Polymerization of Macrocyclic Phospholipid- and Surfactant-Based Vesicles[1a]

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We have recently shown that 1,2-bis(11-mercaptoundecanoyl)-*sn*-glycero-3-phosphocholine can be polymerized as well as depolymerized in vesicle form, via a thiol-disulfide redox cycle.[2] Two intriguing questions regarding the polymerization process are the focus of the present work: Are *nonsymmetrical* lipids necessary for interlipid coupling, i.e., vesicle polymerization? Are the resulting polymers formed under thermodynamic or kinetic control? Phosphatidylcholines, in the bilayer state, are known to prefer a conformation in which the glycerol backbone is approximately perpendicular to the plane of the membrane, where the two fatty acid chains extend unequally.[3] Based on this fact, we have previously suggested that thiol groups positioned at identical carbon atoms in each of the α and β chains would be segregated and that interlipid oxidative coupling would dominate.[2,4] Whether or not this process would be thermodynamically favored, however, was not addressed. We now show that the macrocyclic disulfide analogue **1**, as well as the symmetrical

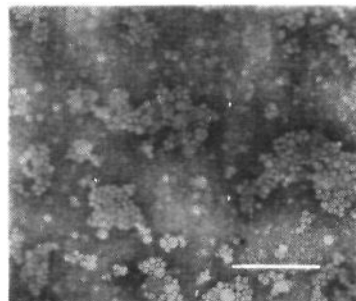

Figure 1. Transmission electron micrograph (2% sodium phosphotungstate stain) of DTT-polymerized vesicles of **1**; bar represents 2500 Å.

conditions (1.4×10^{-4} M) in methanol containing 3.1×10^{-4} M LiOH, under an oxygen atmosphere (72 h), and chromatographic purification afforded **2** (32%) having R_f 0.44 [alumina, $CHCl_3/CH_3OH$ (9/1)]; fast atom bombardment mass spectrometry showed a parent peak at m/z 556 for the ammonium ion with no evidence of contamination by dimer or trimer.[8]

Dispersal of 5 mg of **1** in 5 mL of 10 mM borate buffer (pH 8.5) containing 140 mM NaCl and 2 mM NaN_3 via vortex mixing and sonication (50 °C), using procedures similar to those previously described,[2] produced an opalescent to optically clear dispersion. Dynamic light scattering measurements carried out using a Nicomp 200 instrument, equipped with a helium-neon laser (632.8 nm, scattering angle of 90°) and a computing autocorrelator, indicated particles having diameters ranging between

Electron micrographs of DTT-polymerized **2** confirmed the presence of closed vesicles having a mean diameter of 635 ± 125 Å. In contrast to polymerized **1**, however, open-vesicle membranes were also observed. Whether or not these open structures are artifactual remains to be established.

Studies now in progress are focusing on a comparison of the membrane properties between DTT-polymerized disulfide vesicles derived from **1** and **2** and also their cross-linked analogues. Results of these studies will be reported in due course.

New Semisynthetic Flavoenzymes Based on a Tetrameric Protein Template, Glyceraldehyde-3-phosphate Dehydrogenase

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Recent reports from our laboratory have detailed a promising new strategy directed toward the rational design of artificial enzymes.[1,2] We have convalently modified the active site of papain with reactive analogues of flavin cofactors to yield chemically modified enzymes which are effective catalysts for the oxidation of *N*-alkyl-1,4-dihydronicotinamides. The most effective flavopapains exhibit substrate selectivity, show saturation kinetics, and rival the kinetic efficiency of naturally occurring flavoenzymes. The success of these first-generation semisynthetic enzymes demonstrates both the importance and potential of adapting binding sites of naturally occurring enzymes for the design of effective new catalysts active under mild aqueous conditions. We have now extended our studies to include a new protein template, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which allows for predictable substrate specificity and reaction stereospecificity from a priori considerations of active-site geometry.

GAPDH is a readily available enzyme which has been the subject of extensive physicochemical characterization.[3] The active enzyme is a tetramer composed of four identical subunits (M_r 36 000), each of which contains a discrete active site where both NAD^+/NADH and glyceraldehyde-3-phosphate (GAP) bind. X-ray diffraction studies[4] reveal that the nicotinamide cofactor binds within the pocket so that its pyridinium ring is near Cys-149. This sulfhydryl group, which is essential for catalysis, is believed to form a hemiacetal with GAP and can be selectively alkylated by a variety of reagents without destroying the NAD^+/NADH binding site. In this regard, the observation[5] of Rafter and Colowick that GAPDH promotes the reduction of enzyme-bound dichloroindophenol is particularly relevant.

