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13 ABSTRACT *(Maximum 200 words)*

The molecular mechanisms for thermal tolerance of proteins and other components from hyperthermophilic microorganisms (organisms that grow above 100°C) have yet to be determined. The objective of this work was to identify an approach to understand the role of hydrophobic membrane components (i.e. lipids) in conferring thermal tolerance to a heat-stable membrane-bound enzyme. A tightly-membrane-associated enzyme (hydrogenase) that is a key component of the energy metabolism of the hyperthermophile Pyrodictium abyssi (grows at up to 110°C), can be assayed at temperatures up to 120°C. The specific aims for this project period were to reconstitute partially purified P. abyssi hydrogenase into proteoliposomes to enable assessment of the roles of lipids in conferring stability. This objective was partially achieved, although the amount of enzyme incorporation into liposomes was low, and enzyme activity loss was rapid. The P. abyssi detergent-solubilized enzyme was characterized for some biochemical and physiological properties especially for its function as a membrane-bound respiratory-type uptake hydrogenase enzyme. Another membrane-bound enzyme, sulfur reductase, was partially purified and characterized, and a S°-reducing electron transport chain was partially identified.

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ENCLOSURE 1

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Forward

How proteins and other components from hyperthermophilic microorganisms (organisms that grow above 100°C) tolerate temperature extremes have yet to be determined. This despite the inherent interest in using the unique biomolecules from these organisms in a variety of biodegradative and biotechnological applications. The identification and roles of hydrophobic biological components in permitting enzyme or protein stability at temperatures exceeding 100°C has not been addressed, but is fundamental to understanding high temperature enzymology. The objective of my work was to identify an approach to understand the role of hydrophobic membrane components (i.e. lipids) in conferring thermal tolerance to a heat-stable membrane-bound enzyme. A tightly-membrane-associated enzyme (hydrogenase) that is a key component of the energy metabolism of the hyperthermophile *Pyrodictium abyssi* (grows at up to 110°C), can be assayed at temperatures up to 120°C. The hydrogenase oxidizes H_2 in an energy-generating electron transport chain. My studies showed the enzyme is stable to high temperature conditions *in vivo* (98-110°C for several hours); but the detergent-solubilized form of the enzyme in cell-free extracts, although stable to temperatures of 60-70°C for hours, cannot tolerate "extreme" temperatures of over 100°C for more than a few minutes (see original proposal). The type of detergent used to solubilize the enzyme affects the yield of active enzyme obtained, as well as its thermal denaturation properties. Ample pure hydrogenase from the archaeal organism *P. abyssi* was needed to further address the thermal stable characteristics of the native enzyme. Then investigation of unique membrane components by reconstitution approaches was used to attempt identification the membrane factors permitting in situ thermal tolerance. The individual roles of such biomolecules in conferring thermal protection to the membrane–associated enzyme was to then be assessed.

Long–Range Goal

The primary aim of the work is to identify the membrane factors that permit thermal tolerance of membrane bound enzymes normally active at temperatures exceeding 100°C. Two membrane bound enzymes can be assayed at temperatures exceeding 100°C. These are hydrogenase and sulfur reductase.

Specific Aims

1) To purify enough hydrogenase to be able to carry out initial reconstitution experiments. Active (H_2–oxidizing) proteoliposomes would then be compared to lipid–free and in vivo forms of the enzyme.

2) To reconstitute the pure enzyme into proteoliposomes; then the effects of temperature on individual (lipid) moieties bound to hydrogenase can be addressed.

3) To begin the characterization of another membrane bound activity (sulfur reductase) to begin the study of temperature effects and lipid moieties on its activity.

Achievements

The foundation of this work is based on the differences observed in the thermostability of the membrane bound form of the enzyme to the enzyme in extracts (see figure 1). To rigorously address the factors responsible for stabilizing the enzyme, ample amounts of pure enzyme was needed. Pure enzyme was obtained as described here, and some reconstitution experiments were achieved.

