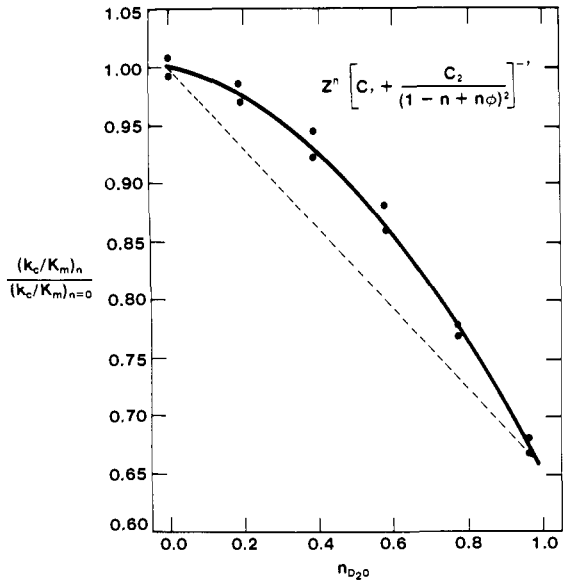

Figure 1. Proton inventory of k_E for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-pNA. Values of k_E were determined as previously described.[12] The solid line passing through the data points was calculated from eq 5 and the parameters $Z = 1.5$, $\phi = 0.54$, $C_1 = 0.46$, and $C_2 = 0.54$. The dashed, straight line connects the points in pure light and heavy water.

To test the generality of the mechanism of eq 5, proton inventories of k_E were determined for the reaction of HLE with a series of peptide *p*-nitroanilide substrates[10] that span a 40-fold range of reactivity toward the enzyme: MeOSuc-Ala-Pro-Ala-pNA (I; K_E = 4500 M^{-1} s^{-1}), MeOSuc-Ala-Ala-Pro-Ala-pNA (II; k_E = 27 000 M^{-1} s^{-1}), MeOSuc-Ala-Pro-Val-pNA (III; k_E = 56 000 M^{-1} s^{-1}), and MeOSuc-Ala-Ala-Pro-Val-pNA (IV; k_E = 182 000 M^{-1} s^{-1}).

All four proton inventories were bowed-upward, as illustrated in Figure 1 for MeOSuc-Ala-Ala-Pro-Val-pNA, and qualitatively confirm our expectations according to eq 5. The data sets were fit to this expression by nonlinear least squares with the parameter constraints[7] that $Z = 1.5$[7] and $\phi = 0.54$.[11] The fits of the experimental to data to eq 5 were all of excellent quality, similar to that of Figure 1, and thus support the generality of the mechanistic model implicit in this expression.[6,7]

The results of the curve-fitting procedures are given in Table I and clearly indicate that increases in substrate specificity, as reflected in k_E, are accompanied by significant changes in the structural features of the virtual transition state of k_E. These changes are indicated by substrate structural-dependent decreases in C_2, increases in C_1, and decreases in the (E:S)' partion ratio k_{-a}/k_b. Inspection of the k_a' and k_b' values of Table I reveals that the substrate structural-dependent change in the virtual transition-state structure is due predominantly to stabilization of the transition state of k_b. The magnitude of k_a is independent of substrate reactivity toward HLE (see Table I) and supports the view that the physical step of k_E is insensitive to substrate structure,[1] at least for tri- and tetrapeptide anilides. These k_a values are very similar to the value of k_2 of 200 s^{-1} obtained by pre-steady-state kinetics for the HLE-catalyzed hydrolysis of MeOSuc-Ala-Ala-Pro-Val-ONP[1] and support an earlier argument[1] that k_2 for this substrate equals k_a.

The ability of the mechanistic model of eq 5 to account for proton inventories of k_E for the four reactions of this study as well as for several other protease-catalyzed reactions[6-8] supports the general importance of solvent reorganization in these associative processes and the existence of a virtual transition state whose properties are dependent on the structure of the substrate.

Table I. Kinetics of HLE Acylation by Peptide *p*-Nitroanilide Substrates[a]

	substrate			
param	I	II	III	IV
k_E[b]	4.5 ± 0.2	27 ± 0.8	56 ± 1.7	182 ± 3.6
$^D(k_E)$	2.21 ± 0.06	2.12 ± 0.02	2.06 ± 0.07	1.56 ± 0.03
C_1[c]	0.05 ± 0.02	0.10 ± 0.03	0.14 ± 0.02	0.46 ± 0.01
C_2[c]	0.95 ± 0.02	0.90 ± 0.03	0.86 ± 0.02	0.54 ± 0.01
k_a'[d]	90 ± 54	270 ± 24	400 ± 58	400 ± 12
k_b'[d]	4.7 ± 0.2	30 ± 2	65 ± 3	340 ± 9
k_a[e]	160 ± 96	380 ± 35	324 ± 50	95 ± 5
k_{-a}/k_b[f]	20	10	6	1.2

