparing the properties and primary sequence of certain enzymes from this organism with their counterparts in mesophilic organisms [29,30]. The hydrogenase from *T. maritima* has been purified and, based on metal analyses, was identified as an Fe-only enzyme [31]. However, the enzyme was reported to be a homotetramer (subunit mass 68 kDa), not to exhibit the rhombic EPR signal characteristic of the oxidized H-cluster, and to contain two [2Fe-2S] and two [4Fe-4S] clusters per subunit [31]. Resonance Raman spectroscopy confirmed the presence of [2Fe-2S] and [4Fe-4S] centers [32]. In a subsequent report, an analysis of the EPR properties of the enzyme revealed an additional [2Fe-2S] center and an unusual high potential Fe-S cluster [33].

In this paper, data are presented to show that what was assumed to be a single subunit of *T. maritima* hydrogenase actually represents two subunits of comparable molecular size, and a third much smaller subunit has also been identified. Hence, the enzyme is a heterotrimer and much more complex than the mesophilic Fe-hydrogenases purified so far. In addition, the genes encoding the three subunits of the *T. maritima* enzyme have been cloned and sequenced.

This has allowed predictions to be made regarding the cofactor content and function for each subunit. However, biochemical analyses of the purified enzyme do not fully conform with these predictions, and the reason for the complexity of this hydrogenase remains unknown.

2. Materials and methods

2.1. Growth of the organism

Thermotoga maritima (DSM 3109) was grown using glucose as the carbon source in 500 l cultures at 80°C in the medium described previously [31] with the following modifications: yeast extract (0.25%, w/v) $CaCl_2$ (0.45 mM), KCl (26.8 mM) and $(NH_4)_2CO_3$ (12 mM) were at the indicated concentrations and NaI, H_3BO_4, $SrCl_2$, KI, and $CaCO_3$ were omitted. After adjusting the pH to 7.0, the medium was sterilized and cooled to 80°C under a continuous flow of Ar before potassium phosphate buffer (from a 1-M sterile stock solution, pH 7.0) was added to a final concentration of 3.7 mM. Cysteine hydrochloride (2.3 mM) or titanium citrate (30 μM) were added to reduce the medium before the inoculum (6%) was added. These reductants supported growth equally well. The pH of the culture was kept constant at 7.0 (80°C) by addition of NaOH from a 5-M stock solution. Cell growth was monitored by the OD_{600} and by determining the protein concentration [34]. Cells were harvested in the late log phase after approximately 16 hs (ΔOD 0.8–1.0), frozen in liquid N_2 and stored at −80°C.

2.2. Analytical methods

Hydrogenase activity was routinely measured by the H_2-dependent reduction of methyl viologen (1 mM) in 50 mM EPPS buffer, pH 8.4, at 80°C as previously described [31] where 1 U is equivalent to 1 μmol H_2 oxidized min^{-1}. When anthraquinone 2,6-disulfonic acid (1 mM) was used as an alternate electron acceptor, the reaction was monitored at 436 nm and a molar absorption coefficient of 3500 M^{-1} cm^{-1} was used [35]. Hydrogenase activity was also measured by H_2 evolution from methyl viologen (1 mM) reduced by sodium dithionite (10 mM) in 50 mM EPPS buffer, pH 8.4 [31]. Activity measurements in the presence of metronidazole were performed as described previously [36]. Hydrogenase was incubated in 50 mM EPPS, pH 8.4, in the presence of 0.1 mM metronidazole in H_2-flushed cuvettes at 80°C. After monitoring the baseline activity, 10-μl aliquots of anaerobically prepared cell extracts before (∼20 mg ml^{-1}) and after centrifugation (∼10 mg ml^{-1}) were added and the reduction of metronidazole was followed at 320 nm. Purified *T. maritima* ferredoxin was also tested in this coupled system using ferredoxin concentrations up to 20 μM. Experiments to determine the functionality of NAD(P) as an electron acceptor were performed at 70°C in 50 mM EPPS buffer, pH 8.4, flushed with H_2. The final NAD(P) concentration was 1 mM and measurements were made both in the presence and absence of 0.8 M KCl and 50 μM of either FAD, FMN or riboflavin. The purified enzyme used in these measurements was preincubated for 30 min under H_2 in EPPS buffer, pH 8.0, at 70°C in the presence of 2 mM sodium dithionite, 0.8 M KCl, and 50 μM of either FAD, FMN or riboflavin [37].

In order to determine whether a small electron transfer protein was involved as an electron acceptor for hydrogenase, cell-free extracts were concentrated anaerobically using an ultrafiltration device with a cutoff of 3 kDa or a cutoff of 100 kDa. In the latter case, the concentrate was washed three times with 20 mM EPPS, pH 8.0, containing 1 M KCl and concentrated again. The concentrated extracts were tested for their ability to reduce NAD(P) as described above. H_2 production in the POR-linked assay was measured in 2 ml 50 mM EPPS, pH 8.4, in the presence of 5 mM pyruvate, 0.1 mM CoA, 2.5 mM $MgCl_2$, and 0.4 mM TPP. *T. maritima* POR (100 μg), purified hydrogenase (160 μg) and purified *T. maritima* ferredoxin up to 20 μM or concentrated cell free extracts were added up to a final concentration of 1 mg ml^{-1}. In a control experiment, methyl viologen (0.25 mM) was added to the assay mixture to verify that electron transfer between the different components could occur when a suitable electron carrier is present. H_2 production was measured by gas chromatography as described [31]. Ferredoxin [38] and POR [39] from *T. maritima* were purified as described in the references.

Protein concentrations were determined using the

microbiuret method [40] after trichloroacetic acid/deoxycholate precipitation [41]. Iron was determined by inductively coupled plasma emission spectroscopy (ICP) and by a colorimetric method using *o*-phenanthroline [42], but with the following modification. Due to the intrinsic stability of the *T. maritima* enzyme, it was necessary to incubate the protein for 30 min at 100°C in order to completely extract the iron from the protein. Acid-labile sulfide was determined according to the methods described previously [43,44]. The purity of protein preparations was determined by denaturing gel electrophoresis using 4–12% NuPage gels (Novex, CA) with MES, pH 7.3, as the running buffer according to the manufacturer's instructions. The gels were stained with colloidal Coomassie (Novex, CA). Peptide fragments of the hydrogenase were obtained by digestion of a 4 mg ml^{-1} sample with 10 μg ml^{-1} *Staphylococcus aureus* V-8 protease in 20 mM Tris, pH 8.0, at 30°C. After 30 min, SDS and β-mercaptoethanol were added to the reaction mixture to a final concentration of 2 and 10%, respectively, and the mixture was subsequently heated at 100°C for 10 min before being loaded onto a 20% Tris/glycine SDS-gel [45]. After electrophoresis, the bands were blotted onto a PVDF membrane using 10 mM CAPS, pH 11.0, containing 10% methanol as the transfer buffer [46], stained with Coomassie Blue R-250 and cut out. The different fragments were sequenced with an Applied Biosystems model 477 sequencer.

The molecular mass of the native holoenzyme and of the separated subunits was determined using a calibrated Superdex S-200 column (10/30) equilibrated in either MES (pH 6.0), CHES (pH 8.0) or CAPS (pH 10.0), each at 20 mM concentration. The presence of flavin in the holoenzyme was assessed using thin layer chromatography. The enzyme (6–15 mg ml^{-1} in 20 mM Tris, pH 8.0) was incubated with 6 M guanidine hydrochloride for 24 h at 25°C and a sample (3 μl) was applied to a silicagel 60, 200-μm TLC plate (Selecto Scientific, Norcross, GA). FMN and FAD in concentrations equimolar to the enzyme were used as the standards. The TLC plate was developed with 5% $NaH_2PO_4 \cdot 12H_2O$ in distilled water, dried and the presence of the cofactor determined by irradiation with UV-light [47].

