Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

Modification of Cyclodextrins for Use as Artificial Enzymes

Valerian T. D'Souza

Online publication date: 13 May 2010

To cite this Article D'Souza, Valerian T.(2003) 'Modification of Cyclodextrins for Use as Artificial Enzymes', Supramolecular Chemistry, 15: 3, 221 — 229
To link to this Article: DOI: 10.1080/1061027031000078220
URL: http://dx.doi.org/10.1080/1061027031000078220

Supramolecular Chemistry, 2003 Vol. 15 (3), pp. 221–229

Modification of Cyclodextrins for Use as Artificial Enzymes

VALERIAN T. D'SOUZA*

Department of Chemistry and Biochemistry, University of Missouri, St. Louis, MO 63121, USA

Received (in Southampton, UK) 30 August 2002; Accepted 11 September 2002

The magical powers of enzymes have been attributed to their ability to bind specific substrates and catalyze reactions of the bound substrate. Artificial enzymes synthetically mimic the binding and the catalytic site to produce molecules that are not only smaller in size but also potentially have similar activity to the real enzymes. The main objective of our research is to create artificial redox enzymes by using cyclodextrins as binding sites and attaching flavin derivatives as the catalytic site. We have developed a strategy to attach a catalytic site to cyclodextrin exclusively at the 2-, 3- or the 6-position. The evaluation of the artificial enzyme in which flavin is attached to the 2-position gives a 647-fold acceleration factor. Although this is modest compared to those of real enzymes (which can have acceleration factors of a trillion), the artificial enzymes allow us to understand the elements that contribute to the incredible catalytic power of enzymes.

INTRODUCTION

Enzymes have been thought of as magical entities due their incredible power to accelerate reactions with high specificity. However, due to the tremendous efforts of talented bio-organic chemists [1], now they have been demystified and their powers have been attributed to two important functions; *binding* and *catalysis* [2]. This dual mode allows enzymes to bring about rate accelerations of 10^{12} on average [3]. *Binding* is not only responsible for the specificity of the reaction but also brings the substrate in close proximity and in the correct orientation to the active site. *Catalysis* lowers the activation energy and thereby accelerates the reaction [4]. However, the main disadvantages of real enzymes are that they are fragile and can be effective primarily in physiological reactions. The artificial enzymes synthetically mimic the binding and the catalytic site to produce molecules that are not only smaller in size but also potentially have similar activity to the real enzymes [5]. The main idea in this area is to attach a suitable catalytic entity to a binding site at the correct distance and orientation and determine the extent to which these can mimic real enzymes.

Cyclodextrins, consisting of 6, 7, or 8 units of α-1,4-linked D-glucopyranoses, have played an important role as binding sites in artificial enzymes [6]. They are doughnut-shaped molecules with secondary hydroxyl groups at the 2- and 3-positions of glucose units arranged in the more open end and primary hydroxyl groups at the 6-position at the other end. The interior of the cavity, consisting of a ring of C–H groups, a ring of glycosidic oxygen atoms, and another ring of C–H groups, is hydrophobic in nature, similar to the binding subsite of many enzymes. The inner diameter of the cavities is approximately 4.5 Å in α-cyclodextrin, 7.0 Å in β-cyclodextrin, and 8.5 Å in γ-cyclodextrin. α- and β-Cyclodextrins fit an aromatic ring snugly with dissociation constants varying from 10^{-2} to 10^{-3} M, depending on the substituent on the ring [6].

A complete artificial enzyme can be synthesized by modifying cyclodextrins to contain a suitable catalytic site attached at an appropriate position. Several such artificial enzymes have been synthesized and evaluated [7–9]. The main objective of our research is to create artificial redox enzymes because redox enzymes have a variety of applications. The design of the artificial redox enzyme consists of a cyclodextrin molecule acting

*E-mail: vally@umsl.edu

ISSN 1061-0278 print/ISSN 1029-0478 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1061027031000078220

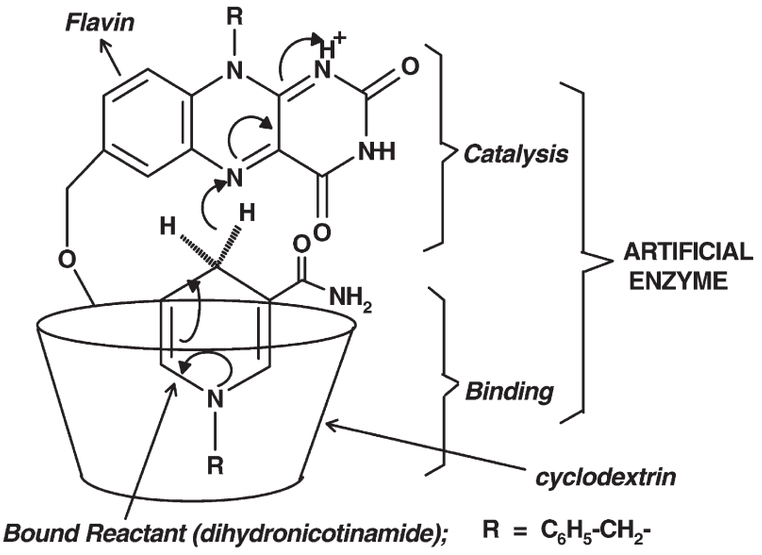

FIGURE 1 Schematic representation of an artificial redox enzyme.

as a binding site covalently attached to a flavin molecule acting as a catalytic site as shown in Fig. 1.

SYNTHESIS OF ARTIFICIAL REDOX ENZYMES

The synthesis of artificial redox enzymes is achieved by reacting 4-methylamino-3-nitrobenzyl chloride (**1**) with cyclodextrin. The product is further reduced and condensed with alloxan to give the desired artificial enzyme as shown in Fig. 2.