I. Extracting the enzyme from membranes, and purification to homogeneity *Pyrodictium abyssi* hydrogenase was successfully solubilized and then purified. This in itself is a major accomplishment as the enzyme is membrane bound and known to be labile to proteolytic breakdown. The pure enzyme consists of a dimer with subunit sizes of 68 and 43 kDa on SDS gels (see figure 2). Some other characterizations such as spectral, inhibitor, and electron acceptor specificity were done on the membrane bound and purified forms.

Pure enzyme was obtained as follows:

A) Solubilization of the enzyme

Cells were broken by passage through a French Press at 1250 lb/in^2 and membranes harvested by centrifugation at 100,000 x g for 1 hr at 4°C. After washing, the membranes were suspended in 50 mM MOPS (pH 7.0) containing 100 mM NaCl, 2 mM EDTA and the detergent CHAPS [3–cholamidopropyl–dimethylammonio]–1 propane sulfonate at 3% w/v. The membranes were allowed to solubilize by rocking on rocking platform for 2 h at room temperature. After pelleting non–solubilized material, the supernatant solution was removed and assayed for H_2 uptake activity recovery by determining H_2 uptake directly amperometrically in the presence of 0.1 mM methylene blue. Solubilization experiments showed that CHAPS solubilization was superior to other detergents, and complete recovery of the initial (membrane) activity was recovered in solubilized form by CHAPS (see table 1). Unfortunately, the stability of the enzyme in the detergents is poor at 98°C (see table 1).

B) Purification

Purification involved two column steps, both based on affinity column chromatographic approaches. The first column was composed of reactive red–agarose, and the hydrogenase on this column was eluted with 50 mM MOPS, 1 M NaCl, 2 mM EDTA. The eluted enzyme was suspended in TA (pH 7.5) buffer containing 0.5 M NaCl and 1% (v/v) Triton X–100, and then applied to a metal–chelate affinity column of iminodiacetic acid–agarose that had been charged with copper (from a $CuSO_4$ solution). The column was extensively washed with TA buffer, pH 8.25 containing 0.5 M NaCl, and hydrogenase was eluted with 10 mM EDTA. The enzyme could be stored at –70°C in 5% glycerol with little activity loss, after removal of EDTA by dialysis. Maintenance of the enzyme in 2% wt/vol of CHAPS at room temperature was preferable to no detergent.

C) Other Progress on Characterizing the Enzyme Activity

The membrane bound enzyme was compared to the hydrogenase of another archael organism (*P. brockii*) for ability to couple H_2 oxidation to a series of electron acceptors (see table 2). The similarity in the two enzymes shows that the hydrogenase of *P. abyssi*, as previously shown for the chemolithotroph *P. brockii*, functions in the "uptake" H_2 direction. Therefore, H_2 plus the hydrogenase enzyme permits a respiratory type metabolism for *P. abyssi*. The likelihood for hyperthermophilic type electron transport enzymes (i.e. cytochromes) involved in H_2/S° respiratory metabolism in *P. abyssi* is therefore high. This possibility should be further investigated in the future, as cytochrome components could be key to understanding thermal stability of proteins compared to their mesophilic counterparts. The pure enzyme (after solubilization and in 2% CHAPS) was able to oxidize H_2 with positive potential acceptors (see table 2 legend).