EPR samples of the holoenzyme or the different subunits were prepared by desalting the different preparations using a Sephadex G-25 Superfine column (0.7×10 cm) equilibrated with 50 mM EPPS, pH 8.0, in an anaerobic glove box (Vacuum Atmospheres, CA). The samples were subsequently oxidized in three different ways: (1) by H_2 evolution, whereby the dithionite-free samples of the holoprotein and the α-subunit were incubated at 50°C for 20 min in anaerobic vials under N_2 and were then transferred to anaerobic EPR tubes and rapidly frozen in a heptane/liquid N_2 mixture; (2) with 2,6-dichlorophenolindophenol (DCIP) (1.4 mM final concentration), wherein the samples were desalted using Sephadex G-25 chromatography and were incubated with the reagent for 30 min at 35°C in the dark, and desalted again prior to freezing; and (3) with thionine (10 mM), wherein desalted protein samples were incubated with the reagent for 10 min at 50°C before freezing. Reduced samples were prepared either by the addition of sodium dithionite (2 mM) followed by incubation at 25°C for 20 min under Ar, or by the addition of sodium dithionite (2 mM) followed by incubation at 50°C for 30 min under H_2 (40 kPa). In both cases, the samples were transferred to EPR tubes in the anaerobic glove box and rapidly frozen. EPR spectra were recorded on a Bruker ER 300E spectrometer equipped with an Oxford instruments ITC flow cryostat and interfaced to an ESP 3220 computer. Spin concentrations were determined by double integration of the first derivative spectra recorded under non-saturating conditions using a microwave power ranging from 10 μW to 1 mW. Spectra of a solution of 0.993 mM copper sulfate and 100 mM EDTA recorded at 10 μW under otherwise identical conditions were used as the standard.

2.3. Purification of hydrogenase and separation of its subunits

The purification of the hydrogenase was performed under strictly anaerobic conditions with sodium dithionite (2 mM) and dithiothreitol (2 mM) present in all the buffers according to the published procedure [31], but with the following modifications. The cell-free extract of 400 g (wet weight) of cells was loaded onto a DEAE Sepharose FF column (10×20 cm) and the hydrogenase was eluted at 30 ml min^{-1} in a linear gradient of 15 l from 0 to 0.5 M NaCl in

20 mM Tris-HCl, pH 8.0. The pooled hydrogenase fractions were loaded onto a Q-Sepharose HP column (3.5×10 cm) equilibrated with 20 mM Tris-HCl, pH 8.0. Adsorbed proteins were eluted at 4 ml min^{-1} with a linear gradient (1 l) from 0 to 0.5 M NaCl in 20 mM Tris-HCl, pH 8.0. The hydrogenase containing fractions were diluted with two volumes of equilibration buffer and loaded onto a hydroxyapatite column (5.0×12 cm) equilibrated in 20 mM Tris-HCl, pH 8.0. The column was developed at 4 ml min^{-1} with a linear gradient (2 l) from 0 to 200 mM potassium phosphate in 20 mM Tris-HCl. The hydrogenase-containing fractions were concentrated using a HiTrap Q column (5 ml) equilibrated with 20 mM Tris-HCl, pH 8.0, at 4 ml min^{-1} and eluted with 0.5 M NaCl in Tris-HCl, pH 8.0. The concentrated fraction was loaded onto a Superdex S-200 gel filtration column (6.0×60 cm) equilibrated in 20 mM HEPES, pH 7.0, containing 150 mM NaCl at 5 ml min^{-1}. Ammonium sulfate (to 1.0 M) was added to the hydrogenase-containing fractions and these were loaded onto a column (3.5×10 cm) of Phenyl Sepharose equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 M $(NH_4)_2SO_4$ and 10% glycerol at 5 ml min^{-1}. The protein was eluted with a linear gradient (1 l) from 1 to 0 M $(NH_4)_2SO_4$. Fractions containing hydrogenase were pooled, loaded on a small Phenyl Sepharose column (1.6×5 cm) equilibrated with 1 M $(NH_4)_2SO_4$ and 10% glycerol at 4 ml min^{-1}, and the enzyme was eluted with 20 mM Tris-HCl containing 10% glycerol at 2 ml min^{-1}. The concentrated enzyme was applied to a Superdex S-200 column (6.0×60 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 150 mM NaCl at 5 ml min^{-1}. Active fractions were pooled and concentrated inside an anaerobic glove box with an Amicon ultrafiltration unit equipped with a PM-30 membrane. The activity of the final preparation ranged from 45 to 75 U mg^{-1} as determined in the hydrogen uptake assay with methyl viologen as the electron acceptor.

The subunits of hydrogenase were separated using a Phenyl Sepharose column (3.5×10 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 M $(NH_4)_2SO_4$ and 10% glycerol at 5 ml min^{-1}. The adsorbed proteins were eluted with a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ containing 4.0 M urea and 10% glycerol. Fractions containing hydrogenase activity and the β-subunit (as determined by gel electrophoresis) were pooled separately. To remove urea, each was loaded on a small Phenyl Sepharose column (1.6×5 cm) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 1 M $(NH_4)_2SO_4$ and 10% glycerol and eluted with 20 mM Tris-HCl, pH 8.0, containing 10% glycerol. The proteins were then applied separately to a Superdex 200 column (3.5×60 cm) equilibrated with 20 mM HEPES, pH 7.0, containing 100 mM NaCl at 4 ml min^{-1}. The fractions containing hydrogenase activity were pooled and concentrated using a HiTrap Q column which was eluted with 20 mM Tris, pH 8.0, containing 0.5 M NaCl. The fractions containing the β-subunit were concentrated in the same way. All final preparations of the proteins were stored as pellets in liquid N_2. Digestion of the isolated subunits was carried out by incubation at 37°C for 30 min with V8 protease from *S. aureus*, α-chymotrypsin, and protease from *Bacillus thermoproteolyticus*. The α- and β-subunit were incubated in sample buffer [45] without SDS at a concentration of 0.6 and 0.9 mg ml^{-1}, respectively. The different proteases were added to a final concentration of 15 μg ml^{-1}. After incubation of the samples for 30 min at 37°C, 2% SDS and 10% β-mercaptoethanol were added, the samples were boiled for 10 min and subsequently loaded onto a 15% Tris/glycine SDS gel.

2.4. Nucleotide sequencing and analysis

The genes encoding the three subunits of *T. maritima* hydrogenase were identified using the available NH_2-terminal and internal sequence information. For the α-subunit two degenerate primers 5′-ATG AAR ATT TAY GTI GA-3′ and 5′-TTR TCR TTT ATT ATI AC-3′ were used to produce a 44-bp PCR fragment coding for its NH_2-terminus. The sequence of this fragment was used to design a non-degenerate primer and this, together with a degenerate primer 5′-CCR CAI GGR CAR TAI GCI ACR CAY TGI CCR CA-3′ based on the internal NH_2-terminal sequence of the α-subunit, yielded a 594-bp PCR product. Two degenerate primers 5′-TTY AAG AAT GCI AAG GA-3′ and 5′-TTG CAI AGI GTT TTI AGY TTR TT-3′ were also used to produce a 57-bp PCR product encoding the NH_2-terminus of the β-subunit. A non-degenerate primer 5′-AAG AAT GCG AAG GAG TTC GTC CAG TAC

GC-3′ designed using the sequence of this PCR product together with a non-degenerate primer 5′-ACG TTC TTC AGC GCT TCA AG-3′ based on the sequence of the previously obtained 594-bp fragment produced a 2-kb PCR product that contained *hydB*. Finally, a degenerate primer 5′-GGA RMG ICA YTT YGA RAA RGT IGA RGA RAT-3′ designed from the NH_2-terminus of the γ-subunit together with a non-degenerate primer 5′-GAG ACC GGA TGC CTT CAC-3′ based on the sequence of *hydB* were used to produce a 1058 bp PCR product that contained *hydC*. The three different PCR products obtained this way were labeled with digoxigenin-UTP using the Genius System (Boehringer Mannheim, IN). These were used to probe a *T. maritima* genomic library, prepared using the λ ZAP II system (Stratagene, CA) and kindly provided by Diversa (San Diego, CA). Three genomic fragments were obtained which together encompassed the hydrogenase gene cluster. All fragments were sequenced in both directions by Sanger dideoxy sequencing using the Sequenase kit purchased from USB (Cleveland, OH) or by automated DNA sequencing using an Applied Biosystems model 373A sequencer.