However, the main complication in this scheme is that the electrophile **1** can react with hydroxyl groups at the 2-, 3- and the 6-positions to give three regioisomers of the artificial enzyme. We have developed a synthetic strategy to selectively attach the desired group exclusively at each of these positions. In order to understand the strategy for selective modification of cyclodextrins, it is important to know the chemistry that is involved in these modifications as described below and shown in Fig. 3 [10].

A cyclodextrin molecule contains hydroxyl groups at the 2-, 3- and 6-positions and a hydrophobic cavity. Thus, the nucleophilicity of the hydroxyl groups and the ability of cyclodextrins to form complexes with the reagents used are the important factors to be considered in selective modification of cyclodextrins. Of the three types of hydroxyl groups present in cyclodextrins, those at the 6-position are the most basic (and often most nucleophilic), those at the 2-position are the most acidic and those at the 3-position the most inaccessible. Thus, under normal circumstances, an electrophilic reagent attacks the 6-position (Fig. 3 and path 2 in Fig. 4). This situation can be reversed by the use of a strong base. Since the hydroxyl groups at the 2-position are the most acidic, they will be the first to get deprotonated [11]. The oxy-anion thus formed is more nucleophilic than the non-deprotonated hydroxyl groups at the 6-position (Fig. 3 and path 1 in Fig. 4). An interesting factor affecting the chemistry of the hydroxyl groups is provided by the ability of cyclodextrins to form complexes. If the electrophilic reagent forms a complex with cyclodextrin, then the orientation of the reagent within the complex introduces an additional factor in determining the nature of the product (Fig. 3 and path 3 in Fig. 4).

The strategy described above is successfully applied to synthesize three regioisomers of cyclodextrin in which 4-methylamino-3-nitrobenzyl group is attached to the 2-, 3- and the 6-position of β-cyclodextrin as shown in Fig. 4. The substitution at the 2-position (path 1 in Fig. 4) was achieved by reacting β-cyclodextrin with a strong base (NaH) and

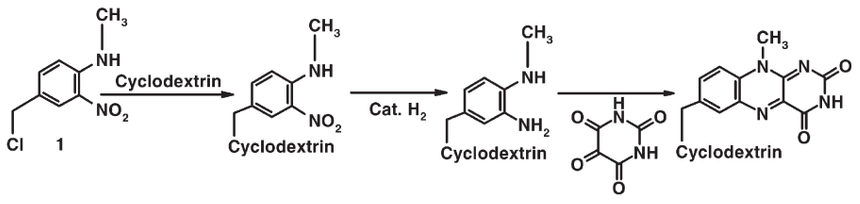

FIGURE 2 Scheme for synthesis of artificial redox enzymes.

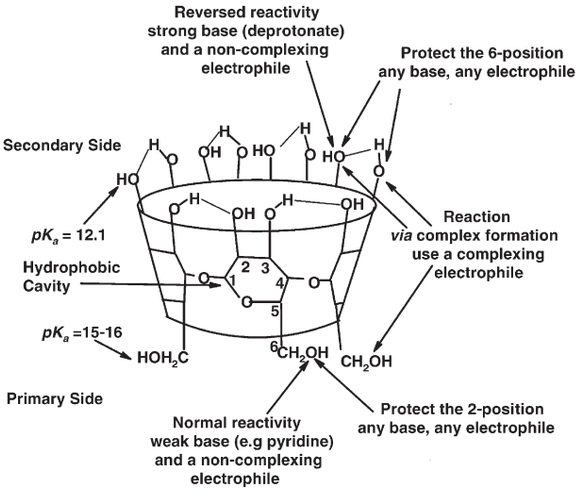

FIGURE 3 Chemistry involved in modification of cyclodextrins.

reacting the resultant oxy-anion with the electrophilic **1** to give the desired 2-substituted cyclodextrin [7]. However, this electrophile does not exclusively afford the 6-substituted product under weakly basic conditions (e.g. pyridine as a solvent) where the normal nucleophilicity would be retained (path 2 in Fig. 4) but always gives a mixture of both the 2- and 6-substituted products. We can explain this only by suggesting that it forms a complex with cyclodextrin such that the chloromethyl group of the reagent is oriented towards the secondary side of the host. The desired exclusively 6-substituted product can only be formed after protection of hydroxyl groups at the 2-position with TBDMS groups. In this protection strategy it is estimated that on an average six out of the seven hydroxyl groups are silylated which is sufficient prevent the incoming electrophile from attacking the secondary side and direct it to the 6-position [12]. Finally, the 3-substituted derivative is obtained by reacting **1** with 6-persilylated cyclodextrin. In this process, the electrophile **1** complexes the protected cyclodextrin in an orientation where the reactive benzyl functionality is directed towards the hydroxyl groups at the 3-position (path 3 in Fig. 4). The subsequent reaction produces a derivative cyclodextrin in which the substituent is at the 3-position [13].

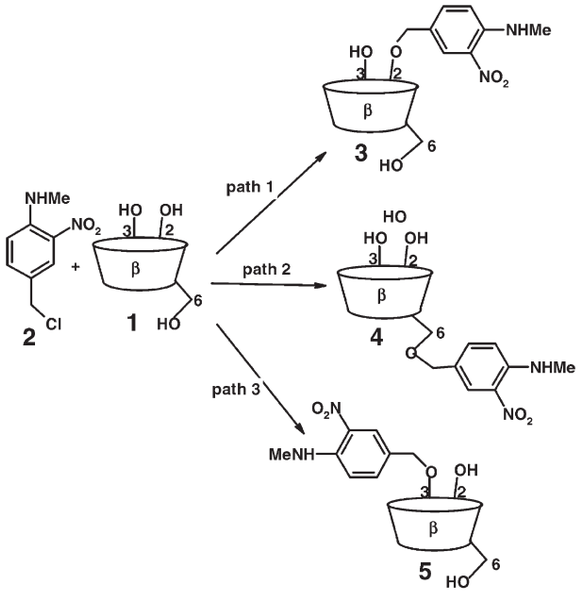

FIGURE 4 Synthesis of three regioisomers in which the 4-methylamino-3-nitrobenzyl group is attached to the 2-, 3- and the 6-position of β-cyclodextrin.

