Rotaxanes

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Enzymatic Synthesis and Photoswitchable Enzymatic Cleavage of a Peptide-Linked Rotaxane**

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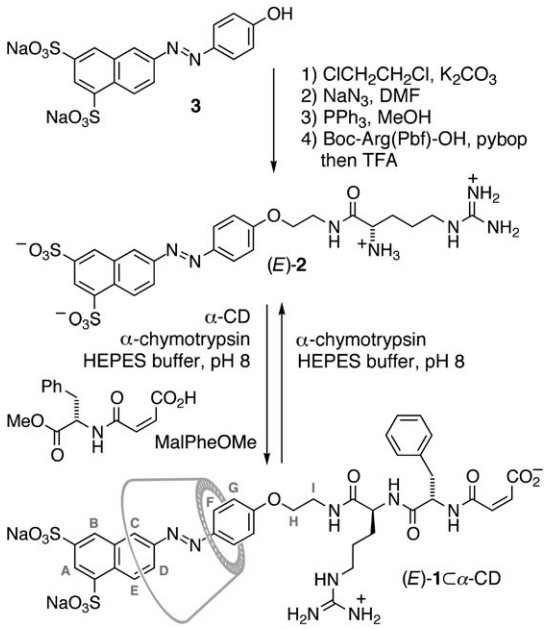
Cyclodextrin inclusion complexes often exhibit useful properties, such as enhanced chemical stability, fluorescence efficiency, and solubility.[1] An inclusion complex of this type can be converted into a kinetically robust rotaxane[2] by attaching bulky stoppers to both ends of the threaded guest, permanently locking it inside the cyclodextrin.[3] The hydrophobic effect is the main driving force for threading, so it is convenient to synthesize these rotaxanes in water, although this limits the range of chemistry available for covalent coupling. Enzymes are uniquely suited for catalyzing covalent-bond formation in water, so it seems surprising that they have not previously been used to catalyze rotaxane synthesis. Herein we report the chymotrypsin-catalyzed synthesis of a peptide-linked azo dye rotaxane, (*E*)-**1**⊂α-CD (CD = cyclodextrin). Rotaxanes of this type generally exhibit reversible photochemical *cis-trans* isomerization, with concomitant relocation of the cyclodextrin along the axle of the rotaxane.[4] Herein we show that photoisomerization of (*E*)-**1**⊂α-CD can be used to prevent its chymotrypsin-catalyzed hydrolysis. Although there are no previous examples of enzyme-catalyzed rotaxane synthesis, enzyme-catalyzed rotaxane cleavage has been studied in detail as an approach to drug delivery in which the enzymes naturally present are used to liberate the components of the rotaxane.[5] Photoswitchable rotaxane cleavage is relevant to this area, because it could enable a drug to be delivered selectively to cells that have a specific enzyme and that are being irradiated with light of the appropriate wavelength.

Protease-catalyzed synthesis of peptides has been investigated extensively because it offers distinct advantages over conventional chemical synthesis, such as high selectivity and lack of racemization.[6] The most common approach is amination of an amino acid ester under kinetic control. The ester acts as an acyl donor and gives an acyl enzyme intermediate, which then acylates the amine to form the peptide bond. A variety of serine and cysteine proteases can be used, and we chose the serine protease α-chymotrypsin because it is readily available and requires no preactivation. To effect efficient peptide coupling, the acyl donor (ester) and acyl acceptor (amine) must be tailored to suit the active site of the enzyme and to match the S_1 and S_1' specificities (S_1 and S_1' are the binding sites for amino acid residues on the donor and acceptor sides of the new amide bond, respectively).[7] For α-chymotrypsin, the S_1 site prefers large hydrophobic side chains, while the S_1' site prefers cationic amino acid residues.[8] Accordingly, a phenylalanine-based donor, MalPheOMe, and an arginine-based acceptor, (*E*)-**2**, were chosen as the substrates for our enzyme-catalyzed rotaxane synthesis. The arginine-substituted azo dye, (*E*)-**2**, was synthesized in four steps from the phenol **3**, as shown in Scheme 1.