3. Results

3.1. Characterization of the enzyme and subunit separation

Purified preparations of the hydrogenase from *T. maritima* when analyzed by SDS gel electrophoresis (using 15%, w/v, acrylamide) gave rise to two proteins bands of approximate equal intensity which migrated closely together with a molecular mass around 70 kDa (see Fig. 1). In addition, a weakly staining protein band with a molecular mass of approximately 19 kDa was apparent. These data are in contrast to the single band of ~68 kDa identified in a previous report [31], which used higher protein concentrations for electrophoretic analyses and an 11% (w/v) acrylamide gel which does not resolve small proteins (<20 kDa). Attempts to separate the three protein species in the hydrogenase sample using different chromatographic materials at pH values between pH 6 and 10 were unsuccessful. In particular, the relative amounts of the three subunits, assessed

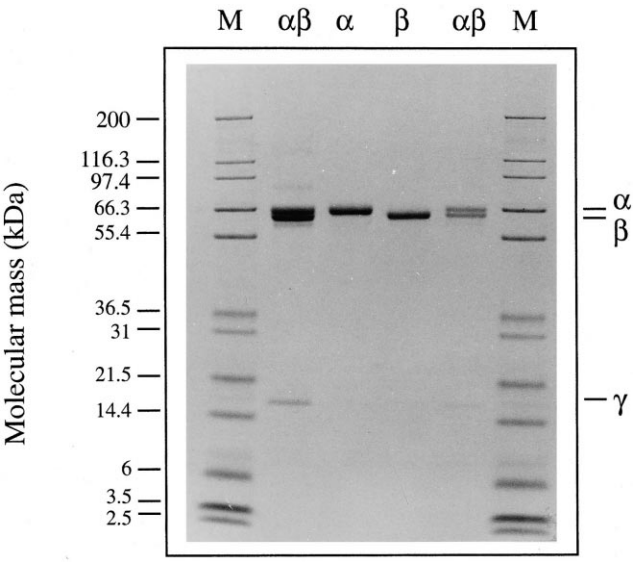

Fig. 1. SDS-PAGE of the *T. maritima* hydrogenase holoprotein and its purified subunits. Lane 1, molecular mass marker mixture; lane 2, 0.7 μg of the holoprotein; lane 3, 0.4 μg purified α-subunit; lane 4, 0.4 μg purified β-subunit; lane 5, 0.35 μg of the holoprotein; lane 6, molecular mass marker mixture.

by scanning of Coomassie-stained denaturing SDS gels, remained the same (1:1:0.9 for α:β:γ). We therefore concluded that the holoenzyme is a heterotrimer.

The overall recovery of hydrogenase activity after purification was 15–20% and the specific activity of the purified preparations varied between 45 and 70 U mg^{-1} in the H_2-consumption assay and 9 and 15 U mg^{-1} in the H_2-production assay using methyl viologen as the electron acceptor or donor, respectively. These values are comparable to those reported previously [31]. The specific activity of the *T. maritima* enzyme is considerably lower than the values reported for hydrogenase I from *C. pasteurianum* or the enzymes from the different *Desulfovibrio* species. Due to the difference in molecular mass it is, however, more appropriate to compare the turnover numbers of the enzymes instead. The turnover number of the *T. maritima* enzyme ranges from 120 to 190 s^{-1} in the consumption assay and 24 to 40 s^{-1} in the production assay, which is well within the range of values reported for hydrogenase II from *C. pasteurianum* and the Fe-only hydrogenases from *M. elsdenii* and *T. vaginalis* (specific activities 120–600 s^{-1} in the H_2-consumption assay and 8–500 s^{-1} in the H_2-production assay) [10,48,49].

Incubation of the purified hydrogenase in 20 mM Tris, pH 8.0, in the presence of 2 or 4 M guanidine-HCl for 20 h at 25°C led to an almost complete loss of activity; however, no activity was lost when the same treatment was carried out with 5.0 M urea. Moreover, it was found that in the presence of urea, the two large subunits could be separated using Phenyl Sepharose chromatography, with the larger 73- kDa subunit (α) eluting after the 68-kDa subunit (β). Both proteins contained Fe-S clusters as judged from their absorbance at 400 nm, but the third subunit (γ) was not detected by this method. An SDS gel of the hydrogenase before and after chromatography in the presence of urea is shown in Fig. 1. During subunit separation, hydrogenase activity also eluted from the Phenyl Sepharose column and this coincided with the second protein peak, the α-subunit. The turnover number of the isolated subunit (160–195 s^{-1}), determined using the H_2 uptake assay, was in the same range as the value obtained for the holoenzyme (see above), indicating that this is the catalytic subunit. To investigate whether the 68-kDa β-subunit was a proteolytic product of the 73-kDa α-subunit, the two proteins were separately digested with three different proteases. The digestion products were run side by side on a SDS gel (data not shown) and from the digestion patterns it was obvious that the two proteins were distinctly different. It was therefore assumed that the two proteins are subunits which, together with the 19-kDa γ-subunit, form the functional enzyme.

3.2. Sequence analysis of T. maritima hydrogenase

The NH_2-terminal sequences of the separated subunits together with internal NH_2-terminal sequences were used to generate oligonucleotide probes for a genomic DNA library from which three overlapping clones were identified. These contained three adjacent ORFs in which NH_2-terminal sequences of the subunits corresponded to the translated amino acid sequences. The gene cluster has a length of 4310 bp and contains *hydC*, *hydB* and *hydA* (486, 1881 and 1938 bp, respectively) which code for the γ-, the β- and the α-subunit, respectively (see Fig. 2). Interestingly, while *hydC* is the first gene in the cluster, it contains TTG as the start codon. Furthermore, there is a 8-bp overlap between *hydC* and *hydB* and a 13-bp intergenic region between *hydB* and *hydA*, suggesting that the different genes are cotranslated. Searches of the available databases revealed that the product of the *hydA* gene has similarity to the sequences of known and putative Fe-hydrogenases, including the recently discovered hydrogenase from *N. ovalis* [20].