II. Reconstitution into Proteoliposomes

A) Reconstitution Approaches

Using procedures described previously, sufficient enzyme was purified to permit some reconstitution studies. As my lab has reported for reconstituting a (mesophilic) hydrogenase, various lipid mixtures were used as a milieu for the hyperthermophilic hydrogenase. These included an *E. coli* lipid mixture and various pure lipids. Detergent solubilized (2% wt/vol octylglucoside) phospholipid (approx 1 mg) was mixed with purified hydrogenase (26 μg) and incubated for 60 min. The mixture was then dialyzed (M_r cut off at 12,000) against 50 mM

potassium phosphate pH 7.0 containing 2.5 mM $MgCl_2$. The buffer was continuously sparged with Ar to reduce oxidative damage to the lipids. The vesicles were subjected to three freeze–thaw cycles (quick–frozen in liquid N_2, thawed at room temperature in a water bath). An increase in vesicle formation (i.e. turbidity) was noted by the freeze thaw procedure, thus it can be assumed this procedure had a positive affect on proteoliposome formation. Hydrogenase activity incorporation was noted by comparing the amounts of sedimentable versus supernatant hydrogenase activity. Hydrogenase was incorporated into lipids by these methods. However, the percent of enzyme incorporation was low based on methylene–blue dependent H_2 uptake assays. The poor incorporation (reconstitution) of the enzyme into lipid mixtures made experiments designed to test the temperature–dependent rate of enzyme loss impossible.

B) Various Lipid Mileus: Affects on Thermal Tolerance

The affects of various lipid environments (*E. coli* lipids) DOPC, DOPE, and DOPG were tested (see Table 3). For details of proteoliposome preparation and other information, see Ferber and Maier, Anal. Biochem. vol. 203, pp. 235-244, (1992). The hypothesis that the lipid environment plays a role in hyperthermophily could not be rigorously tested, as the degree of incorporation yielded insufficient amounts of proteoliposome material to enable rigorous thermal stability studies. The system is a challenging one, perhaps not amenable to liposome-dependent biochemical approaches. Nevertheless, the hydrogenase activity incorporated was not removed by a 1M NaCl wash, so the enzyme was well-incorporated. In one experiment the 50% DOPC/50% DOPG liposome containing hydrogenase were heated to 90°C for 30 min, and compared to a sample kept at 30°C for the 30 min. The 10 min hydrogenase assay at 80° was about the same for both samples, so this lipid mixture did not seem to "protect" the enzyme from thermal denaturation.

III. Sulfur Reductase

A) Partial Purification

The sulfur reductase was partially purified by use of (Zn-charged) metal chelate affinity chemotography and elution performed by use of a pH gradient. This step was accomplished after solubilizing the cystamine trisulfide dependent phenosafranine oxidation activity in 2% (wt/vol) octylgluside. Characterization for subunit composition, metals analysis and other properties will be done when sufficient enzyme can be obtained. For example, preliminary difference absorption spectral studies (O_2–oxidized minus dithionite–reduced) on the

most purified fractions are indicative of a *c*–type cytochrome being a component of sulfur reductase.

B) An H_2 Oxidizing and S° Reducing Membrane Bound Electron Transport Chain

To study the H_2-oxidizing sulfur reducing pathway of P. abyssi, the inhibitor HQNO was used (see Figure 3). Membrane particles of P. abyssi that were washed free of sulfur, were capable of oxidizing H_2 at rates of about 1 µmol H_2 oxidized per min per mg protein, and producing H_2S from S° at a rate of nearly 0.5 µmol H_2S per min per mg protein. The utilization of both substrates (H_2 and S°) was inhibited similarly by HQNO, with a 50% inhibition for both activities at concentrations of about 1×10^{-7} M HQNO (see Figure 3). The results indicate that P. abyssi utilizes H_2 and S° in a energy-conserving respiratory pathway that involves an obligate respiratory factor (likely to be a quinone) between H_2 and S°. These findings cause us to propose that P. abyssi is a chemolithotrophic autotroph, using H_2 and S° in a respiratory electron transport pathway.