Scheme 1. Enzymatic synthesis and hydrolysis of the rotaxane (*E*)-**1**⊂α-CD. Boc-Arg(Pbf)-OH = N_α-*tert*-butoxycarbonyl-N_ω-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine; DMF = *N*,*N*-dimethylformamide; HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Mal = maleyl; pybop = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TFA = trifluoroacetic acid.

The α-chymotrypsin-catalyzed acylation of (*E*)-**2** was monitored by HPLC (Figure 1). Use of excess acyl donor (10 equiv) and excess α-cyclodextrin (15 equiv) gave 87% conversion into the rotaxane (*E*)-**1**⊂α-CD after 20 min. However, at longer reaction times the enzyme hydrolyzes the rotaxane back to (*E*)-**2** by selective cleavage of the PheArg link. Stopping the reaction after 20 min and purification by reversed-phase and anion-exchange chromatography enabled the rotaxane to be isolated in 37% yield. The same reaction in the absence of cyclodextrin gave the dumbbell peptide azo dye (*E*)-**1** in 48% yield. When a dumbbell with non-equivalent ends, such as (*E*)-**1**, is threaded through a cyclodextrin with non-equivalent rims, there is the

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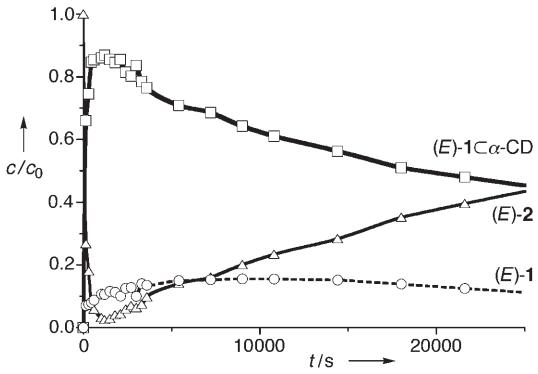

Figure 1. HPLC data showing the formation of rotaxane (*E*)-**1**⊂α-CD over time, as in Scheme 1. All concentrations are normalized by dividing by the initial concentration of (*E*)-**2**. The conversion into (*E*)-**1**⊂α-CD peaks at 87% after 20 min, whereas the concentration of (*E*)-**1** peaks at 16% after 150 min, and the concentration of (*E*)-**2** reaches a minimum of 2.5% after 20 min. The concentration of MalPheOMe (not plotted here) becomes less than 1% of its initial value after 20 min. Initial concentrations: [(*E*)-**2**] = 1.57 mM, [MalPheOMe] = 15.7 mM, [α-CD] = 23.6 mM, [α-chymotrypsin] = 0.3 μM. Buffer: HEPES 0.1 M, NaCl 0.2 M, $CaCl_2$ 0.02 M, pH 8.

possibility of forming two stereoisomers. HPLC analysis indicates that rotaxane (*E*)-**1**⊂α-CD is formed as a single isomer, and this observation was supported by ^{1}H and ^{13}C NMR spectroscopic analysis. ^{1}H NMR NOESY experiments showed that the wide 2,3-rim of the cyclodextrin is directed towards the sulfonate groups, while the narrower 6-rim is directed towards the peptide. For example, H_C shows a strong NOE interaction with 3-H of the cyclodextrin (and a weak one with 5-H, but none with 6-H or 6′-H), whereas H_F shows strong NOE interactions with 5-H, 6-H and 6′-H, but none with 3-H. Surprisingly, NOE investigations of the (*E*)-**2**⊂α-CD inclusion complex revealed that the dominant isomer of this complex has the opposite cyclodextrin orientation,[9] implying that the enzyme determines the stereochemistry of the rotaxane by kinetic selection of the less stable isomer of this inclusion complex.