Having synthesized three derivatives, **3**, **4** and **5**, of cyclodextrin in which the same group (4-methylamino-3-nitrobenzyl) is attached to a different position as described above, it is important to unambiguously establish the regiochemistry of each of these compounds. This is accomplished by NMR spectroscopy as shown in Fig. 5 [14].

Starting from an absence of any information on the regiochemistry of **3** (spectra **3** in Fig. 5), **5** (spectra **5** in Fig. 5) and **4** (spectra **4** in Fig. 5), a selective INEPT experiment is carried out on these compounds by selective excitation of the benzyl methylene signals at 4.70 ppm in **3**, at 4.96 ppm in **5**, and at 4.41 ppm in **4**. These INEPT spectra indicate that the substituted carbon has a chemical shift of 80.6, 75.8 and 71.3 ppm for compounds **3**, **5** and **4** respectively. The identity of **4** can be assigned with confidence from an examination of the DEPT spectra. In these, the signal for the substituted carbon in compounds **3** (80.6) and **5** (75.8) are in-phase, whereas, that for **4** (71.3 ppm) is anti-phase relative to the methyne (–CH–) signals, indicating that the substituent in **4** is attached to a methylene group at the 6-position of cyclodextrin.

Conclusive evidence for the substitution pattern in **3** and **5** can be obtained from the multiplicity of the proton signals extracted as one-dimensional projections from two-dimensional HSQC experiments. The HSQC projection for **3** gives a doublet-like proton signal at 3.52 ppm. This compound can now be assigned to the one in which the substituent is at the 2-position because in **3**, the H-2 is in the axial position of a $^4C_1$ conformation glucose ring of β-cyclodextrin. This proton has two coupling partners, the axial H-3 and the equatorial H-1. The axial–axial homonuclear coupling constant (between H-2 and H-3) is large (around 7.5 Hz). However the axial–equatorial coupling constant (between H-2 and H-1) has a smaller magnitude (around 3.5 Hz). These two couplings result in a double doublet structure on the detected proton signal. Since the signal is extracted from a two-dimensional heteronuclear spectrum, the low resolution washes away the smaller coupling, resulting in a single doublet like structure.

The HSQC projection for **5** gives a triplet proton signal at 3.99 ppm. This compound can now

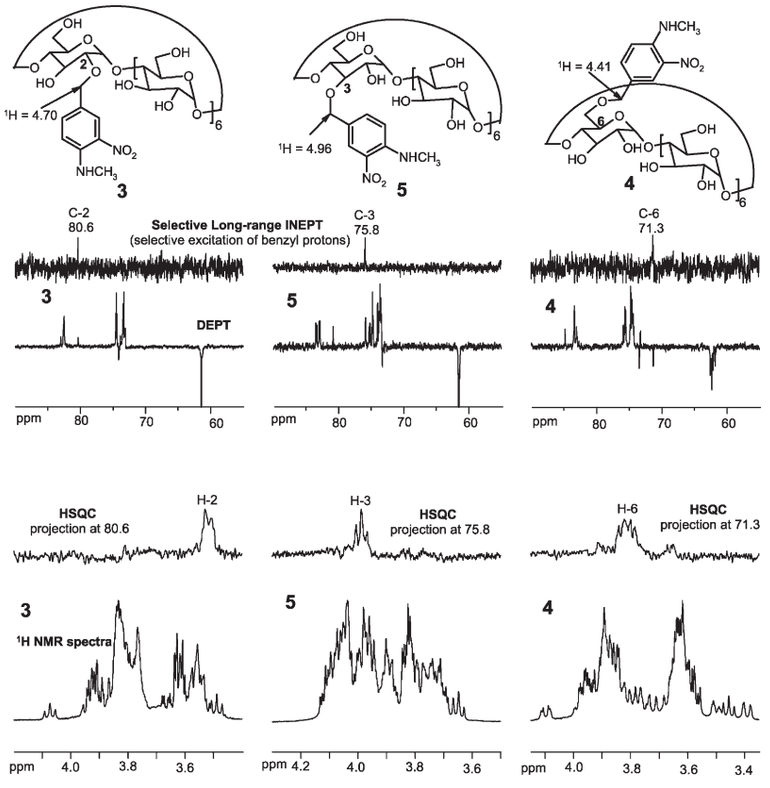

FIGURE 5 Unambiguous evidence for the structure of regioisomers of β-cyclodextrin.

be assigned as the one in which the substituent is at the 3-position because in **5**, the axial H-3 proton also has two coupling partners, the axial H-4 and the axial H-2. These two protons contribute equally (two large axial–axial couplings) to the resultant multiplet structure. The overall appearance of the detected signal, therefore, is a triplet.

The HSQC projection for **4** is a quartet at 3.79 ppm. This compound can now be assigned as the one in which the substituent is at the 6-position because this signal accommodates two proton signals of the methylene group. There is a large geminal coupling (around 9 Hz) between these signals. The coupling constant to H-5 is very small on both H-6 signals, and cannot be measured from the proton spectrum. The overall appearance of the detected signal, therefore, is a quartet.

The cyclodextrin derivatives synthesized using the strategy described above were converted to flavocyclodextrins as described in Fig. 2 [15]. We have synthesized the eight flavocyclodextrins shown in Fig. 6 using the methodology described above.

