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p-Octiphenyl β-Barrels with Ion Channel and Esterase Activity

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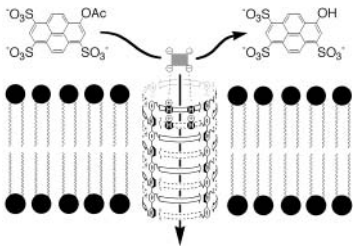
ABSTRACT



Design, synthesis, and esterase and ion channel activity of a novel barrel-stave supramolecule with hydrophobic exterior and histidine-rich interior are reported. Voltage-dependent binding of pyrenyl-8-oxy-1,3,6-trisulfonates by histidines within *p*-octiphenyl β-barrels (and *not* monomers) via ionic (and *not* hydrophobic) interactions (K_D, K_I, K_M < 1 μM) is the basis for superb esterolytic proficiency up to ($k_{cat}/K_M)/k_{uncat} = 9.6 \times 10^5$ in water and bilayer membranes. The conductance of labile ion channels formed in planar bilayer membranes is shown to be reduced by 8-hydroxypyrene-1,3,6-trisulfonate on the single- and multichannel level.

The creation of "man-made enzymes" beyond the classical setting has attracted scientific curiosity for decades.[1] Pertinent work in this dynamic area highlights binding proteins (abzymes), oligonucleotides (ribozymes), organic polymers (synzymes), de novo α-helix bundles, supramolecular architectonics, steroids, cyclodextrins, crown ethers, and calixarenes as priviledged scaffolds.[1,2] Most of these archetypal motifs appear in artificial receptors[1] and ion channels as well.[3,4] Synthetic supramolecular architectonics able to form ion channels *and* to recognize and transform molecular guests have, however, not yet been reported. Here, we describe design, construction, and characteristics of the first synthetic

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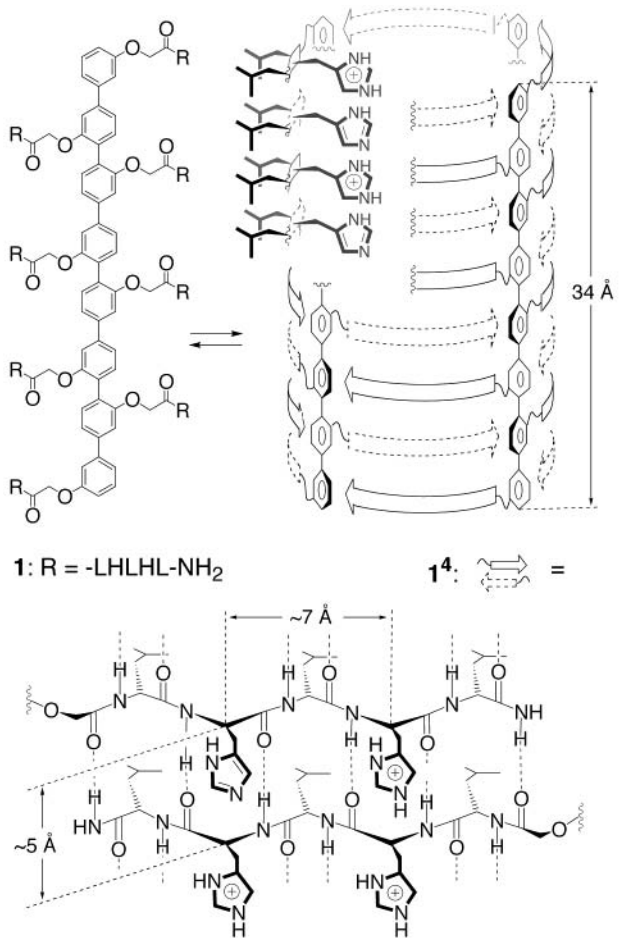


Figure 1. Structure of *p*-octiphenyl **1** and designed cutaway suprastructure of rigid-rod β-barrel $\mathbf{1}^4$ with pertinent distances estimated from molecular models. The extent of protonation of internal histidines depends on pH and is indicated arbitrarily. Four histidine residues from two β-strands forming a rectangle of ~5 Å × ~7 Å are named "H-quartet" for convenience only (bottom).