The $E \rightarrow Z$ photoisomerization of (*E*)-**1**⊂α-CD and (*E*)-**1** in aqueous solution was monitored by HPLC and ^{1}H NMR spectroscopy. Irradiation at 368 nm gave photostationary mixtures dominated by the *Z* isomers, whereas irradiation at 445 nm rapidly shifted the composition back towards the *E* isomers. This photochemistry is cleanly reversible and no by-products were detected by HPLC or NMR spectroscopy. The rate of dark thermal $Z \rightarrow E$ isomerization was measured for both the rotaxane (*Z*)-**1**⊂α-CD and the dumbbell (*Z*)-**1** in aqueous solution at 297 K and 277 K. In each case, the reaction is first order, and isomerization is faster in the rotaxane ($t_{1/2} = 5.4 \pm 0.3$ h at 297 K and 72 ± 10 h at 277 K) than in the dumbbell ($t_{1/2} = 34 \pm 3$ h at 297 K and > 200 h at 277 K). Thus the presence of the cyclodextrin destabilizes the *Z* isomer of the azo dye and decreases the activation energy for thermal isomerization (as noticed in similar rotaxanes by Nakashima and co-workers[4b]). NOE analysis of (*E*)-**1**⊂α-CD and (*Z*)-**1**⊂α-CD in D_2O showed that $E \rightarrow Z$ photoisomerization of the rotaxane results in relocation of the α-cyclodextrin from the azo region to the phenoxyethyl moiety adjacent to the dipeptide stopper. For example, in (*Z*)-**1**⊂α-CD, H_C shows no NOE interactions with cyclodextrin protons, whereas H_F and H_H show NOE interactions with 3-H and 5-H, respectively (Figure 2a).

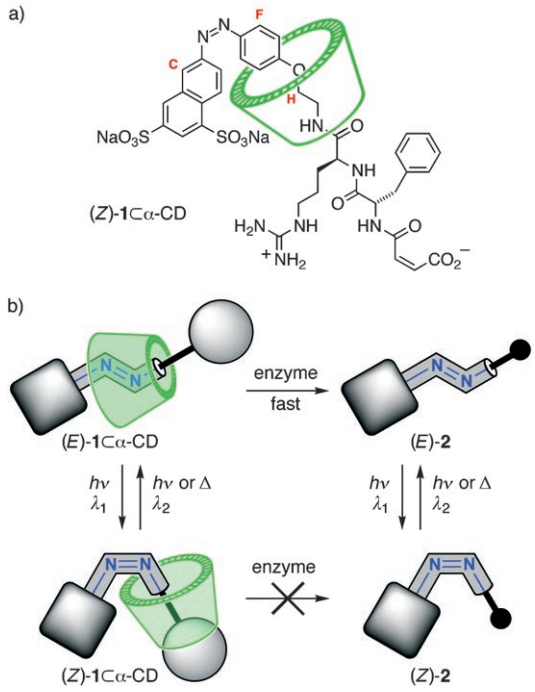

Figure 2. a) Co-conformation of (*Z*)-**1**⊂α-CD deduced from NOE measurements. b) Schematic representation of photoisomerization and enzyme-catalyzed cleavage of the PheArg peptide bond, which decreases the bulk of the end group and results in unthreading of the cyclodextrin ($\lambda_1 = 368$ nm, $\lambda_2 = 445$ nm).

The relative rates of enzyme-catalyzed hydrolysis of (*E*)-**1**⊂α-CD, (*Z*)-**1**⊂α-CD, (*E*)-**1**, and (*Z*)-**1** were tested by monitoring the hydrolysis of a mixture of these four compounds simultaneously by HPLC, to test whether photoswitching can be used to control enzymatic hydrolysis (Figure 2b). The reaction was carried out at 277 K in the dark at high enzyme concentration to minimize thermal or photochemical $Z \rightarrow E$ isomerization. The data from this experiment were fitted to first order decay curves to give the pseudo-first-order rate constants indicated in Figure 3.[10] The *E* and *Z* isomers of the dumbbell **1** are hydrolyzed at the same rate, whereas (*E*)-**1**⊂α-CD is hydrolyzed more than 80 times faster than (*Z*)-**1**⊂α-CD. The slower hydrolysis of the *Z* rotaxane shows that when the cyclodextrin shifts towards the peptide link it hinders interaction with the enzyme; thus the reactivity of the peptide can be controlled by photoisomerization. (*Z*)-**1**⊂α-CD is hydrolyzed so slowly that, under the conditions of this experiment, thermal $Z \rightarrow E$ isomerization dominates, and we can only estimate an upper limit to its rate of hydrolysis.