Conclusions

Progress toward the ultimate aims was clear, as procedures to solubilize and purify the enzyme were developed. Successful storage of the protein was achieved; this is crucial as the enzyme will need to be stored, in order to accumulate sufficient amounts for reconstitutions, and to permit time for liposome preparation. Initial reconstitutions were successful, but insufficient to rigorously test the effects of each lipid mileu on thermostability. Perhaps native archael lipids are needed to successfully reconstitute ample hydrogenase enzyme, or perhaps proteolytic activity degrades the native enzyme during the reconstitution process. Larger amounts of the membrane bound enzymes (hydrogenase and sulfur reductase) may need to be obtained by recombinant approaches. Nevertheless, relatively pure enzyme was obtained, and some important enzyme characterizations were achieved. Further reconstitution experiments are in order, with lipids from archael organisms, to address the thermal stability issues. The membrane bound nature of the *P. abyssi* hydrogenase and its functioning in the "uptake H_2" direction lead me to conclude that this enzyme is key to respiratory energy metabolism in this 100°C organism. The obligate coupling of H_2 oxidation to sulfur reductase is important to connect the respiratory chain through reductant (H_2) and oxidant (S°). The electron transport pathway likely involves a membrane bound quinone (inhibited by HQNO) between hydrogenase and sulfur reductase, and this quinone will no doubt be a key factor in energy generation by the organism. This result was unexpected from the proposed studies, and needs follow-up work.

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List of Publications

Maier, R.J. Respiratory Metabolism in Hyperthermophilic Organisms: Hydrogenases, Sulfur Reductases, and Electron Transport Factors That Function at Temperatures Exceeding 100°C. Advances in Protein Chemistry, 48:36-99 (1997).

Maier, R.J. The Membrane Bound H_2 Oxidizing S° Reducing Electron Transport Chain of *Pyrodictium abyssi*. In preparation.

List of Participating Scientific Personnel

Graduate Students

Marci Surpin, Ph.D. received March, 1996
James Kolonay, Ph.D. received December, 1997
Research Technician (part time) - Susan Maier

Figure 1. Comparison of Thermal Stabilities of Membrane-Bound Hydrogenase Activity to that in Detergent Solubilized Form