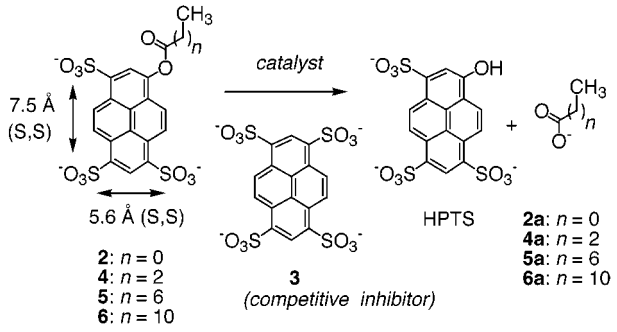
barrel-stave supramolecule with ion channel and esterase activity, i.e., rigid-rod β-barrel $\mathbf{1}^4$, Figure 1.

The design of rigid-rod β-barrels, developed in our lab during the past 2 years, is based on the self-assembly of *n* rigid-rod staves (here **1**) into barrel-stave supramolecules (here $\mathbf{1}^n$).[4] Interdigitation of lateral pentapeptide strands gives multiple antiparallel β-sheets. Their precise topology places all hydrophobic leucine side chains at the outer barrel surface to interact with lipid bilayers, while the hydrophilic histidine residues point inward to form a transmembrane channel. The hypothetical rectangle of ~5 Å × ~7 Å formed by four histidine residues from two β-strands is in the following referred to as "H-quartet" for convenience only. Because we were so far unable to determine the precise barrel stoichiometry experimentally, we decided to assume, supported by molecular models,[4f] previous results,[4] and the magnitude of the single channel conductance (vide infra), a tetrameric supramolecule (i.e., $\mathbf{1}^4$) throughout the manuscript with the only intention to simplify calculations and text.

p-Octiphenyl peptide **1** was synthesized and characterized following established protocols (see Supporting Information). Structural studies of peptide rods **1** by atomic force microscopy suggested that barrel-stave supramolecule $\mathbf{1}^n$ self-assembles spontaneously in aqueous media at concentrations below 3 μM, whereas β-fibrillogenesis occurs above 3 μM.[5] These insights limit functional studies on $\mathbf{1}^4$ to the nanomolar range and hamper conventional structural studies at elevated concentrations.

Esterolytic activity of barrel $\mathbf{1}^4$ was assessed using CB-ester substrates (CB = Cascade Blue = pyrenyl-8-oxy-1,3,6-trisulfonate,[6] Scheme 1). The planar, trianionic CB motif was

Scheme 1



envisioned for molecular recognition because the distances between the three negative charges and those between the three positive charges of protonated histidine residues in an H-quartet are similar (Figure 1).

Esterolysis was monitored by sensitive fluorescence kinetics following the increase in emission of product HPTS as a function of time.[7] The original trace with 1.0 μM CB-acetate **2** and 1.35 nM barrel $\mathbf{1}^4$ exemplifies turnovers of >120 substrates per barrel $\mathbf{1}^4$ (i.e., >30 per rod **1**, >2 per histidine, Figure 2*a*). The bell-shaped pH profile with maximal initial velocities around pH 5.5, indicative for importance of partial but not full histidine protonation for activity, was suggestive for presence of nucleophilic or base catalysis (Figure 2*b*).

The esterolytic activity of rigid-rod β-barrel $\mathbf{1}^4$ at optimized pH 5.5 was independent of presence and absence of spherical bilayer membranes composed of egg yolk phosphatidylcholine (Figure 2*c*).[8] Application of the Michaelis−Menten model gave $K_M = 0.7$ μM, $k_{cat} = 0.13$ min^{-1} (0.0021 min^{-1} per histidine) and a catalytic efficiency $k_{cat}/K_M = 3064$ M^{-1} s^{-1} (48 M^{-1} s^{-1} per histidine) for tetramer $\mathbf{1}^4$. Autohydrolysis

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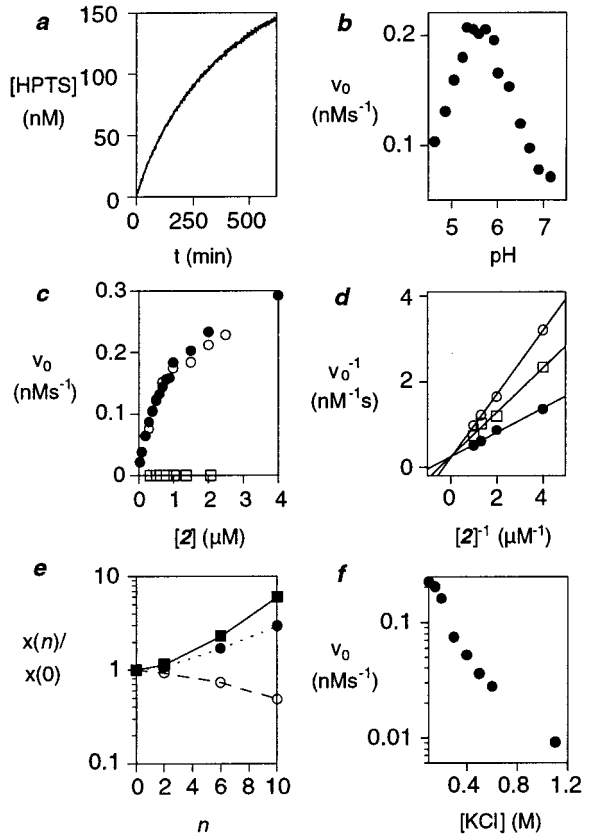

