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Hydrogen Production by a Hyperthermophilic Membrane-Bound Hydrogenase in Water-Soluble Nanolipoprotein Particles

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Several membrane proteins have been identified as attractive candidates for enabling the conversion of biomass and/or solar energy to renewable fuels.[1−4] Among these promising enzymes are membrane-bound hydrogenases (MBHs), which are microbial metalloenzymes that catalyze the reversible reduction of protons to hydrogen using organic matter and/or light as energy sources in vivo.

The use of isolated hydrogenases in ex vivo synthetic enzymatic reactions has been shown to produce nearly theoretical yields of hydrogen from glucose or starch and is therefore a potentially economically viable method of renewable hydrogen production.[5−7] The strategy will be significantly improved when oxygen-stable hydrogenases that preferentially evolve hydrogen can be immobilized at high density on surfaces for reactant cycling and prevention of catalyst poisoning from reaction byproducts.[5,8]

The MBH from the hyperthermophile *Pyrococcus furiosus,* a microorganism that grows optimally at 100 °C, has properties that are extremely attractive for ex vivo hydrogen production. This MBH retains a significant fraction of its initial activity in air over several days, and the eventual oxidation of the active site is reversible.[9] Given the stability of the enzyme in oxygen, the MBH has an unusual propensity for catalyzing hydrogen production from protons (as opposed the reverse reaction, hydrogen oxidation) in vivo[1,10] Despite these attractive properties, a major roadblock in utilizing this and other biocatalytic membrane proteins is the fact that MBHs are associated with the water-insoluble cell membrane, and the practical challenge of solubilizing the enzyme while maintaining its activity must be overcome to enable the use of MBHs either in the solution phase or immobilized on surfaces for heterogeneous catalysis.

Two common methods for handling membrane proteins involve the use of artificial vesicles or detergent micelles. Vesicles are largely insoluble and structurally unstable.[11] Because vesicles are spherical and therefore contain both exposed and buried leaflets, a large fraction of the enzyme active sites are inaccessible. Detergent micelles do not typically retain the activity of the native membrane protein.

Here we report the first successful demonstration of biocatalysis by a bioenergy-related membrane protein incorporated into water-soluble nanoparticles called nanolipoprotein particles (NLPs). NLPs are discoidal nanoparticles formed when an apolipoprotein "scaffold protein" directs the self-assembly of a population of phospholipids into nanoscale lipid bilayers.[12,13] These bilayers closely mimic the cell membrane, allowing membrane proteins to be incorporated and functional inside the nanoparticles.[14] The NLP scaffold protein provides a handle for the eventual immobilization of the enzyme on surfaces[15] for heterogeneous catalysis.

Briefly, cellular membranes from *P. furiosus* lysates were separated from cellular debris and washed using centrifugation (Figure 1). A suspension of the membranes was added to a mixture

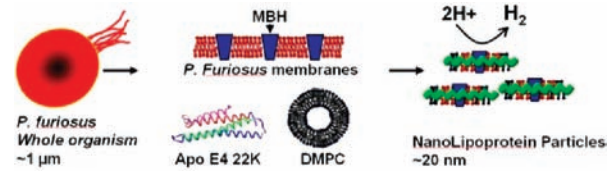

Figure 1. Overview of the process for generating MBH−NLPs.

of the phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), which mimics cell-membrane phopholipids, a truncated amphiphilic apoliprotein E with a mass of 22 kD (Apo E422k) to serve as the scaffold protein, and cholate, a surfactant used to aid NLP self-assembly. The mixture was thermally cycled above and below the transition temperature of DMPC to facilitate NLP self-assembly. Excess DMPC and cholate were removed by dialysis. The NLPs were separated from unincorporated proteins and lipids by size-exclusion chromatography (SEC) under anaerobic and reducing conditions in order to prevent MBH active-site degradation, and the resulting fractions were characterized for size and homogeneity by native and denaturing gel electrophoresis and atomic force microscopy (AFM). The resulting NLPs were tested for hydrogen production using an established gas chromatography (GC) assay.[10]

Three assembly compositions were compared to elucidate (1) the effects of *P. furiosus* MBH-containing membranes on nanoparticle size and (2) the effects of scaffold-protein-directed NLP self-assembly on membrane solubility and hydrogenase activity. Assembly A contained the components required for incorporation of MBH into NLPs: lipid, surfactant, scaffold protein, and MBH-containing membranes. Assembly B lacked the structure-directing scaffold protein. The third ("empty") NLP assembly was prepared without MBH-containing membranes. After synthesis, assembly A was clear and colorless, indicating solubilization of the MBH via NLP formation, while assembly B was gray and turbid. Gel electrophoresis of the SEC fractions from these assemblies was used to identify the NLP- and/or MBH-containing fractions requiring further characterization, and the results supported the formation of NLPs containing *P. furiosus* membrane proteins in assembly A (see Figure S1 in the Supporting Information).