α
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  1 MKIYVDGREVIINDNERNLLEALKNVGIEIPNLCYLSEASIYGACRMCLV
 51 EINGQITTSCTLKPYEGMKVKTNTPEIYEMRRNILELILATHNRDCTTCD
101 RNGSCKLQKYAEDFGIRKIRFEALKKEHVRDESAPVVRDTSKCILCGDCV
151 RVCEEIQGVGVIEFAKRGFESVVTTAFDTPLIETECVLCGQCVAYCPTGA
201 LSIRNDIDKLIEALESDKIVIGMIAPAVRAAIQEEFGIDEDVAMAEKLVS
251 FLKTIGFDKVFDVSFGADLVAYEEAHEFYERLKKGERLPQFTSCCPAWVK
301 HAEHTYPQYLQNLSSVKSPQQALGTVIKKIYARKLGVPEEKIFLVSFMPC
351 TAKKFEAEREEHEGIVDIVLTTRELAQLIKMSRIDINRVEPQPFDRPYGV
401 SSQAGLGFGKAGGVFSCVLSVLNEEIGIEKVDVKSPEDGIRVAEVTLKDG
451 TSFKGAVIYGLGKVKKFLEERKDVEIIEVMACNYGCVGGGGQPYPNDSRI
501 REHRAKVLRDTMGIKSLLTPVENLFLMKLYEEDLKDEHTRHEILHTTYRP
551 RRRYPEKDVEILPVPNGEKRTVKVCLGTSCYTKGSYEILKKLVDYVKEND
601 MEGKIEVLGTFCVENCGASPNVIVDDKIIGGATFEKVLEELSKNG
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β
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  1 MFKNAKEFVQYANKLKTLREKKLNGVSIYVCVGTGCTAKGALKVYSAFEE
 51 ELKKRNLLGQVTLEKIDDDKVTLNRTGCCGRCSSGPLVKIMPYRFFYSNV
101 APEDVPEIVDRTVLKGEPIERLFLTDPLTGEKVPRIEDTTLFKNQDFYIM
151 EAIGESECDSIEDYIARSGYESLVKALTSMTPEEIIETVKASGLRGRGGG
201 GFPTGLKWEFTRKAQGDIKFVVCNGDEGDPGAFMNRTLLERDPHLVLEGM
251 IIAGYAVGAQKGYAYIRAEYPFAVKMFKKAIEDARKLGLLGENILGTGFS
301 FDLEVKEGAGAFVCGEETALLASIEGKRGMPRPKPPFPAQSGLWGKPTLI
351 NNVETYANIPRILRDGVENYRKRGTENSPGTKMFSVAGPLKATGIIEVEF
401 GTTLRDIIYNICGGFVEGEEFKAVQIGGPSGACLSEDFIDMPLDYDTLKK
451 ADAMVGSGGIVVITKKTCMVEVARFFLDFTKRESCGKCVPCREGTMQAYN
501 ILEKFTHGKATYEDLKTLEHLSKTIKTASLCGLGKTAPNPILSTLKLFRE
551 EYIAHIEGECPSGMCTAFKKYVINPDICKGCGLCARSCPQNAITGERGKP
601 YTIDQEKCVKCGLCASKCPFKAIELV
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γ
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  1 MERHFEKVEEILKKYGYKRENLIKILLEIQEIYRYLPEDVINYVSTAMGI
 51 PPAKIYGVATFYAQFSLKPKGKYTIMVCDGTACHMAGSPEVLKAIEEETG
101 LTPGNVTEDLMFSLDQVGCLGACALAPVMVINGEVYGNLTADKVKEILRK
151 IKEKERESANV
```

Fig. 2. Translated amino acid sequence of the different subunits of the Fe-hydrogenase from *T. maritima*. Amino acid sequences in bold indicate NH_2-terminal sequences of the separated subunits. Asterisks indicate the cysteine residues. The putative NAD and FMN binding domains are underlined with a dashed and a solid line, respectively.

As shown in Fig. 3, Fe-hydrogenases can generally be divided into two types, with one (~45 kDa) smaller than the other (~60 kDa). The two types have high sequence similarity (typically 35% identity), and they correspond to the C-terminal part of

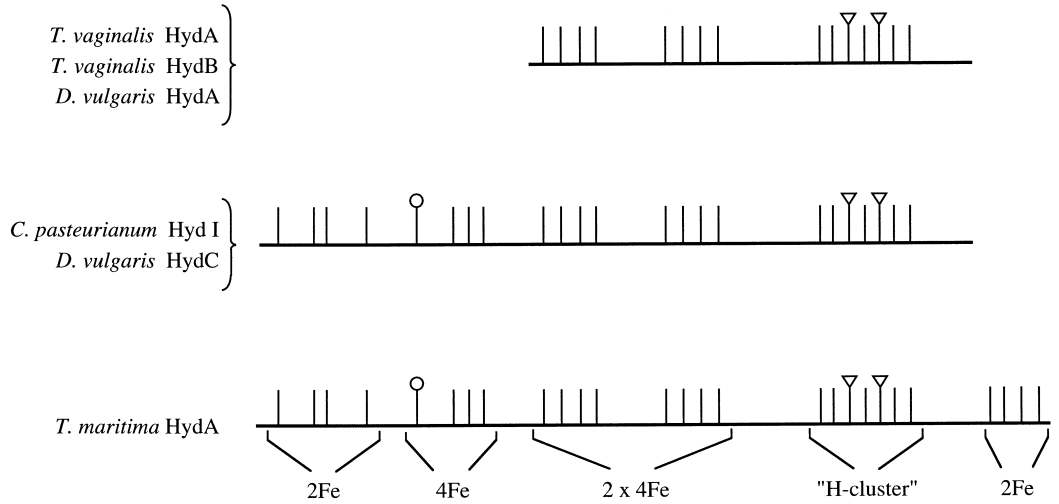

Fig. 3. Alignments of the conserved cysteines (vertical lines), methionines (triangles) and histidines (circles) of the α-subunit of *T. maritima* hydrogenase and of the indicated Fe-hydrogenases (modified from [1]). The sources of the data are as follows: *T. vaginalis* (HydA and B; [19]), *D. vulgaris* (HydA and C; [13,15]), *D. fructosovorans* (HndD; [16]), *C. pasteurianum* (Hyd I; [14]) and *C. acetobutylicum* (HydA; [17,18]).

the α-subunit of *T. maritima* hydrogenase with approximately 30% identity (43% similarity) (see Fig. 3). The high degree of sequence similarity, especially in the regions known to be involved in cluster binding, suggests that the α-subunit of the *T. maritima* enzyme is structurally very similar to the enzyme from *C. pasteurianum*. Besides the H-cluster, which is coordinated by four cysteine residues at positions 295, 350, 482, and 486, the α-subunit contains the regions which bind three [4Fe-4S] clusters, one of which contains a histidine as a ligand, and one [2Fe-2S] center. With a length of 645 amino acids, the α-subunit of *T. maritima* hydrogenase would be the largest Fe-hydrogenase currently known. Compared to the clostridial or the putative *Desulfovibrio* enzymes, it has an additional sequence at its C-terminal that is not present in the other enzymes (see Fig. 3). This extension contains an additional cysteine motif, $CX_4CX_{31}CX_3C$, which is similar to that which coordinates a [2Fe-2S] cluster in HoxE of *Synechocystis* spp. and in NuoE of *Escherichia coli* [50–52], and to the motif proposed to bind a 2Fe cluster in the γ-subunit of *T. maritima* hydrogenase (see below). It has been argued that Fe-only hydrogenases have a modular architecture such that the domains that bind 2Fe- and 4Fe-clusters are structurally similar to 2Fe- and 4Fe-type ferredoxins [21]. Support for this comes from a study in which the isolated N-terminal fragment of *C. pasteurianum* hydrogenase, expressed in *E. coli*, was shown to fold in a manner similar to plant type ferredoxins and to coordinate a [2Fe-2S] cluster [53,54]. It is, therefore, likely that the C-terminal extension in the α-subunit of the *T. maritima* enzyme is a [2Fe-2S] cluster containing domain. While the exact function of this additional cluster remains to be established, it is likely to play a role in intersubunit electron transfer involving the β- and γ-subunits. Thus, based on sequence analyses, it can be postulated that the α-subunit of *T. maritima* hydrogenase contains two [2Fe-2S] and three [4Fe-4S] clusters. In addition, it should contain the active site 'H-cluster', in accordance with the results on the separated subunits which show that the α-subunit in isolation retains catalytic activity.

Sequence analysis of the γ-subunit shows that its four Cys residues are arranged in a motif with high similarity (36% identity, 58% similarity) to motifs in *E. coli* NuoE, *Synechocystis* sp. PCC6803 HoxE and *D. fructosovorans* HndA, all of which are thought to bind a [2Fe-2S] cluster ([51,52,55,56]; see below). Furthermore, as shown in Fig. 4, the β-subunit of *T. maritima* hydrogenase encoded by *hydB* shows similarity to the gene product of one of the subunits of the putative NADP-reducing Fe-hydrogenase from *D. fructosovorans* (HndC, 55% identity, 70%

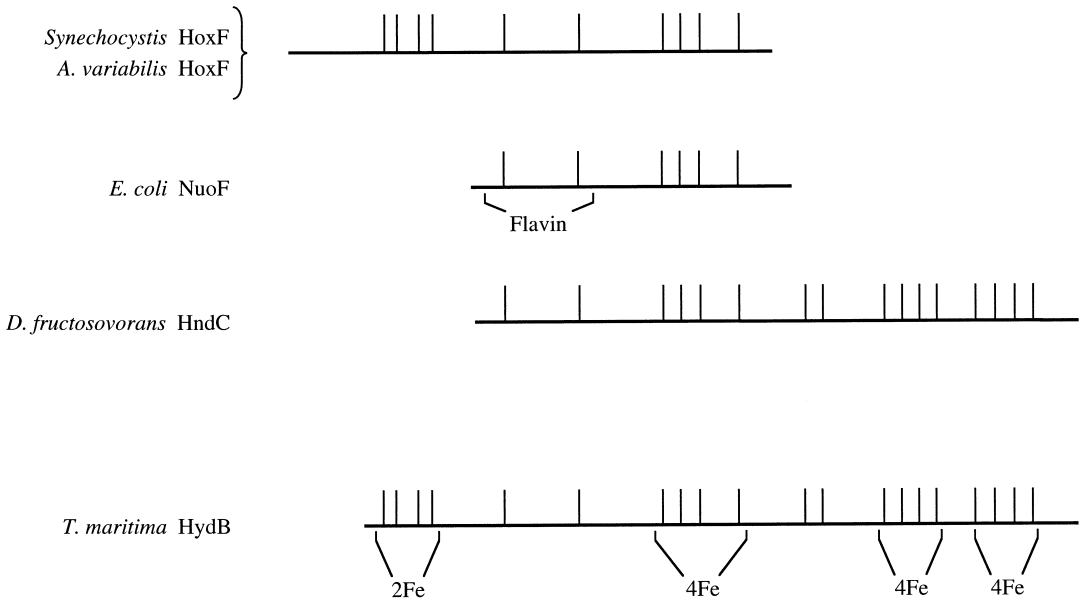

Fig. 4. Schematic comparison of the conserved cysteinyl residues in the sequences of the β-subunit from *Thermotoga maritima* (HydB, this work) with the HydC subunit of *Desulfovibrio fructosovorans* [16], HoxF of *Alcaligenes euthrophus* [58] and *Synechocystis* sp. PCC6803 [52], and NuoF of the NADH dehydrogenase of *Escherichia coli* [51].