Figure 2. Esterolytic activity of β-barrels **1**^{4}. (*a*) Increase in product concentration (HPTS, λ_{em} = 510 nm, λ_{ex} = 415.5 nm; controls, 404 nm, 455 nm) as a function of time (1 μM **2**, 1.35 nM **1**^{4}, pH 5.5). (*b*) Initial velocities of product formation as a function of pH. (*c*) Substrate concentration in the presence (●) and absence (○) of EYPC bilayer membranes (10 mM EYPC) compared to 540 nM MeIm (□). (*d*) Lineweaver−Burk plots in the presence of 0 μM (●), 0.5 μM (□), and 1.0 μM (○) inhibitor **3**, (*e*) hydrophobicity plot of kinetic constants x (n) = K_M (■), k_{cat} (●), and k_{cat}/K_M (○) for substrates **2** (n = 0, x (0)), **4** (n = 2), **5** (n = 6), and **6** (n = 10). (*f*) Initial velocities of product formation as a function of KCl concentration. Conditions, if not varied as indicated: 135 nM β-barrel **1**^{4}, 2 μM **2**, 10 mM MES, 100 mM KCl, pH 5.5, rt, dark.

of CB-acetate **2** and catalysis by 4(5)-methylimidazole (MeIm) at identical concentrations is very slow at pH 5.5. Therefore, a second-order k_{MeIm} = 0.0032 M^{-1} min^{-1} was measured at millimolar MeIm concentrations and used to calculate comparable velocities (Figure 2*c*, □) and the proficiency $(k_{cat}/K_M)/k_{MeIm}$ = 9.6 × 10^5 of tetramer **1**^{4} (1.5 × 10^4 per histidine). Lineweaver−Burk plots for esterolysis of CB-acetate **2** catalyzed by barrel **1**^{4} in the presence of 0 μM (●), 0.5 μM (□), and 1.0 μM (○) pyrene-1,3,6,8-tetrasulfonate **3** gave separate straight lines that intersected on the y-axis (Figure 2*d*). This indicated that anion **3** influenced substrate binding (K_M) rather than substrate conversion (v_{max}), i.e., acts as a competitive inhibitor with K_I = 0.5 μM.

The influence of substrate hydrophobicity was determined by comparing the characteristics of CB-butyrate **4**, CB-caprylate **5**, and CB-laurate **6** with those of CB-acetate **2** (Figure 2*e*). Fractional changes of K_M (■), k_{cat} (●), and k_{cat}/K_M (○) were plotted as a function of the number n of additional methylene groups in the substrate (Scheme 1). All observed changes were within 1 order of magnitude. Corroborating the absence of hydrophobic substrate binding pockets in barrel **1**^{4}, the K_M actually *increased* with increasing substrate hydrophobicity up to 4.2 μM for CB-laurate **6**. Decreasing initial velocities of product formation in the presence of increasing concentrations of KCl corroborated the expected strong influence of ionic strength on dominant electrostatic interactions (Figure 2*f*).

Ion channel formation was studied in planar EYPC-bilayers doped with 0.06 mol % of rod **1**. At pH 5.0, an ohmic single-channel conductance level of g = 1.23 nS, compatible with the expected internal diameter of β-barrel **1**^{4}, was dominant (Figure 3*a*). A mean lifetime τ = 4.5 ms

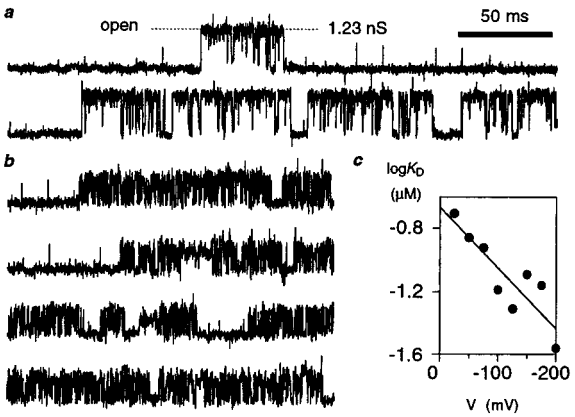

Figure 3. Ion channel activity of β-barrels **1**^{4}. Representative traces at −25 mV before (*a*) and after (*b*) addition of 50 μM HPTS. (*c*) Voltage dependence of the K_D (μM) of HPTS from multichannel dose response isotherms.