CATALYTIC REACTIONS OF ARTIFICIAL REDOX ENZYMES

Flavoenzymes catalyze a variety of reactions using flavin co-enzyme as the catalytic group. Similarly, the artificial redox enzyme whose synthesis is described above catalyzes all these reaction since it has a flavin as the co-enzyme. Most of these reactions were found to follow a second order kinetics indicating that the reaction catalyzed is not of the substrate bound in the cavity. These reactions were comparable to reactions of riboflavin (ribloflavin is referred to as Rfl). However, oxidation of benzyl mercaptans to corresponding disulfide and photooxidation of benzyl alcohols to benzaldehyde were found to follow saturation kinetics indicating complex formation prior to the reaction similar to the ones found in natural enzymes. The study of these two reactions is described here in detail.

Oxidation of Mercaptans

The oxidation of mercaptans was carried out in 10% (v/v) methanol aqueous buffer ($NaHCO_3$–NaOH buffer, pH 10.0) with a calculated ionic strength of 0.24 M (adjusted with KCl) at 25.0°C. When a solution of 2-flβCD (**6**) was mixed with an 80-fold excess of benzyl mercaptan under anaerobic conditions a slow decrease of the absorbance of 2-flβCD (**6**) at 440 nm was observable and after several hours, a spectrum characteristic of reduced flavin remained. The spectrum of 2-flβCD can be completely restored by re-oxidation of the reaction mixture with air.

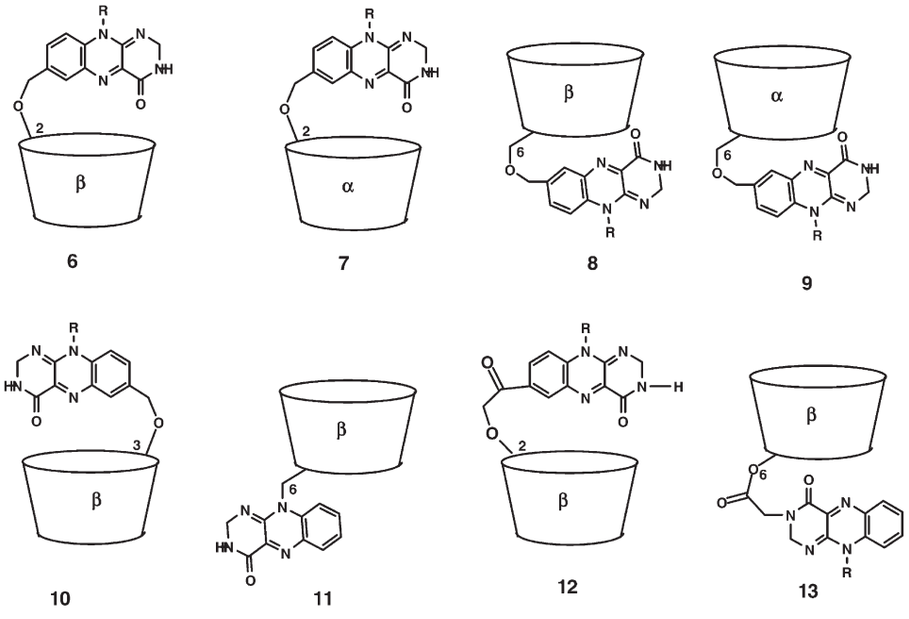

FIGURE 6 Structures of artificial redox enzymes synthesized using the strategy described in this paper.

Compared with the oxidation of mercaptans by flavins, the decomposition of flavins is so slow that it can be ignored. With excess benzyl mercaptan, the absorbance of 2-flβCD and Rfl as a function of time gave good first-order plots under anaerobic conditions, but the reaction with 2-flβCD was faster than that with Rfl. When the concentration of benzyl mercaptan (still in excess) was varied a plot of the initial rates for the reaction of Rfl with benzyl mercaptan vs. the concentrations of mercaptan gave a straight line showing the reaction to be overall second order. The second-order rate constant can then be obtained from this plot and is shown in Table I. In contrast to Rfl, similar plots for the reactions of 2-flαCD and 2-flβCD with benzyl mercaptan showed the saturation kinetics. The K_{diss} and k_{cat} calculated from these plots are listed in Table I [16].

The oxidations of substituted benzyl mercaptans and α-naphthyl mercaptan by Rfl and flavocyclodextrins were monitored by the decrease in absorbance at 440 nm due to flavin moiety. The kinetic measurements were performed in $NaHCO_3$–NaOH buffer (pH 10.0) containing 30% (v/v) methanol at 25.0°C at a calculated ionic strength of 0.24 M. The oxidation of substituted benzyl mercaptans catalyzed by Rfl followed second-order kinetics, whereas saturation kinetics was observed for the oxidation catalyzed by flavocyclodextrins. The rate constants for these reactions are listed in Table I.

The oxidations of benzenethiol, phenethyl mercaptan and cyclohexyl mercaptan by Rfl and flavocyclodextrins were also investigated in a similar manner. The oxidations either by Rfl or by flavocyclodextrins were either very slow, or did not proceed at all because the plots of absorbance vs. time for these

TABLE I The rate constants for the oxidation of benzyl mercaptan and substituted benzyl mercaptans by flavins*