similarity [16]), to HoxF of *Anabaena variabilis* [57], *Synechocystis* sp. PCC 6803 [52] and *Alcaligenes eutrophus* [58] (49% identity, 69% similarity), to NuoF of the NADH ubiquinone oxidoreductase of *E. coli* (41% identity, 60% similarity [51]) and to the C-terminal half of the putative hydrogenase from *N. ovalis* (24% identity, 38% similarity [20]). The C-terminal part of the β-subunit contains Cys motifs that could bind three [4Fe-4S] clusters, although note that of these, only the putative 4Fe-center motif nearest the N-terminal has actually been shown to coordinate an Fe-S cluster [59]. The β-subunit also contains four more Cys residues at its N-terminus and these are conserved in the HoxF subunits (Fig. 4) in which they are thought to be involved in binding a [2Fe-2S] cluster [52]. Based on these analyses, the β-subunit of the hydrogenase should contain one [2Fe-2S] cluster and three [4Fe-4S] clusters, while the γ-subunit should contain one [2Fe-2S] cluster.

In addition to the Fe-S cluster-binding motifs, there are two stretches of sequence within the β-subunit which are also conserved in (putative) NAD-binding subunits of other hydrogenases and NADH dehydrogenases. The first stretch of sequence, found between residues 190 and 227, is similar to residues 54–91 of *D. fructosovorans* HndC which were reported to follow the ADP binding βαβ-fold defined by Wieringa et al. [60] using structural comparisons of different NAD binding proteins. Although neither hydrogenase contains exactly the same motif, there is a high similarity between their two sequences and corresponding sequences in known NAD-binding proteins, such as *E. coli* NuoF and *A. eutrophus* HoxF [51,58]. Moreover, there is an acidic residue at the end of the fingerprint region which has been implicated in determining specificity for the utilization of NAD rather than NADP [60]. The second stretch of sequence lies between residues 307 and 327 in the β-subunit of the hydrogenase. Inspection of the sequence by hand indicates that this contains 14 of the 16 predicted residues typically present in an FMN-binding site [55].

Thus, the sequence analyses suggest that the β-subunit of *T. maritima* hydrogenase is an Fe-S-containing flavoprotein that uses NADH as an electron donor. Presumably the γ-subunit, which is postulated to contain a 2Fe-center, has a redox function and transfers electrons from the β-subunit to the α-subunit, which contains the H-cluster, the site of H_2 production. The extent to which these predictions are corroborated by the biochemical analyses is addressed below.

3.3. Molecular properties

From the sequence analyses, the minimum molecular mass for *T. maritima* hydrogenase is 158 949 Da, which corresponds to an αβγ structure. However, the size of the holoenzyme as determined by gel filtration chromatography was dependent on the pH. At pH 6.0 (in buffer containing 100 mM NaCl), the enzyme eluted as a single peak with an estimated molecular mass of at least 500 kDa, while at pH 10.5, a single peak was observed corresponding to a mass of 175 kDa. The latter value is in reasonable agreement with the size of a heterotrimeric holoenzyme as calculated from the translated gene sequences, or estimated from SDS-gel analysis (160 kDa). At an intermediate pH value (8.0), two peaks were observed in the chromatogram, one with a molecular mass >500 kDa and one with a value of 160 ± 5 kDa ($n=4$). It therefore appears that near neutral and acidic pH conditions favor oligomerization of the heterotrimeric holoenzyme. Under all of these pH conditions, the α- and β-subunits after separation each ran as single peaks with estimated molecular masses of 76 and 69 kDa, respectively, compared to 72 248 and 68 676 Da based on the sequence analysis. Incubation of stoichiometric amounts of the two subunits for 30 min at 25°C before chromatography did not result in a shift of their elution patterns. A summary of these results is included in Table 1.

Temperature and stability profiles of the holoprotein and the separated α-subunit are compared in Fig. 5. Although there is an obvious difference in the optimal temperature for the activity of the two proteins, the slopes of the corresponding Arrhenius plots (inset of Fig. 5A) are the same and correspond to a value of 44 kJ mol^{-1}. A more dramatic effect was observed when the thermal stability of the two proteins was compared. As shown in Fig. 5B, the holoprotein was stable for several hours at 80°C while the α-subunit rapidly lost its activity under these conditions. Addition to the α-subunit of stoichiometric amounts of the β-subunit prior to the incubation at 80°C did not affect its stability, indicating that their separation is irreversible, at least in the absence of the γ-subunit. This is consistent with the results obtained by gel filtration.

The results of iron analyses of the holoprotein and the α- and β-subunits are presented in Table 1. The values obtained for the holoprotein are, within experimental error, the sum of the values obtained for the separate subunits indicating that no significant loss of Fe-S clusters has taken place under the conditions used for the separation. ICP analysis of the holoprotein did not indicate the presence of any met-

Table 1
Physicochemical data on the hydrogenase of *T. maritima*

Property	α-Subunit	β-Subunit	γ-Subunit	Holoprotein
Fe content (mol mol^{-1})[a]	n.d.	n.d.	n.d.	32.2 ± 0.5 ($n=2$)
Fe content (mol/mol^{-1})[b]	20.1 ± 2.0 ($n=4$)	14.6 ± 0.7 ($n=5$)	n.d.	31.1 ± 2.8 ($n=7$)
S^{2-} content (mol/mol^{-1})	n.d.	n.d.	n.d.	28.2 ± 0.5 ($n=4$)
[2Fe-2S][c]	0.7	2.4	n.d.	3.1 [3.3]
[2Fe-2S][d]	2	1	1	4
[4Fe-4S]+[2Fe-2S][c]	3.3	3.3	n.d.	8.1 [7.3]
[4Fe-4S][d]	3	3	0	6
'H-cluster'[c]	n.d.	n.d.	n.d.	∼0.1
Molecular mass (SDS)	73 kDa	68 kDa	19 kDa	n.d.
Molecular mass (sequence)	72 248 Da	68 676 Da	18 025 Da	158 949 Da
Molecular mass native	76 kDa	69 kDa	n.d.	160 kDa

The holoprotein was separated as described in the text and the properties of the α- and the β-subunit were determined in separate experiments; n.d., not determined; The values in brackets are included for comparison and were taken from [31] after correction for the change in molecular mass.

[a] Measured by inductively coupled plasma emission spectroscopy (ICP).

[b] Measured by the *o*-phenanthroline method.

[c] Cluster content is expressed as spins mol^{-1}.

[d] Based on analysis of cluster binding motifs.

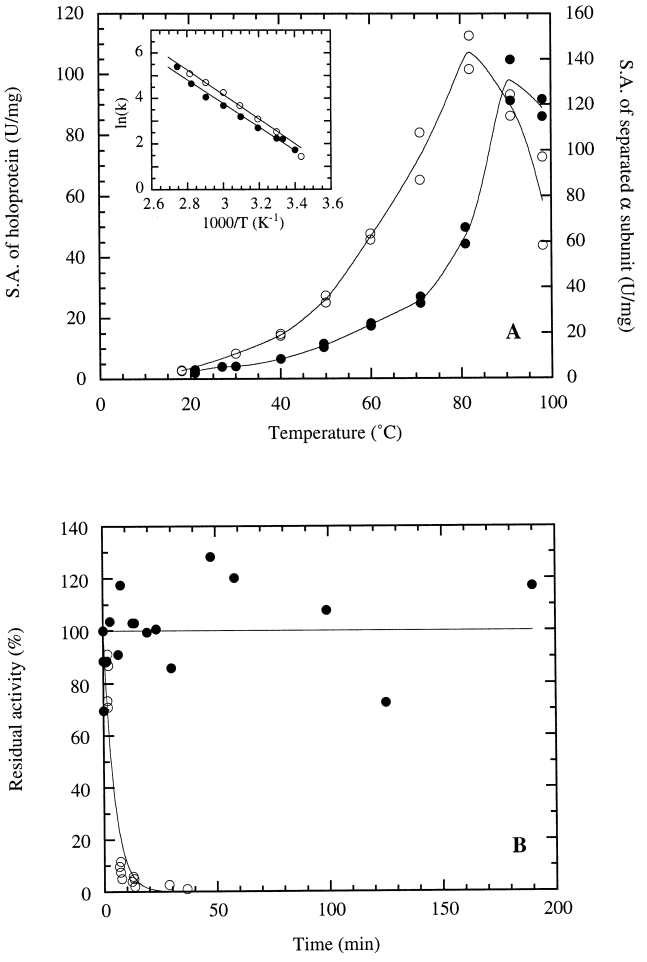

Fig. 5. (A) Temperature dependence of *T. maritima* hydrogenase (●) and the separated α-subunit (○). Activities were measured at the indicated temperatures in 50 mM EPPS, pH 8.0, using the hydrogen uptake assay with methyl viologen as the electron acceptor. Inset: the corresponding Arrhenius plots. (B) Stability of the holoprotein (●) and the separated α-subunit (○) during incubation at 80°C. Solutions of 0.4 mg ml^{-1} hydrogenase in 50 mM EPPS, pH 8.0, containing 2 mM sodium dithionite were incubated and 5 μl aliquots were withdrawn at the indicated times. The residual activity was measured at 80°C using the hydrogen uptake assay with methyl viologen as the electron acceptor.