was calculated. An additional, more stable low-conductance level (g = 0.26 nS) was observed occasionally at pH 5.0 and frequently at pH ≥ 5.5 (not shown). Addition of 50 μM HPTS reduced both the apparent conductance (g = 0.69 nS) and the mean lifetime (τ = 0.69 ms) of the main single-channel conductance level (Figure 3*b*). The periods of 50−200 ms with predominant open channels remained, however, similar in the presence and absence of HPTS. The lability of the system was incompatible with quantitative analysis of HPTS binding on the single-molecule level.[9]

Addition of HPTS to multiple open channels caused significant reduction of the current. Thus, apparent dissocia-

(8) Control experiments without barrels **1**^{4} confirmed that spherical EYPC-bilayers do not influence the rate of esterolysis. One referee suggested that a 2-times reduced esterolytic activity in the presence of spherical EYPC-bilayers could be expected because one barrel terminus is not as accessible as in water. Compensation of this conceivable effect would then imply that activity in bilayer membranes may be up to 2 times higher than in water (because of, e.g., transmembrane substrate concentration gradients).

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tion constants (K_D's) of HPTS were determined from dose response isotherms, and log K_D's were plotted as a function of applied voltage (Figure 3*c*). Although weak, the observed voltage dependence indicated that the CB-binding site is located within the ion-conducting pathway of **1**[4]. By applying the Woodhull equation

$$\log K_D = \log K_D\,(0\ \text{mV}) - (\delta zFV)/(2.303RT)$$

a distance $l_A = 2.7$ Å was calculated (z = charge of the blocker (−3 for HPTS), $\delta = l_A/l$, l = channel length = 34 Å, l_A = distance from entrance to active site).[10] Although the meaning of l_A values smaller than the size of the blocker can be questioned, it is intriguing to note that $l_A \approx 2.7$ Å is exactly the value one would expect for CB-binding to the first accessible H-quartets at the barrel entrance as rate-limiting process.

In summary, rigid-rod β-barrel **1**[4] can act as artificial ion channel on one hand and as artificial esterase on the other. Strong dependence of esterase activity on ionic strength (Figure 2*f*), near independence on substrate hydrophobicity (Figure 2*e*), competitive inhibition by tetraanion **3** (Figure 2*d*), submicromolar K_M (Figure 2*c*) and bell-shaped pH-profile maximal at pH 5.5 (Figure 2*b*) *all* argue in favor of powerful electrostatic binding of planar CB-substrates by topologically matching H-quartets within aqueous or membrane-bound *p*-octiphenyl barrel **1**[4]. As a specific example for this global interpretation, near independence on substrate hydrophobicity is supportive for β-barrel **1**[4] (rather than monomer **1**) as catalyst because only β-sheet conformation permits proper separation of the hydrophobic leucine from active, partially protonated histidine residues in LHLHL-pentapeptides. Comparison of k_{cat} and K_M suggests that this powerful molecular ground-state recognition (rather than transition-state stabilization) accounts for the nearly 10^6-fold increase of the second-order rate constant.[2d] Near independence on presence and absence of bilayer membranes implied existence of similar suprastructures in water and bilayers (Figure 2*c*).[8]

Some interdependence of functional plasticity was observed as well. Ion channel activity is affected on both the single and multichannel level by the presence of CBs, and CB-binding to ion channels **1**[4] is affected by external voltage applied to planar bilayer membranes. Evidently, ion channels **1**[4] have esterolytic activity, because no reduction of catalytic activity occurred under conditions where ion channel activity is observed (Figure 2*c*). In principle, it should thus be possible to observe hydrolysis of one single ester on the way across an ion channel with esterase activity directly in planar bilayer conductance experiments.[11] However, the stability of single channels formed by rigid-rod β-barrels **1**[4] needs to be increased to attempt meaningful approaches toward single-molecule catalysis. Studies in this direction as well as on the expansion of substrate diversity[12] and the construction of alternative internal recognition motifs are ongoing.[13]

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Supporting Information Available: Experimental procedure and characterization for all new compounds and protocols for ion channel and esterase assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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