It seems amazing that the bulky rotaxane (*E*)-**1**⊂α-CD is hydrolyzed 2.6 times faster than the corresponding dumbbell (*E*)-**1** (Figure 3). This implies that the cyclodextrin increases the affinity of the peptide for the active site of chymotryp-

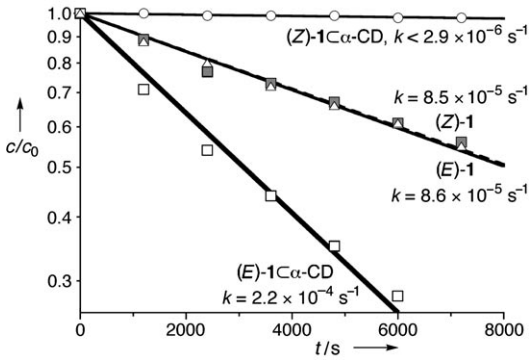

Figure 3. Plot of normalized concentration (c/c_0) of (*E*)-**1**⊂α-CD (□), (*Z*)-**1**⊂α-CD (○), (*E*)-**1** (△), and (*Z*)-**1** (■) against time (*t*) during competitive α-chymotrypsin-catalyzed hydrolysis, showing values of pseudo-first-order rate constants. The reaction was monitored by HPLC at 277 K in the dark; initial concentrations: [**1**⊂α-CD] = 1.2 mM, [**1**] = 1.2 mM and [α-chymotrypsin] = 12 μM.[10] These rate constants have error margins of about 30%, but their relative values are accurate to ±5% owing to the nature of the competition experiment.

sin.[11] This conclusion is supported by the selective formation of this stereoisomer of the rotaxane (*E*)-**1**⊂α-CD, despite the fact that the other stereoisomer of the inclusion complex (*E*)-**2**⊂α-CD predominates in solution. We carried out molecular-mechanics calculations on the α-chymotrypsin/(*E*)-**1**⊂α-CD complex (with the PheArg peptide bound to the active site of the enzyme) to gain insight into the factors stabilizing this complex. The energy-minimized structure (Figure 4) shows that the cyclodextrin lies close to the surface of the enzyme and indicates a hydrogen-bonding interaction between the OH group of tyrosine 146 and O6 on the narrow rim of the cyclodextrin.[12] Interactions of this type may explain why the presence of the cyclodextrin increases the affinity of the peptide for the enzyme.

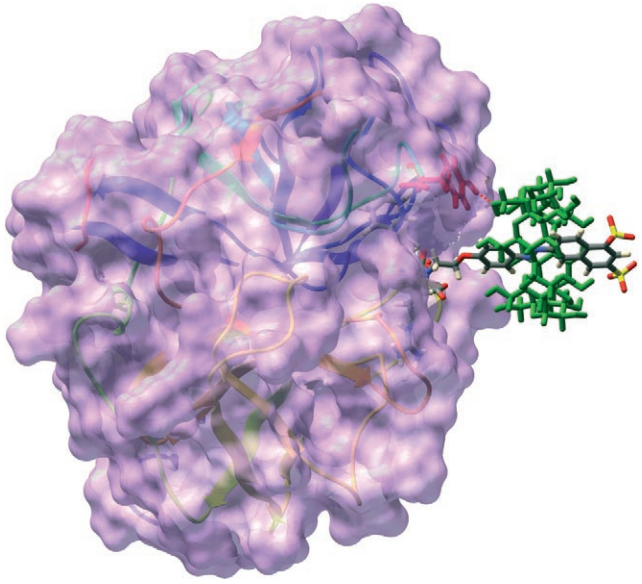

Figure 4. Calculated structure of the α-chymotrypsin/(*E*)-**1**⊂α-CD complex, with PheArg bound in the active site of the enzyme. The tyrosine-146 residue (shown in red, top left of rotaxane) is hydrogen bonded to O6 of the cyclodextrin. (Other protein residues not displayed; enzyme surface: 1.4-Å probe radius; α-cyclodextrin green.)[12]

In conclusion, we have shown that enzymes can be used to synthesize cyclodextrin rotaxanes rapidly and selectively under mild conditions. This chemistry should be useful for the synthesis of encapsulated dyes[3] and insulated molecular wires.[13] The observation that photoisomerization of (*E*)-**1**⊂α-CD turns off its reactivity towards enzyme-catalyzed hydrolysis can also be regarded as a first step towards photoswitchable rotaxane-mediated drug delivery.