al other than iron. Thin layer chromatography of the enzyme denatured in 6 M guanidine HCl using fluorescent or non-fluorescent plates did not reveal the presence of FAD, FMN or any other water-soluble fluorescent cofactor. That such cofactors if present in the enzyme in stoichiometric amounts would have been detected was verified by analyzing samples containing FAD or FMN as an internal standard.

3.4. EPR spectroscopy

EPR spectra of the holoprotein and of the separated α- and β-subunits in their reduced states are shown in Fig. 6. The holoprotein and the α-subunit were reduced with either H_2 only or a combination of H_2 and sodium dithionite (both at 50°C), but no differences were observed in the resulting EPR spectra. The β-subunit was reduced with sodium dithionite. The spectra recorded at high temperature (35 K) indicate the presence of slowly relaxing [2Fe-2S] clusters, as reported previously [31]. The EPR spectrum of the holoprotein represents two distinct paramagnetic species, one of which gives rise to a rhombic spectrum with *g*-values of 2.00, 1.96 and 1.92 reminiscent of bacterial type [2Fe-2S] ferredoxins. Each subunit indeed contains a sequence with a cysteine

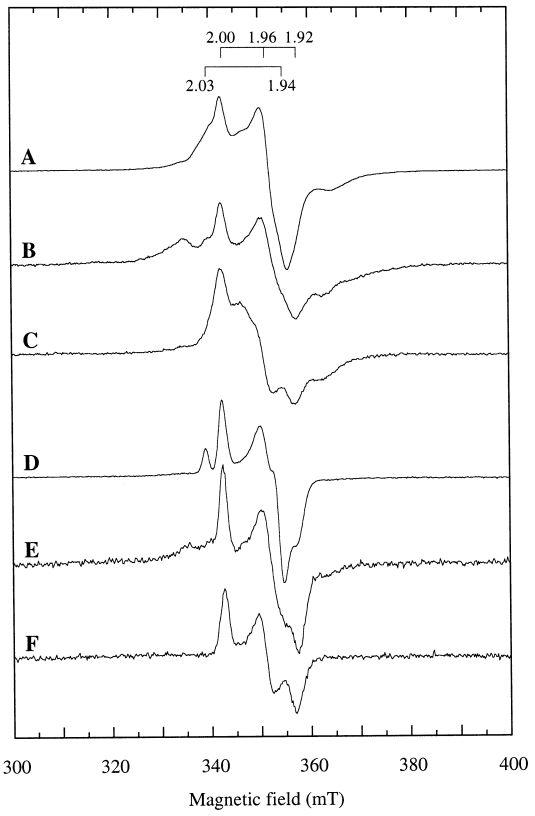

Fig. 6. EPR spectra of the holoprotein and its separated subunits at 9 and 35 K. The samples were prepared as described in Section 2. Traces A/D, B/E and C/F represent the holoprotein, the separated α-subunit and the separated β-subunit, respectively, at a temperature of 9/35 K. EPR spectrometer settings: frequency, 9.590 GHz; modulation amplitude, 0.68 mT; microwave power, 0.01 mW (at 9 K) or 1 mW (at 35 K).

pattern similar to the one observed in NQO2 from *Paracoccus denitrificans* or HndA from *D. fructosovorans* which have both been shown to coordinate a bacterial type [2Fe-2S] cluster [56,61]. In contrast, the second paramagnetic species gives rise to a more axial signal with *g*-values of 2.025 and ~1.94 which are typical for plant or mammalian type [2Fe-2S] clusters, e.g. in adrenodoxin [62]. This signal is most likely caused by a [2Fe-2S] cluster present in the N-terminal domain of the α-subunit. Separate expression of this domain from the *C. pasteurianum* hydrogenase resulted in a [2Fe-2S] containing protein which displayed a nearly axial signal with apparent *g*-values of 2.047, 1.954, and 1.911 [53]. Although this domain does not contain a canonical cysteine motif its sequence is highly conserved between the two hydrogenases suggesting that they have a similar structure. The observation of this signal in spectra of the holoprotein suggests that this [2Fe-2S] cluster is magnetically isolated in the *T. maritima* enzyme, unlike the situation in the *C. pasteurianum* enzyme, where this EPR resonance is obscured due to weak intercluster spin–spin interactions (see [53]). The axial signal, however, was not evident in spectra of the separated α-subunit, even when spectra were recorded at high microwave power (100 mW). Instead, this subunit as well as the β-subunit gave rise to only rhombic-type spectra with *g*-values equivalent to the rhombic signal present in the spectrum of the holoprotein. Spin quantitations of the EPR signals from the different protein samples indicated the presence of one, two and three [2Fe-2S] centers in the α-subunit, β-subunit, and holoprotein, respectively (see Table 1).

At low temperatures (8 K), the holoprotein and the α-subunit gave rise to complex spectra with broad wings indicative of weak dipolar interactions between paramagnetic centers. The spin quantitation for the holoprotein amounted to 8.1 spins mol^{-1} while for the α-subunit a value of 3.3 spins mol^{-1} was obtained. The spectrum of the β-subunit at 8 K was not as broad as the spectra obtained for either the α-subunit or the holoprotein, suggesting that the clusters in the β-subunit do not interact with each other to the same extent as those in the α-subunit and, therefore, that they are likely to be more than 10 Å apart. The quantitation of the spectrum from the β-subunit accounted for 3.3 spins mol^{-1}. The

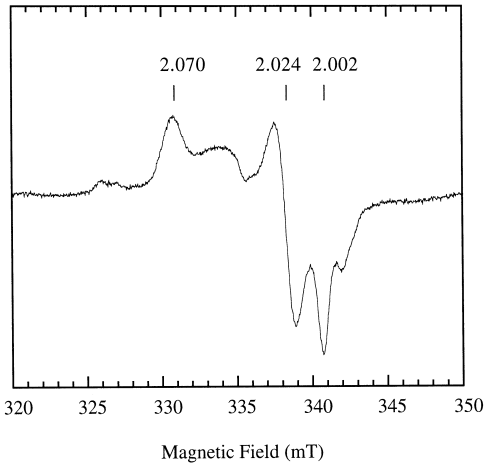

Fig. 7. 'H-cluster' EPR signal of the DCIP-oxidized holoprotein recorded at 5 K. EPR spectrometer settings: frequency, 9.595 GHz; modulation amplitude, 0.68 mT; microwave power, 10 mW.

spin values obtained from spectra recorded at 8 K represent both reduced [4Fe-4S] as well as reduced [2Fe-2S] clusters. By subtracting the values for the 2Fe-centers obtained at 35 K, one can estimate the number of [4Fe-4S] clusters present in the proteins. Such an analysis indicates the presence of three, one and five [4Fe-4S] centers in the α-subunit, β-subunit, and holoprotein, respectively (see Table 1).

All the Fe-hydrogenases that have been purified from mesophilic bacteria, when oxidized either by autooxidation (by H_2 evolution) under an inert gas atmosphere or by the anaerobic addition of thionine, exhibit a unique rhombic EPR spectrum (with typical *g*-values of 2.07, 2.03, 2.00) from a slow-relaxing Fe-S cluster which is assigned to the catalytic H-cluster (see [2]). In contrast, the oxidized hydrogenase of *T. maritima* was reported not to exhibit this EPR signal after dye oxidation, rather, an isotropic signal with a *g*-value of 2.01 was observed [31,33]. However, upon autooxidation of the *T. maritima* enzyme (for 20 min at 40°C under Ar), we could clearly detect at 30 K part of a rhombic signal with estimated *g*-values of 2.070 and 2.024 (data not shown, see below). The third *g*-value could not be determined since the enzyme was only partially oxidized, and this part of the spectrum was obscured by resonances from other $S = 1/2$ centers, (presumably the [2Fe-2S] clusters based on their relaxation properties). The midpoint potentials for the [2Fe-2S] centers in this enzyme are around −390 mV [31] indicating