	Substrate	Flavin	$K_{diss} \times 10^3$ (M)	$k_{cat} \times 10^3$ (s^{-1})	k_{cat}/K_{diss} ($s^{-1} M^{-1}$)	$k_2 \times 10^2$ ($M^{-1} s^{-1}$)	$(k_{cat}/K_{diss})/k_2$‡
1	Benzyl mercaptan†	2-flβCD (**6**)	1.89 ± 0.23	1.11 ± 0.13	0.587		53
2	Benzyl mercaptan†	2-flαCD (**7**)	2.38 ± 0.09	0.585 ± 0.022	0.246		22
3	Benzyl mercaptan†	Rfl				1.11 ± 0.06	
4	*p*-Chlorobenzyl mercaptan	2-flβCD (**6**)	2.91 ± 0.17	1.20 ± 0.07	0.412		21
5	*p*-Chlorobenzyl mercaptan	6-flβCD (**11**)				11.8 ± 0.8	
6	*p*-Chlorobenzyl mercaptan	Rfl				1.93 ± 0.1	
7	*o*-Chlorobenzyl mercaptan	2-flβCD (**6**)	1.53 ± 0.11	0.178 ± 0.013	0.116		4
8	*o*-Chlorobenzyl mercaptan	Rfl				2.82 ± 0.12	
9	*m*-Chlorobenzyl mercaptan	2-flβCD (**6**)	8.85 ± 1.02	3.53 ± 0.40	0.400		16
10	*m*-Chlorobenzyl mercaptan	Rfl				2.45	
11	α-Naphthyl mercaptan	2-flβCD (**6**)				13.2 ± 1.8	

*The kinetics measurements were carried out in $NaHCO_3$–NaOH buffer (pH10.0) containing 30% methanol (v/v) at 25.0 ± 0.1°C at calculated ionic strength of 0.24 M. †$NaHCO_3$–NaOH buffer containing 10% (v/v) methanol. ‡Second-order rate constants of the oxidation of mercaptans by riboflavin (Rfl).

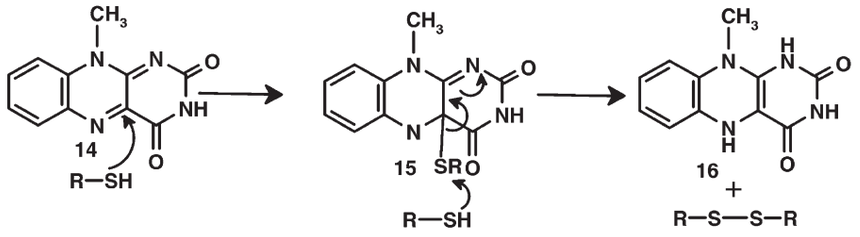

FIGURE 7 Mechanism for oxidation of mercaptans by flavins.

reactions were almost the same as the decomposition of flavins.

Table I gives all results for the oxidation of benzyl mercaptans by flavins. First of all, the oxidation of benzyl and substituted benzyl mercaptans by 2-flavocyclodextrins shows saturation kinetics, i.e., reactions proceed by the complex formation between 2-flavocyclodextrins and mercaptans. In contrast to 2-flavocyclodextrins, 6-flavo-β-cyclodextrin (6-flβCD, **11**) gives the second-order kinetics in the oxidation of *p*-chlorobenzyl mercaptan (entry 5 in Table I), which is similar to the oxidation of mercaptans by Rfl. A computational chemistry study [17] of the conformations of the inclusion complexes between benzyl mercaptan and 2-flβCD indicates that the conformation in which the thiol group is oriented towards the secondary side of cyclodextrin is more stable than the conformation where the thiol group is oriented towards the primary side of cyclodextrin. The difference in energy between these two conformations is 44 kcal/mol. The preferred conformation brings the thiol group of the substrate close to the catalytic functional group of 2-flavocyclodextrins. In the case of 6-flβCD, the complex formation brings the thiol group of the substrate away from the catalytic functional group. Therefore, the flavin moiety of 6-flβCD only reacts with unbound *p*-chlorobenzyl mercaptan.

Entries 1, 4 and 9 in Table I give the rate constants of the oxidation of benzyl mercaptan, *p*-chlorobenzyl mercaptan and *m*-chlorobenzyl mercaptan by 2-flβCD. The stability of the complexes formed between benzyl mercaptans and 2-flβCD is directly related to the reaction rate. The more stable the complex (smaller K_{diss}), the higher the rate acceleration factor.

The oxidation of benzyl mercaptan by 2-flβCD (entry 1) is faster than by 2-flαCD (entry 2). Because the dissociation constants of these two reactions are close, the difference in rate is caused mainly by the difference of catalytic rate constant k_{cat}. Model studies for the oxidation of mercaptans to disulfides by oxidized flavins suggest that thiolate anions add to the C(4a)-position of oxidized flavins, producing an adduct (**15**) as shown in Fig. 7. The reaction of a second molecule of mercaptan with the adduct gives the disulfide and reduced flavin [18,19]. It has been shown that the rate-determining step in the oxidation of thiols by flavins is the formation of the C(4a) adduct. Therefore, the distance between C(4a) of the catalytic functional group of the host and the sulfur atom of the thiol group of the guest [*d*S–C(4a)] (Fig. 8) is very important for the catalytic reaction. Because the cavity size of α-cyclodextrin and β-cyclodextrin differs, the *d*S–C(4a) will be different in the complexes of benzyl mercaptan with 2-flαCD and 2-flβCD. This difference is further transferred to the reaction rate. For the same reason, the oxidation of α-naphthyl mercaptan by 2-flβCD shows second-order kinetics. Although α-naphthyl mercaptan can form a complex with β-cyclodextrin [20], the *d*S–C(4a) of the complex with 2-flβCD is too long to undergo reaction. The artificial enzyme 2-flβCD only reacts with unbound α-naphthyl mercaptan and the reaction follows second-order kinetics.