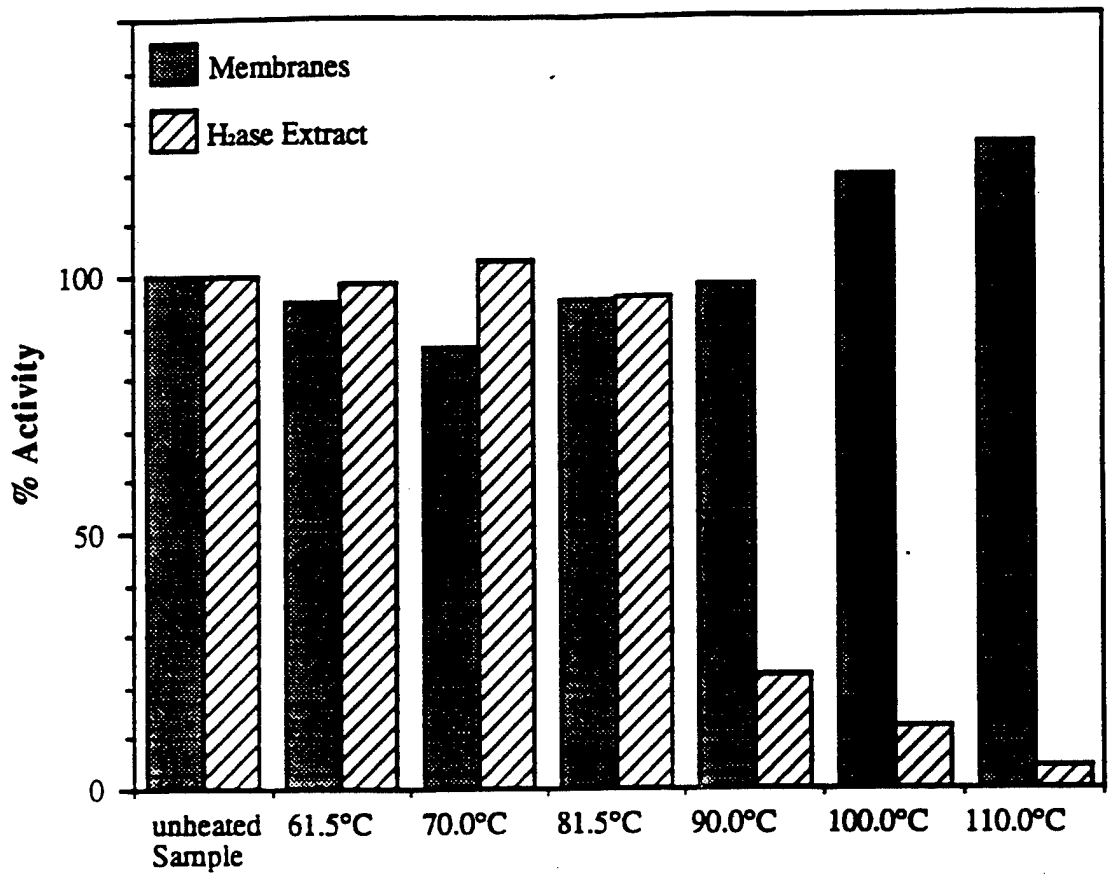


Figure 2. SDS PAGE of the purified *Pyrodictium abyssi* hydrogenase. The (15% acrylamide) gel was silver stained, and 18 μg of protein was loaded. The bars show the migration of the molecular weight standards, given in Daltons.

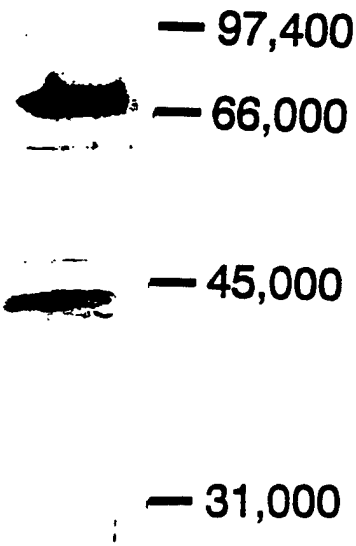
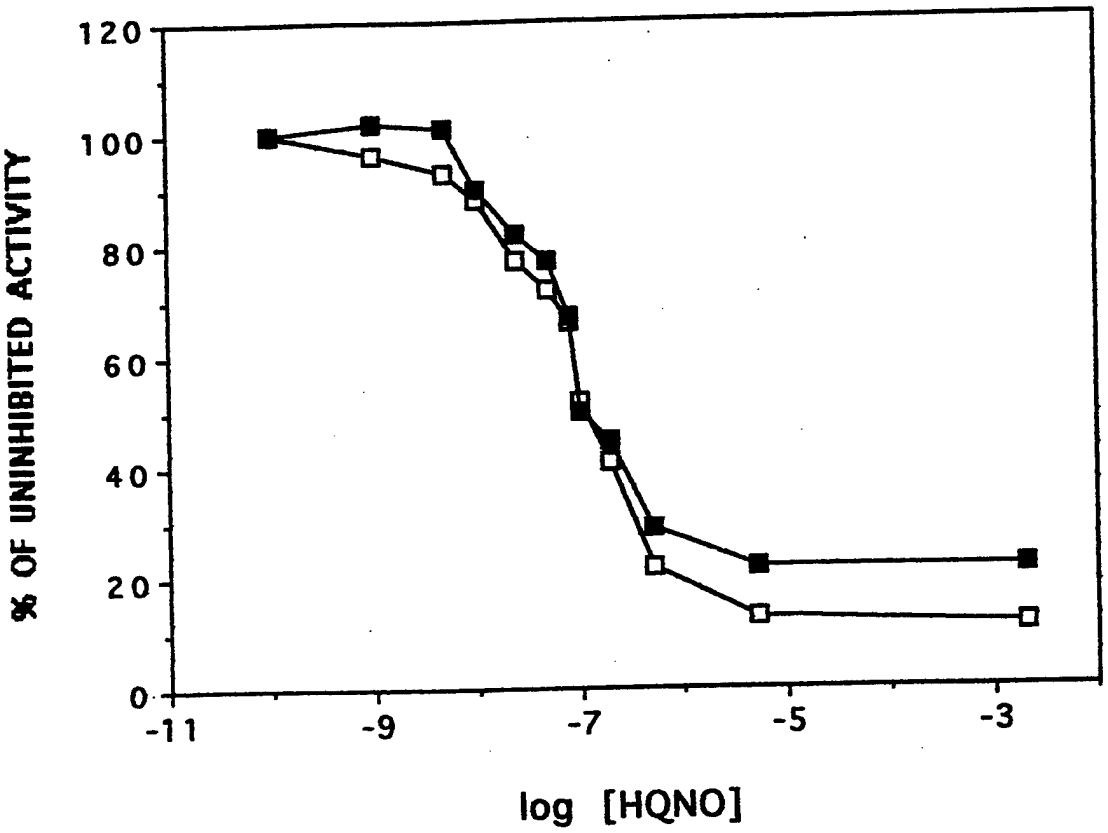