that the overall redox state of the enzyme could not have been above −350 mV. The same rhombic signal was also evident in the spectrum of the separated α-subunit even though the sample was prepared in the continuous presence of sodium dithionite (2 mM) suggesting that the protein is in a very slow equilibrium with chemical reductants, e.g. dithionite at low or intermediate temperatures. This is consistent with the low rates of hydrogen evolution when assayed in the absence of redox dyes using dithionite as the reductant. Nevertheless, the presence of the H-cluster in the α-subunit is consistent with Fe analyses, i.e. it contains ∼6 Fe atom mol^{-1} not accounted for by 2Fe- and 4Fe-centers, and by its catalytic activity. Incubation of the holoenzyme with thionine or DCIP resulted in an oxidized sample exhibiting only the EPR signal characteristic of the H-cluster (see Fig. 7). However, the spectrum represented only ∼0.1 spin mol^{-1}. With mesophilic Fe-hydrogenases, values between 0.4 and 1.0 spins mol^{-1} have been reported for the integrated intensity of the EPR signal of the H-cluster, and it is not clear why substoichiometric amounts are often present [2,63,64]. Attempts to find conditions to increase the spin content of the signal in the *T. maritima* enzyme are underway.

3.5. Electron carrier specificity

T. maritima grows by glucose fermentation, wherein the excess reductant, via NADH (from glyceraldehyde-3-phosphate dehydrogenase or GAPDH; [65]) and reduced ferredoxin (from pyruvate ferredoxin oxidoreductase, or POR; [39]), is disposed of ultimately as H_2 by hydrogenase [29,66]. Analyses of cytoplasmic extracts of *T. maritima* revealed that only one major form of hydrogenase [31] and ferredoxin [38] are present, but it was reported previously [31] that dithionite-reduced ferredoxin from *T. maritima* [38] could not function as an electron donor for H_2 evolution by the hydrogenase of this organism. This was confirmed by using purified POR to reduce the ferredoxin (with pyruvate as the electron donor); no H_2 was detected when the hydrogenase was also present. We were also unable to identify a second ferredoxin, flavodoxin or related cofactors in cell-free extracts that would serve as electron acceptors for the hydrogenase. In this case, metronidazole, which is irreversibly reduced by electron carriers such as ferredoxin or flavodoxin, but not by hydrogenase, was used as the reporter molecule [36]. Thus, using a system consisting of pure *T. maritima* hydrogenase and a 10-fold concentrated cell-free extract (obtained using an Amicon YM-3 ultrafilter), no H_2-dependent metronidazole reduction could be detected.

In addition, the pure hydrogenase did not catalyze the H_2-dependent reduction of NAD or NADP, even in the presence of FMN or FAD, and no H_2 evolution activity could be detected with the pure enzyme using NADPH or NADH as the electron donor. On the other hand, cell-free extracts did catalyze the H_2-dependent reduction of NAD at a significant rate (∼0.3 μmol NAD reduced min^{-1} mg^{-1}). Moreover, when the same experiment was performed with a 'washed' cell-free extract (nominally containing molecules only of 100 kDa and above), the specific activity of H_2-dependent NAD reduction remained unchanged. Similarly, oxygen-treated extracts which lacked H_2-dependent methyl viologen reduction activity, were capable of complementing purified hydrogenase and enabled the reduction of NAD by H_2 (0.4 μmol NAD reduced min^{-1} mg^{-1}). In both cases, the ratio of the activities with NAD and methyl viologen remained the same. These results suggest that an electron transfer pathway exists between H_2 and NAD in *T. maritima* and that this pathway is not dependent on low molecular weight redox factors, such as ferredoxin or flavodoxin.

Interestingly, the pure hydrogenase did catalyze the H_2-dependent reduction of a quinone, namely, anthraquinone 2,6-disulfonic acid (AQ; $E_m = -228$ mV at pH 7.0 and 80°C); [67]. This appeared to be an efficient and specific reaction as the apparent K_m for the quinone was 0.69 mM with a V_{max} value of 61 μmol H_2 oxidized min^{-1} mg^{-1}. These data compare to a K_m and a V_{max} value for methyl viologen of 2 mM and 45 μmol H_2 oxidized min^{-1} mg^{-1}, respectively [31]. Moreover, AQ was a very poor substrate for the isolated α-subunit. For example, the ratio of the specific activities using methyl viologen and AQ as the substrates was 0.7 for the holoprotein and 7.5 for the α-subunit, indicating a role for the β- and/or γ-subunits in quinone interaction. In a control experiment, the NiFe-hydrogenase of the archaeon *Pyrococcus furiosus* [68] was found to have negligible

H_2 oxidation activity with AQ as a substrate. Furthermore, extracts of *T. maritima* have considerable NADH:AQ oxidoreductase activity (1.8 U mg^{-1}) indicating that *T. maritima* contains additional enzymes with affinity for this substrate. This was in contrast to the situation in *P. furiosus* extracts, as these showed no measurable activity under the same assay conditions.

4. Discussion

4.1. Molecular properties of hydrogenase

The hydrogenase of *T. maritima* is the first complex Fe-hydrogenase to be purified and its complexity raises two important questions. First, what is its relationship to previously purified and much simpler, mesophilic Fe-hydrogenases, and second, what is the physiological purpose for this complexity? The results presented herein indicate that the α-subunit of the *T. maritima* enzyme is analogous to mesophilic Fe-hydrogenases, as this is catalytically active in isolation and appears to contain a catalytic H-cluster (by EPR and Fe analyses). Furthermore, the high degree of similarity between the α-subunit of *T. maritima* hydrogenase and other Fe-only hydrogenases suggests that this class of enzymes is extremely conserved throughout the different branches of the bacterial domain. A relationship between Fe-hydrogenases, NiFe hydrogenases and components of the NADH dehydrogenase complex is now clearly established [69,70]. With the current divisions of the domains of life in mind [71] and with the available genomic sequence information, one can speculate that the narrow distribution of Fe-hydrogenases in the bacterial domain (i.e. the genome sequence of *Aquifex aeolicus*, which is another slowly evolving hyperthermophilic bacterium, does not indicate the presence of an Fe-hydrogenase; instead, it contains NiFe-hydrogenases [72]) and their apparent absence from the domain of archaea, implies that they have evolved from a NiFe-containing ancestor. The Fe-hydrogenases from clostridial and *Desulfovibrio* spp. have subsequently lost their ability to use NAD(P) as the direct electron donor and rely instead on a ferredoxin or low potential cytochrome as part of the electron transfer chain. This scenario would also imply that the relationship between Fe-hydrogenase, NADH dehydrogenase, and NiFe-hydrogenase is the result of evolution along two independent lines.