The reaction rates for the oxidation of different benzyl mercaptans by 2-flβCD can be alternately explained by the *d*S–C(4a) distance. Computational chemistry studies were performed to determine

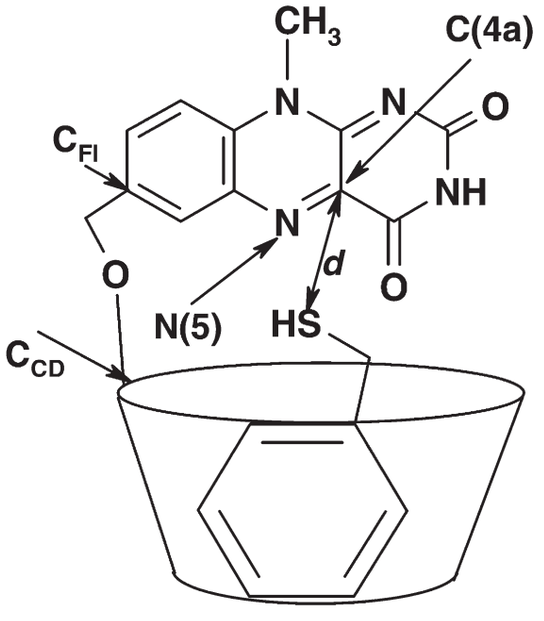

FIGURE 8 Important distances in the oxidation of mercaptans by flavins.

TABLE II The relationship between *d*S–C(4a) and the catalytic rates

Substrate	ΔE (kcal/mol)	$d_{S-C(4a)}$ (Å)	$k_{cat} \times 10^3$ (s^{-1})
Benzyl mercaptan	−24.77	3.531	1.11
p-Chlorobenzyl mercaptan	−26.87	3.322	1.20
m-Chlorobenzyl mercaptan	−47.94	3.300	3.53

the relationship between the experimental catalytic rates and the *d*S–C(4a) distance. The results shown in Table II lead to the conclusion that the shorter the distance *d*S–C(4a), the faster the oxidation of the mercaptans by the artificial enzyme.

The results described provide evidence that the mercaptan first binds to the artificial enzyme and then reacts with the catalytic functional group of the artificial enzyme. Saturation kinetics observed experimentally in these reactions indicates that oxidation of mercaptans by the artificial enzyme proceeds via formation of an enzyme–substrate complex. This reaction path allows the artificial enzyme to catalyze the reaction 53 times faster than riboflavin.

Photo-oxidation of Benzyl Alcohols

Irradiation of an air-saturated aqueous solution containing 2-flβCD, benzyl alcohol, and $HClO_4$ with visible light of $360\,nm < \lambda < 440\,nm$ resulted in the formation of benzaldehyde. The reaction was monitored by detecting the concentration of benzaldehyde with reverse phase HPLC. The molar ratio of the product formed after 674 min to the initial amount of 2-flβCD used is 103 (turnover), demonstrating an efficient recycle of 2-flβCD in the photo-oxidation of benzyl alcohol. When Rfl was used as a photocatalyst instead of 2-flβCD, the reaction was much slower than that catalyzed by 2-flβCD (Fig. 9). Only 6 turnovers were observed after 718 min for Rfl. The yield of benzaldehyde is lower in the case of Rfl than of 2-flβCD, indicating a lower catalytic efficiency of the former.

Complex Formation Between 2-Flβ CD and p-tert-Butylbenzyl Alcohol

Cyclodextrins form inclusion complexes in solution with a variety of organic substances, and it is likely that increase in the rate of oxidation of substituted benzyl alcohols catalyzed by 2-flβCD compared with those catalyzed by Rfl involved the formation of 2-flβCD-benzyl alcohol complex. Efforts to get direct experimental evidence for the formation of complexes were made. The dependence of the catalytic action of flavins on the concentration of the substrate was examined. A plot of the initial rates vs. substrate concentration for the oxidation of *p-tert*-butylbenzyl

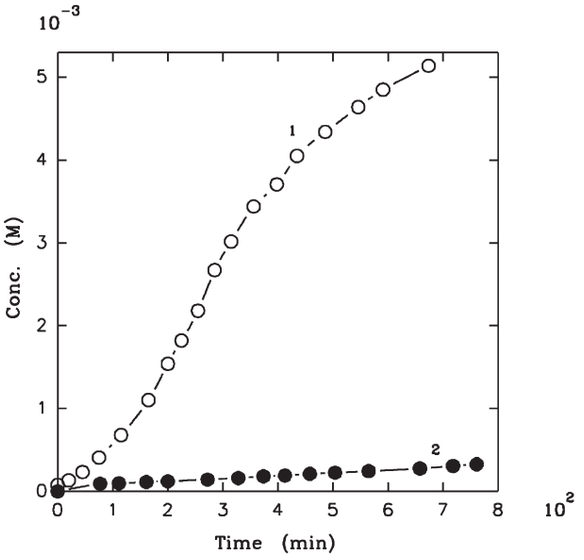

FIGURE 9 The concentration of benzaldehyde vs. irradiation time for the oxidation of benzyl alcohol by oxygen catalyzed by 1) 2-flβcd and 2) Rfl.

alcohol catalyzed by Rfl gives a straight line with zero intercept, indicating a first-order dependence on the substrate [21]. A similar plot for the same reaction catalyzed by the artificial enzyme [i.e., 2-flβCD] shows saturation kinetics. Lineweaver–Burk treatment of these data gives an excellent straight line with a slope of $K_{diss}/V_{max} = 88.92$ min and a Y intercept equal to $1/V_{max} = 2.46 \times 105\,M^{-1}$ min (Fig. 10). This result indicates binding of the substrate to the catalyst prior to the reaction, which