[a] Reaction conditions: 0.1 M HEPES, 0.5 M NaCl, pH 7.79 (and pD equivalent for solvent isotope effects and proton inventories), 3.3% Me_2SO, 25 ± 0.1 °C. [b] k_E, k_a', and k_b' are expressed in units of mM^{-1} s^{-1}, while k_a is in units of s^{-1}. [c] Values of C_1 and C_2 were determined by nonlinear least-squares fit of the dependence of k_E on mole fraction solvent deuterium, n, to eq 5 of the text. Z and ϕ were constrained to 1.5 and 0.54, respectively. The proton inventories consisted of duplicate or triplicate k_E determinations at six values of n and were of the same general quality of the data shown in Figure 1. [d] k_a' and k_b' were calculated according to eq 6 and 7, respectively. [e] $k_a = k_a'/K_s$. K_s values I, 1.8 mM; II, 1.4 mM; III, 0.81 mM; IV, 0.24 mM.[10] [f] $k_{-a}/k_b = C_1/C_2 = k_a'/k_b'$.

Supplementary Material Available: Derivation of eq 5 (3 pages). Ordering information is given on any current masthead page.

Rhodopsin in Polymerized Bilayer Membranes

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Rhodopsin (Rh), the major integral protein of the light-harvesting and energy-transducing portion of the rod cell,[1] can be reconstituted into phospholipid membrane bilayers with retention of its chemical regenerability,[2] photochemical functionality,[3] and ability to activate an enzyme cascade that results in the hydrolysis of $>10^5$ cyclic GMP molecules per photon.[4] Current reports indicate that this enzyme cascade directly modulates the sodium permeability of the rod plasma membrane, which results in visual excitation.[5] We describe here the incorporation of Rh into partially polymerized bilayer membranes with retention of its chemical, photochemical, and enzymatic functionality. This functional protein behavior demonstrates that sensitive vertebrate membrane proteins can be usefully incorporated into membrane bilayers that have been modified by polymerization reactions.

Membrane bilayer-forming polymerizable amphiphiles introduced in recent years[6] include those with diacetylene,[7-9] meth-

(10) Substrates were prepared by Drs. H. Horl and J. C. Powers (Georgia Institute of Technology). A complete kinetic characterization of the HLE-catalyzed hydrolyses of these and related substrates is underway as part of a collaboration with this laboratory.

(11) The use of the squared term in the proton inventory of eq 5 for all four substrates is justified by observations of simpler proton inventories of k_2 for I[10] and k_3 for III and IV.[2,10] These proton inventories are "bowed-downward" and are fit by the expression $k_n/k_0 = (1 - n + n\phi)^2$.

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acryloyl,[10-12] dienoyl,[13,14] and vinyl[15] groups. For this study, we used 1,2-bis(octadeca-2,4-dienoyl)-*sn*-glycero-3-phosphorylcholine (DENPC) (prepared by the Eibl method[16]). DENPC absorbs at 260 nm (ϵ 3 × 10^4), which allows efficient photoinitiation of the polymerization of the lipid in membrane bilayers above or below the lipid phase transition at 22 °C. Dienoyl lipids form unilamellar vesicles upon careful hydration, and polymer forms by the 1,4 addition of the diene units of dienoyl compounds, as shown by ^{13}C NMR spectroscopy, IR spectroscopy, X-ray crystallography,[17] and studies of the lipid-phase behavior.[18]

First, membrane vesicles were prepared by hydration and sonication of a homogeneous thin film of DENPC and dioleoylphosphatidylcholine (DOPC) (1:1.2 mol ratio) in an isotonic Tris buffer, pH 7.0. Then the deoxygenated suspension was photolyzed to reduce the DENPC absorbance at 260 nm to 7% of the starting value. Conversion to polymer was followed by thin-layer chromatography on silica gel. Polymerization of dienoyl lipids enhances the formation of phase-separated domains in mixed lipid bilayers.[18] Finally, the Rh was inserted into the polymerized vesicles by a procedure adapted from Albert,[19] who inserted Rh into egg PC vesicles. The Rh-containing membranes were purified by sucrose density-gradient ultracentrifugation. About 60% of the Rh was incorporated into membrane vesicles, which are homogeneous in density and 0.2–0.9 μm in diameter (negative-stain EM). The characteristics of the purified Rh:polyDENPC/DOPC (1:100) membranes were compared with those of natural rod outer segment (ROS) membranes and reconstituted membranes prepared by detergent dialysis of Rh:egg phosphatidylcholine/egg phosphatidylethanolamine, Rh:PC/PE (1:50/50).[4]