We have utilized rabbit muscle GAPDH[6] for our studies to date and have carried out the alkylation of Cys-149 with 7α-(bromoacetyl)-10-methylisoalloxazine (**1**).[7] Because NAD^+ copurifies with GAPDH and is known to inhibit alkylation of the active-site thiol,[8] apoenzyme[9] (NAD^+ free, 1.5×10^{-5} M) was treated with a 25-fold excess of **1** in aqueous buffer for 1 h (50 mM pyrophosphate, pH 8.0, 25 °C). By use of this protocol, 0.9 ± 0.05 flavins are incorporated per subunit as estimated by anaerobic titration of the flavoprotein with dithionite[10] and Biuret protein determination.[11] The enzyme-bound flavin has λ_{max} 434 nm (ϵ 1.04×10^4 M^{-1} cm^{-1}); this is red-shifted by 7 nm relative to the λ_{max} of the analogous flavin free in solution.[7]

NADH is a good substrate for the flavo-GAPDH enzyme. Saturation kinetics were observed for the oxidation of this substrate in air-saturated buffer (pH 8.0, 25.0 °C, monitored at 340 nm), giving an apparent K_m value of 25.4 μM, while the product of the reaction (NAD^+) was determined to be a competitive inhibitor with a K_i value of roughly 330 μM. Under these conditions the apparent bimolecular rate constant $(k_{cat}/K_m)_{app}$ is 83 times larger than the second-order rate constant (k_2) for the oxidation of NADH promoted by the model compound 7-acetyl-10-methylisoalloxazine (**2**) (Table I). However, the observed rate acceleration depends on the concentration of oxygen in the medium, since both k_{cat} *and* k_{cat}/K_m increase with increasing amounts of oxygen, while k_2 for the model is oxygen independent. In oxygen-saturated buffer (25.0 °C), for example, the apparent bimolecular rate constant for the enzymatic reaction increases to 1550 M^{-1} s^{-1} which represents a 120-fold rate enhancement over the model system. This finding indicates that the catalytic efficiency of 7-acetylflavo-GAPDH, like that of flavopapain,[12] is limited by the flavin reoxidation step and, further, that flavin reoxidation can occur with substrate/product bound at the active site.[13] The turnover number is still small in oxygen-saturated buffer (0.0360 s^{-1}), although larger values of k_{cat} may be attainable if suitable artificial electron acceptors can be found.[12]

In contrast to the results obtained with NADH, significant rate accelerations were not observed for the oxidation of NADPH or simple *N*-alkyl-1,4-dihydronicotinamides (Table I). Although saturation kinetics were seen in the reactions promoted by the semisynthetic enzyme, the K_m values are greater than 150 μM for each of these substrates. Such substrate specificity (NADH > NADPH ~ BNAH ~ PNAH) was expected, given the known specificity of GAPDH itself,[3] and provides evidence that the flavin moiety is selectively incorporated into the active site of the protein.

Alkylation of GAPDH with the flavin derivative 8α-(bromoacetyl)-10-methylisoalloxazine (**3**)[1] yields an isomeric flavoprotein with properties very similar to those of 7-acetylflavo-GAPDH. For example, in air-saturated buffer the $(k_{cat}/K_m)_{app}$ value for NADH oxidation (475 M^{-1} s^{-1}) is 86-fold larger than the second-order rate constant for the model reaction in which 8-acetyl-10-methylisoalloxazine is the catalyst (5.5 M^{-1} s^{-1}). The differences in the rate constants for the oxidations of *N*-propyl- and *N*-benzyldihydronicotinamides catalyzed by the enzyme and the model systems are less than 2-fold. Flavopapains, on the other hand, exhibit a strong preference for the nonpolar *N*-alkyl-1,4-dihydronicotinamides as substrates over NADH,[1,7] and the specific enzymatic activity shows a greater dependence on the structure of the flavin analogue than is evident with the flavo-GAPDH's.[1,7]

We have also examined the stereochemistry of hydrogen transfer from chirally deuterated NADH molecules. The *pro-S* hydrogen of NADH is selectively transferred to diphosphoglycerate in the native protein,[3] and we predicted that a similar preference would be manifest in the reactions catalyzed by 7-acetylflavo-GAPDH. As the kinetic and product analysis data presented in Table II show, 7-acetylflavo-GAPDH does exhibit substantial *si*-face stereoselectivity. In contrast, transfer of the *pro-R* hydrogen is favored in the oxidation of NADH catalyzed by both the nonenzymatic model system[15] and flavopapain.[16] We are currently using stopped-flow techniques in order to determine the intrinsic

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