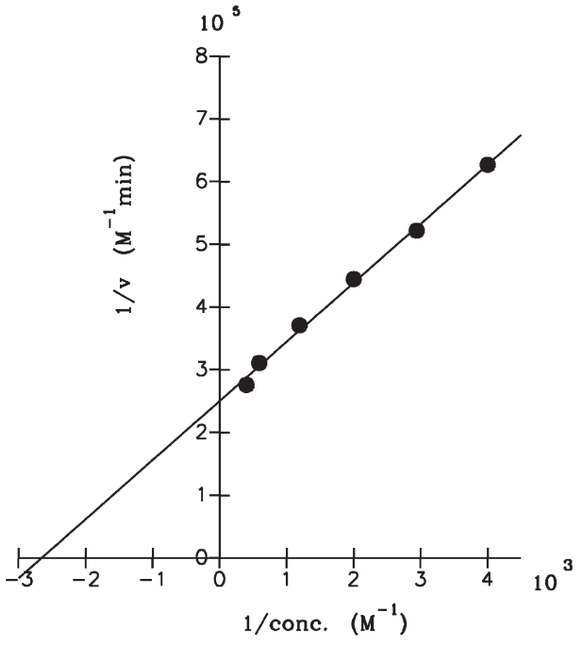

FIGURE 10 Double reciprocal plot of the oxidation of *p-tert*-butylbenzyl alcohol by the artificial enzyme.

TABLE III A comparison of the catalytic activity of artificial redox enzymes

Enzyme	k_{cat} (s^{-1})	K_{diss} (10^{-4} M)	k_{cat}/K_{diss} ($s^{-1}M^{-1}$)	k_2 ($s^{-1}M^{-1}$)	$(k_{cat}/K_{diss})/k_2$
2-flβCD*	1.36×10^{-3}	3.61	3.77	5.83×10^{-3}	647
2-flβCD†	1.36×10^{-3}	2.32	5.86		
Flavo-crown ether‡	3.65×10^{-5}	1.01	0.361	1.26×10^{-2}	29
Flavocyclodextrin¶	0.5	4	1250	117.1§	11

*The reaction of 2-flβCD with *p-tert*-butylbenzyl alcohol was performed at 25.0°C with error limits in k_{cat} and K_{diss} ± 2.8%. †The reaction of 2-flβCD with *p-tert*-butylbenzyl alcohol was performed at 70.0°C with error limits in k_{cat} ± 5.6% and K_{diss} ± 9.7%. ‡The reaction of crown ether flavin with N^3-dodecyl-1-[*p*-(ammoniomethyl)benzyl]-1,4-dihydronicotinamide is from Ref. [20]. ¶The reaction of 6-(8α-S-riboflavo)-α-cyclodextrin with 1-(1-hexyl)-1,4-dihydronicotinamide is from Ref. [21]. §pH 7.0, 25°C.

is similar to the reaction scheme followed by enzymes. The turnover constant (k_{cat}), the dissociation constant (K_{diss}), and the enzyme efficiency (k_{cat}/K_{diss}) for this system are given in Table III. Similar studies were also performed at high temperature (70°C) and the calculated K_{diss} and k_{cat} (Table III) are almost the same as those obtained at 25°C. This indicates that the temperature of the reaction is not important in photochemical reactions.

The oxidation of *p-tert*-butylbenzyl alcohol catalyzed by 2-flβCD was compared with that catalyzed by Rfl and 7,10-dimethylflavin (referred to as Dmfl) to determine the contribution of structural differences (the differences of redox potentials) between Rfl and 2-flβCD. The HPLC chromatograms of the reaction mixtures (Fig. 11) and the plots of product concentrations vs. irradiation time (Fig. 12) indicate that the oxidation catalyzed by 2-flβCD is much faster than those catalyzed by Rfl and Dmfl. Because the structure of the flavin moiety of 2-flβCD is identical with the structure of Dmfl, these results demonstrate clearly that the rate acceleration by 2-flβCD is due to complex formation. The differences in reaction rates caused by change in flavin structure (the difference of redox potentials) as in the cases of Rfl and Dmfl is very small (plots 2 and 3 in Fig. 12).

The oxidation of *p-tert*-butylbenzyl alcohol under irradiation conditions catalyzed by 2-flαCD is only slightly slower than that catalyzed by 2-flβCD but is considerably faster than that catalyzed by Rfl and Dmfl. We interpret this by the fact that both 2-flαCD and 2-flβCD have binding sites (cyclodextrins) to form complexes with the substrate and thus this accelerates the reaction. The flavin moiety of 2-flαCD and 2-flβCD is identical, and the difference of the reaction rates is caused by the different binding energies between 2-flαCD and 2-flβCD with the substrate. This observation further supports the conclusion that the rates of the oxidation of benzyl alcohols are accelerated by the complex formation between flavocyclodextrins and substrates.

It is interesting to compare this artificial enzyme with previously published non-proteinic enzyme models (Table III): flavo-crown ether [22] and 6-(8α-*S*-riboflavo)-α-cyclodextrin [23]. While the dissociation constants (K_{diss}) for all these systems are in the same range, the turnover constant (k_{cat}) for

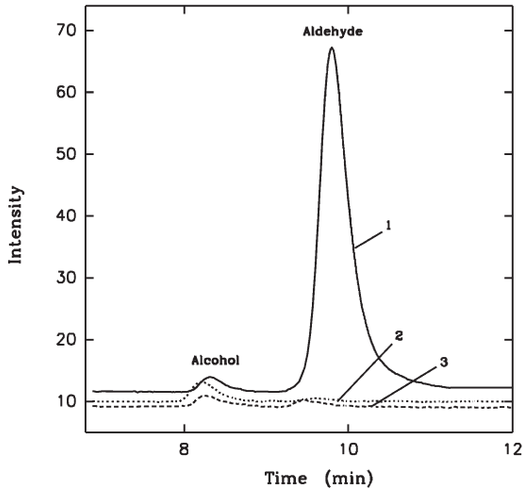

FIGURE 11 HPLC chromatograms for analysis of the reaction mixture in the oxidation of *p-tert*-butylbenzyl alcohol by flavins. 1. Catalyzed by 2-flβCD for 53 min; 2. Catalyzed by Rfl for 51 min; 3. Catalyzed by Dmfl for 76 min.