The morphology and size distribution of particles in NLP-containing SEC fractions in assembly A were further characterized

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with AFM. Figure 2a shows a representative AFM image of NLPs from SEC fraction 3. Round disk-shaped particles with diameters

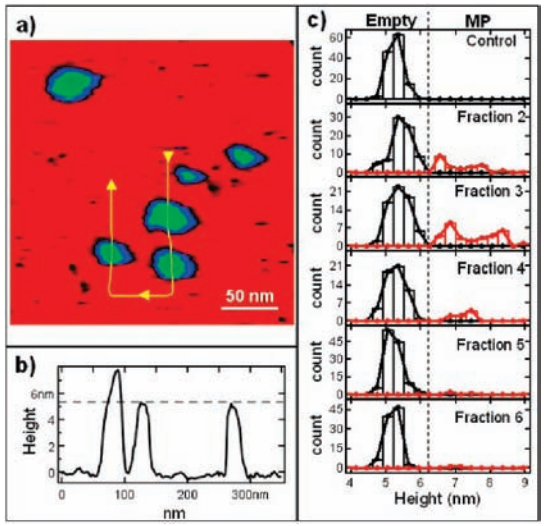

Figure 2. AFM of MBH incorporated NLPs. (a) AFM image of NLPs purified by SEC (fraction 3). Bright-green regions indicate particles that are higher than 6.5 nm. (b) Cross section of the three particles along the line trace in (a), showing height differences between the NLPs. (c) Histogram of heights observed for "empty" NLPs (assembled without *P. furiosus* membrane) and SEC fractions 2−6 from assembly A.

of 20−30 nm were observed with varied height profiles. The height variations in three representative particles are depicted in cross section in Figure 2b. When the height populations in the NLP-containing fractions from assembly A were counted, two distinct populations emerged, as depicted in the histograms in Figure 2c. For comparison, the top histogram represents the height distribution of empty NLPs. The empty NLPs displayed a Gaussian height distribution with a mean height consistent with that of a lipid bilayer (4.9 ± 0.2 nm). In contrast, assembly A fractions 2, 3, and 4 contained two populations of NLPs, one having a height profile similar to that of the empty NLPs and the other exhibiting larger heights than the empty-NLP subset. Because *P. furiosus* membranes have associated membrane proteins (including MBH) that can both span and extend beyond the cell membrane, the subset of higher NLPs likely contains MBH.

The assembly-A SEC fractions that contained higher particles were found to generate hydrogen in the presence of methyl viologen, a nonphysiological electron carrier, using sodium dithionite, an electron donor, as determined by GC analysis of the head space above the solution. Figure 3 shows the hydrogen-producing activity of each NLP-containing fraction (left axis) and the corresponding population of membrane-protein-containing NLPs according to AFM (right axis). The amount of hydrogenase activity in each fraction correlated closely with the proportion of apparent *P. furiosus* membrane-protein-containing NLPs.

Incorporation of MBH into the NLPs both stabilized the enzyme in a water-soluble form and preserved the enzymatic activity. The total hydrogenase activity added to the MBH−NLP assembly mixture was 0.10 ± 0.01 U (in μmol/min), and the total hydrogenase activity *recovered* from the purified assembly fractions 2, 3, and 4 totaled 0.13 ± 0.07 U (0.042, 0.058, and 0.031 U, respectively; see Figure 3). The difference between the crude membrane and MBH−NLP activities was found to be statistically insignificant. The activity and gel data combined with the topographical images strongly suggest that the MBH was properly folded and inserted

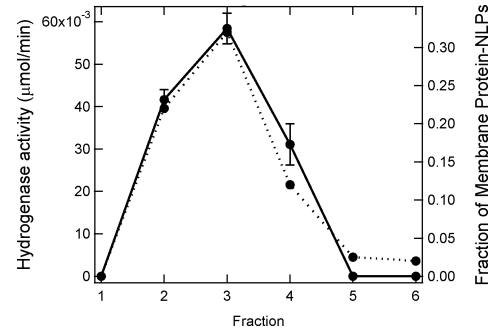

Figure 3. Hydrogenase activity as a function of NLP fraction. The hydrogenase activity (solid line) closely corresponded with the fraction of NLPs containing membrane proteins (dotted line), as assessed by AFM.

into the NLPs in assembly A. In contrast, the SEC fractions from assembly B, which lacked scaffold protein, had no measurable hydrogenase activity, indicating that NLP formation was required to preserve the hydrogenase activity in this process.

The incorporation of active MBHs into lipid nanoparticles will enable MBHs to be immobilized on high-surface-area porous supports for continuous reactant cycling and to be tested in solution-phase synthetic enzyme pathways for ex vivo hydrogen production from biomass. We have demonstrated that NLPs can be used as a tool to solubilize complex membrane biocatalysts from crude membrane preparations while preserving the initial catalytic activity, opening the door to using membrane proteins as biocatalysts for renewable energy production.

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Supporting Information Available: Experimental details, discussion of the preparation and characterization of hydrogen-producing NLPs, Figure S1 showing gel electrophoresis of SEC fractions, and complete ref 13 (as SI ref 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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