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[9] UV/Vis titration of (*E*)-**2** and α-cyclodextrin, carried out under the same conditions as the enzymatic synthesis (HEPES buffer, pH 8, 22 °C), gave data which fit well to a 1:1 binding isotherm with a binding constant of $K = 2.5 \pm 0.2 \times 10^3\ \text{M}^{-1}$. ^{1}H NMR titration showed the formation of two 1:1 complexes, in a ratio of 1:10. These complexes undergo slow exchange on the chemical shift timescale, with each other and with excess (*E*)-**2**, but exchange cross-peaks were observed during NOESY experiments, and the complexes appeared to form immediately on addition of the cyclodextrin; hence the exchange rate is probably in the 1-s^{-1} regime. The dominant isomer of the (*E*)-**2**⊂α-CD inclusion complex gave NOE interactions of H_C and H_D with both 5-H and 6-H, H_F with both 3-H and 5-H, and H_G with 3-H, indicating that it has the opposite cyclodextrin orientation to the (*E*)-**1**⊂α-CD rotaxane. The ^{1}H NMR spectrum of the minor isomer of the (*E*)-**2**⊂α-CD inclusion complex is very similar to that of the (*E*)-**1**⊂α-CD rotaxane, leading us to conclude that these two species have the same cyclodextrin

orientation, and this is supported by the observation of NOE interactions of H_C with 3-H, and H_G with 6-H, 6′-H, and 5-H.

[10] The pseudo-first-order rate constants marked in Figure 3 are simply the gradients of the straight-line fits. Under these conditions the rate constants for thermal $Z \rightarrow E$ isomerization of (*Z*)-**1** and (*E*)-**1**⊂α-CD are $< 1 \times 10^{-6}\ s^{-1}$ and $(2.7 \pm 0.4) \times 10^{-6}\ s^{-1}$, respectively; hence, thermal isomerization makes no significant contribution to the loss of (*Z*)-**1**, but it accounts for at least 90% of the loss of (*Z*)-**1**⊂α-CD.

[11] The presence of the cyclodextrin in (*E*)-**1**⊂α-CD may accelerate enzymatic hydrolysis by lowering the Michaelis constant K_M or by increasing the turnover number k_{cat}. We plan to carry out Michaelis–Menten analysis to address this issue.

[12] The α-chymotrypsin/(*E*)-**1**⊂α-CD complex was modelled by using the Maestro software (from Schrödinger LLC) starting from the crystallographic coordinates of α-chymotrypsin (PDB ID: 6CHA; A. Tulinsky, R. A. Blevins, *J. Biol. Chem.* **1987**, *262*, 7737–7743) and of the methyl orange α-CD complex (K. Harata, *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1493–1501). The structure was minimized by using MacroModel (Schrödinger LLC), with the MMFFs force field, with water as the solvent, and with the Polak–Ribier Convergence Gradient Method at a convergence threshold of 0.005. During this calculation the geometry of the azo group was constrained to be planar, and the carbonyl of the Phe residue of the rotaxane was constrained to be within hydrogen-bonding distance of Ser-195 and Gly-193. The calculated structure (Figure 4) features the following intercomponent hydrogen bonds: Gly193-NH to C=O of Arg_{rotax} (2.2 Å); Arg-$NH_2(1)_{rotax}$ to C=O of Phe-41 (2.0 Å); Arg-NH-(Guan.)$_{rotax}$ to C=O of Phe-41 (1.8 Å); Arg-$NH_2(2)_{rotax}$ to C=O of Cys-58 (1.9 Å); Phe-NH_{rotax} to N-imidazole of His-57 (2.8 Å); Phe-NH_{rotax} to OH of Ser-195 (2.7 Å); Arg-NH_{rotax} to OH of Ser-195 (1.7 Å); α-CD 2-OH to 3-SO_3 (1.8 Å); Tyr-146-OH to α-CD 6-OH (1.7 Å). Molecular-mechanics calculations were also performed on both isomers of the (*E*)-**2**⊂α-CD inclusion complex bound to the enzyme. Similar interactions were predicted for both isomers; however, the level of these calculations is not sufficient to probe the origins of the exclusive formation of one isomer of the rotaxane.

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