Thermolysin proteolysis of native ROS[20] and of Rh:polyDENPC/DOPC (1:100) membranes completely cleaves the 38-kdalton monomer of Rh to smaller fragments. In contrast, proteolysis of the Rh:PC/PE (1:50/50) cleaves 60–65% of the Rh. Since thermolysin (37.5 kdalton) is too large to penetrate the membrane, proteolysis occurs preferentially outside the vesicles. Thus essentially all the Rh in the insertion membranes is in the normal orientation, with the carboxy terminus accessible on the outside surface of the vesicles, whereas detergent dialysis membranes have Rh in normal and retrograde orientations.[2]

High chemical regenerability of bleached Rh to Rh by the addition of 11-*cis*-retinal is a characteristic of native Rh, and the yields depend on the molecular environment of the protein.[2] We found the regenerability of photolyzed Rh to be 42% for the Rh:polyDENPC/DOPC (1:100). Thus about half of the Rh inserted into the polyDENPC/DOPC vesicles is in a lipid environment that maintains the native configuration of Rh upon bleaching.

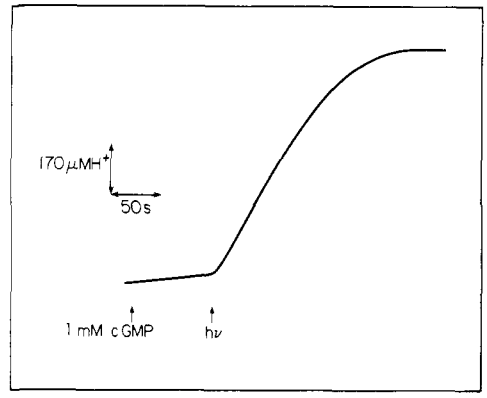

Figure 1. Time course of pH for light-stimulated PDE activity of purified Rh:polyDENPC/DOPC (1:100) (2 μM Rh) membrane vesicles recombined with purified ROS G protein (0.8 μM) and PDE (0.02 μM) in 120 mM NaCl, 2 mM $MgSO_4$, 1 mM DTT, 10 mM Hepes, pH 7.9, and 250 μM GTP at 25 °C. An aliquot of substrate was added in the dark to give 1 mM cGMP. The sample was then exposed to a 1-ms light flash, which bleached 8% of the Rh. The sample pH changed until the cGMP was hydrolyzed. Controls without GTP, cGMP, or enzymes did not change pH upon light exposure of the Rh membranes.

The photochemical functionality of the insertion membranes was compared with that of freshly prepared ROS membranes by determination of the relaxation for the metarhodopsin I intermediate after flash excitation with a 530-nm Nd:YAG laser.[3] The ROS data show a single exponential decay of meta I with k_1 = 140 ± 5 s^{-1} (25 °C). The data for the polymerized membranes show rapid formation of the 470-nm intermediate (meta I) and a multiexponential relaxation of meta I. The flash-excitation-induced increase in the meta I absorbance of these membranes was 0.7 that observed with ROS membranes, which shows that some of the Rh in the polymerized membranes does not readily form meta I. The initial relaxation had k_1 = 160 s^{-1}, similar to that observed with the ROS. The ROS-like component accounts for 55–60% of the signal. Since the membranes are uniform in the mole ratio of lipid to protein, the multiphasic relaxation kinetics suggests that the Rh is inserted into lipid domains of different sizes.

The ability of light-stimulated Rh in recombined membranes to catalyze the activity of the ROS phosphodiesterase (PDE) is a crucial test of its functionality. Reconstitution of the light-induced PDE activity requires the recombination of two membrane surface-associated proteins, the G protein and the PDE, in isotonic media with Rh–lipid membranes.[4,21] Furthermore, the G protein must interact with bleached Rh and bind GTP to communicate with the PDE associated with the phospholipid membrane surface. Comparable amounts of freshly purified PDE in isotonic buffer bind to the surface of membrane vesicles of both Rh:polyDENPC/DOPC (1:100) and Rh:PC/PE (1:50/50); therefore, the bilayer polymerization did not significantly alter the membrane surface binding. Prior to light stimulation, the PDE activity for the Rh:polyDENPC/DOPC (1:100) (Figure 1) was low and increased upon a millisecond flash excitation to a V_m of 4.9 μM H^+ s^{-1} (μM Rh)$^{-1}$. The observed V_m is similar to that found with other Rh–lipid membranes.[4] A V_m of 4.7 μM H^+ s^{-1} (μM Rh)$^{-1}$ was found for Rh:PC/PE (1:50/50) membranes recombined with the same amount of enzymes. Thus part of the Rh in the polymerized membranes can catalyze GTP binding upon light excitation and activate the ROS PDE. This enzyme cascade produced an amplification (>10^5 cGMP per bleached Rh) similar to that observed with natural ROS membranes.

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