Figure 3. HQNO Inhibition of S°-dependent H_2 Oxidation and Sulfur Reductase of P. abyssi Membranes



□ Sulfur-dependent H_2 oxidation

■ H_2-dependent H_2S production

Table 1. Activity Recovery and Temperature Stability of Detergent Solubilized *P. abyssi* Hydrogenase

Detergent	Total Activity recovered*	Activity after 1 hr at 98°C**
Lubrol PX	97%	14.1
Na–cholate	61%	9.7
Triton X–100	77%	8.5
Octylglucoside	79%	12.2
CHAPS	112%	16.0
Zwittergent	110%	14.6

* The amount of detergent–treated activity recovered compared to the initial native membrane
** Percent compared to the initial activity in (native) membranes

Table 2. Comparison of *P. brockii* and *P. abyssi* membrane–bound hydrogenase for ability to couple H_2 oxidation to various electron acceptors[a]

Electron acceptor	E_o' (mV)	Relative activity (%)	
		P. brockii	*P. abyssi*
Methylene blue (200 µM)	11	100	100
Phenazine methosulfate (400 µM)	80	40	62
Ferricyanide (1.5 mM)	360	30	35
DCIP (200 µM)	217	55	52
Cytochrome *c* (100 µM)	250	6	9
FMN (1 mM)	-122	2	4
Benzyl viologen (1 mM)	-360	5	6
Methyl viologen (1 mM)	-440	2	5

[a] Relative activity is hydrogen uptake activity in comparison to that with methylene blue. Acceptors are at saturating concentrations, and each value is the average of three amperometric determinations. The 100% value for membranes was 2.9 µmol (*P. brockii*) and 2.4 µmol (*P. abyssi*) of H_2 consumed per min per mg of protein. DCIP, Dichloroindophenol; FMN, flavin mononucleotide. The purified *P. abyssi* hydrogenase (see above) was able to oxidize H_2 with methylene blue (100%), phenazine methosulfate (152%), DCIP (82%) and ferricyanide (60%). No H_2 uptake by pure enzyme was observed with viologen dyes provided as acceptor.

Table 3. Effects of different phospholipid compositions on Hydrogenase Reconstitution Efficiency

Source of Lipid	Percent of Total Activity Recovered after the Reconstitution Procedure	Percent of Total Activity Sedimented
E. coli lipid	8.8	15
100% DOPC	10.4	18
50% DOPC/50% DOPE	11.9	26
50% DOPC/50% DOPG	11.7	29

All assays and reconstitution experiments followed the procedures of Ferber and Maier, 1992.