The hydrogenase gene cluster of *T. maritima* is organized with *hydC* upstream of *hydB* and *hydA*, respectively. This arrangement is similar to the operon structure found in *D. fructosovorans*, but switched compared to the hydrogenase gene found in *N. ovalis* [20]. The functional elements within these different hydrogenases are, however, extremely conserved and it will therefore be interesting to explore the phylogenetic relationship between the different species in light of the current discussion on the origin of hydrogenosomes and mitochondria [19,73]. The significance or consequence of the use of TTG as an initiation codon for this operon in *T. maritima* is not clear [74]. It has been reported that the affinity of the fMet-tRNA for TTG is significantly decreased compared to its affinity for ATG as an initiation codon resulting in a lower level of translation. This can be compensated for by an increased level of transcription and, therefore, both translation and transcription efficiencies have to be considered to determine how protein levels in the cell will be affected [74]. Unfortunately, information on promoter elements in *T. maritima* is scarce, which precludes a further analysis at this point. The stoichiometry of the subunits in the purified protein was approximately 1:1:1, suggesting that the different subunits are cotranslated and that the γ-subunit forms an integral part of the hydrogenase in vivo. It is therefore assumed that the use of TTG as the start codon for this key enzyme in the metabolism of *T. maritima* is part of an as yet unknown regulatory mechanism.

EPR analyses of *T. maritima* hydrogenase after separation of its subunits indicated the presence of two [2Fe-2S] and one [4Fe-4S] clusters in the β-subunit (Table 1). This seems to be an underestimation since one would expect the presence of one [2Fe-2S] and three [4Fe-4S] based on the putative binding domains present in the sequence of this subunit. Such an assignment is corroborated by the metal analysis of this subunit. The catalytic α-subunit was found to contain one [2Fe-2S] and three [4Fe-4S] clusters. This leaves about 6Fe mol^{-1} in the α-subunit which can be assigned to the H_2-activating H-cluster (see [21]). However, the reduced holoenzyme gave rise to an axial-type EPR signal at 35 K

that was not seen with the individual subunits. The species which gives rise to the axial spectrum is presumably a [2Fe-2S] center present in the N-terminal part of the α-subunit, as discussed previously. Subunit dissociation could cause a change in the local environment of this cluster resulting in a change in the magnetic interactions between the different centers in the α-subunit. Note that the spectrum of this cluster has not been identified in *C. pasteurianum* hydrogenase due to spin–spin interactions (see [53]). It is not yet clear whether the disappearance of this axial signal upon subunit separation of the *T. maritima* enzyme is reversible, but this will be assessed in future studies by assembly of the holoprotein from its isolated subunits.

We now turn to the issue of why the Fe-hydrogenase in the hyperthermophile *T. maritima* is more complex than the well-characterized, mesophilic, clostridial and *Desulfovibrio* enzymes. Separation of the subunits of *T. maritima* hydrogenase had a marked effect on the properties of the enzyme with respect to its stability and temperature dependence. Thus, while the β- and γ-subunits of the enzyme stabilize the catalytic α-subunit at high temperatures, the presence of multiple Fe-S centers in the β-subunit suggests that this is not its primary function. Instead, the β-subunit of *T. maritima* hydrogenase probably functions to oxidize a specific electron donor and then transfer electrons to the catalytic α-subunit. However, neither NADH nor reduced ferredoxin, which are generated during sugar fermentation [66], functioned in vitro as electron donors to the holoenzyme for H_2 production. *T. maritima* can also dispose of excess reductant during growth by reducing elemental sulfur (S°) or thiosulfate to H_2S [75], but the hydrogenase (and its β-subunit) do not appear to be involved in these reactions. For example, the enzyme exhibits very low S° reduction activity, comparable to that observed with the monomeric Fe-hydrogenase of *C. pasteurianum* [76] and the reduction of thiosulfate in *T. maritima* is catalyzed by an integral membrane-bound enzyme (Verhagen, M.F.J.M., unpublished data).

The observation that *T. maritima* hydrogenase catalyzes the H_2-dependent reduction of a quinone (anthraquinone 2,6-disulfonic acid) with high efficiency in a reaction that requires the β- and/or γ-subunit supports the conclusion that these subunits are involved in transferring electrons to and from the α-subunit and are involved in substrate recognition. The physiological significance of this reaction is unclear, especially since quinones are generally located within membranes, and there is no evidence that the hydrogenase is membrane associated. It is important to note that the separated α-subunit loses its ability to reduce the quinone, and in this regard the *T. maritima* enzyme is analogous to the membrane-bound NiFe hydrogenase from *Wolinella succinogenes*. This enzyme can be purified in two forms either with or without a small 23-kDa subunit, but the subunit is required for reduction of 2,3-dimethyl-1,4-naphthoquinone, a substrate used as the hydrophilic equivalent of menaquinone [77].

Sequence analysis of the β-subunit indicates a possible FMN-binding site and the absence of FMN in the purified enzyme suggests that this cofactor is lost during purification. Reconstitution experiments in the presence of different flavin cofactors have not yet been successful, although it was found that a high molecular weight protein (nominally >100 kDa), present in cell extracts, can partially restore the H_2-dependent NAD reduction activity of the purified protein in the presence of FMN. While the mechanism of this reaction is currently under investigation, it is reasonable to assume that *T. maritima* hydrogenase indeed uses NADH as a primary electron donor. The complexity of the enzyme is thus a consequence of the nature of its electron donor which depends on the presence of the β- and γ-subunit to transfer electrons to the catalytic α-subunit.

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