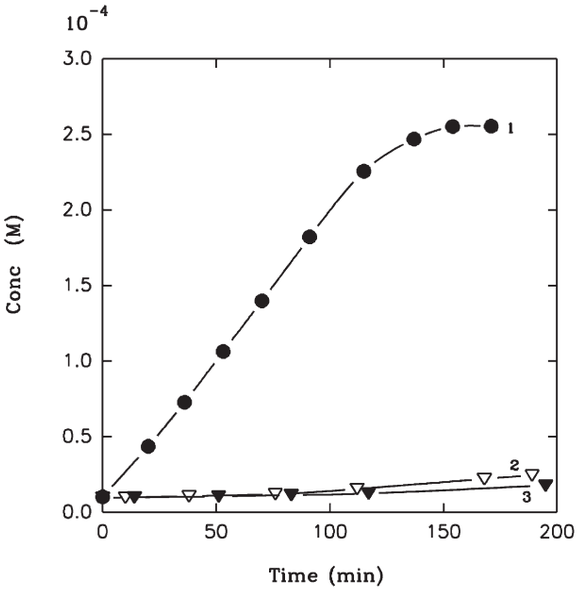

FIGURE 12 The plots of product concentration vs. irradiation time for the oxidation of *p-tert*-butylbenzyl alcohol catalyzed by 1) 2-flβCD; 2) Dmfl; 3) Rfl.

2-flβCD is higher than flavo-crown ether. The turnover constant for 6-(8α-*S*-riboflavo)-α-cyclodextrin is the highest because the bimolecular rate constant (k_2) for this reaction catalyzed by riboflavin is the highest among all these systems. The efficient electron transfer reported for flavo-crown ether and 6-(8α-*S*-riboflavo)-α-cyclodextrin is a property of the reactive substrate used in the reaction rather than the advantage offered to it through binding of the substrate by the artificial enzyme. The highest acceleration factor $[(k_{cat}/K_{diss})/k_2 = 647]$ exhibited by 2-flβCD over riboflavin offers clear evidence for the importance of binding in a reaction scheme.

CONCLUSION

We have offered a general method for synthesis of artificial redox enzymes and studied some of the catalytic properties of these systems. Although these artificial enzymes adhere to the same mechanistic pathway as enzymes (binding the substrate prior to the reaction) their efficiency leaves much to be desired. A thorough investigation including computational chemistry which is currently underway is focused on the reasons for such a disparity between artificial enzymes (efficiency $\sim 10^3$) and natural enzymes (efficiency 10^{12}).

Acknowledgements

Heartiest congratulations are due to Professor Roberto Cao and Dr Alex Fragoso of University of Havana for organizing a superb workshop in Santa María, Cuba. I also thank my graduate students, post-doctoral fellows and funding agencies for making this investigation possible.

References

[1] Siverman, B. *The Organic Chemistry of Enzyme-Catalyzed Reactions*; Academic Press: New York, 2002.
[2] D'Souza, V. T.; Bender, M. L. *Acc. Chem. Res.* **1987**, *20*, 146–152.
[3] Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman: San Francisco, 1979.
[4] Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman: New York, 1985; p 29.
[5] Breslow, R. *Acc. Chem. Res.* **1995**, *28*, 146.
[6] Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Springer Verlag: New York, 1978.
[7] Breslow, R.; Dong, S. D. *Chem. Rev.* **1998**, *98*, 1997.
[8] Murakami, Y.; Kikuchi, J.; Hisaeda, Y.; Hayashida, O. *Chem. Rev.* **1996**, *96*, 721.
[9] *Advances in Supramolecular Chemistry*; Gokel, G. W., Ed.; JAI Press: Stamford, CT, 2000; Vol. 6.
[10] Khan, A.; Forgo, P.; Stine, K. J.; D'Souza, V. T. *Chem. Rev.* **1998**, *98*, 1977.
[11] Rong, D.; D'Souza, V. T. *Tetrahedron Lett.* **1990**, *31*, 4275.
[12] Tian, S.; D'Souza, V. T. *Tetrahedron Lett.* **1994**, *35*, 9339.
[13] Tian, S.; Forgo, P.; D'Souza, V. T. *Tetrahedron Lett.* **1996**, *37*, 8309–8312.
[14] Forgo, P.; D'Souza, V. T. *Tetrahedron Lett.* **1999**, *40*, 8533–8537.
[15] Rong, D.; Ye, H.; Boehlow, T. R.; D'Souza, V. T. *J. Org. Chem.* **1992**, *57*, 163.
[16] Ye, H.; Tong, W.; D'Souza, V. T. *J. Chem. Soc., Perkin Transactions 2* **1994**, 2431–2437.
[17] Tong, W.; Ye, H.; Rong, D.; D'Souza, V. T. *J. Comput. Chem.* **1992**, *13*, 614.
[18] Yokoe, I.; Bruice, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 450.
[19] Loechler, E. L.; Hollocher, T. C. *J. Am. Chem. Soc.* **1975**, *97*, 3235.
[20] Harata, K. H.; Uedaira, H. *Bull. Chem. Soc. Jpn.* **1975**, *48*, 375.
[21] Ye, H.; Tong, W.; D'Souza, V. T. *J. Am. Chem. Soc.* **1992**, *114*, 5470.
[22] Shinkai, S.; Ishikawa, Y.; Shinkai, H.; Tsuno, T.; Makishima, H.; Ueda, K.; Manabe, O. *J. Am. Chem. Soc.* **1984**, *106*, 1801.
[23] Tabushi, I.; Kodera, M. *J. Am. Chem. Soc.* **1987**, *109*, 4734.