Flavocyclodextrins as Artificial Redox Enzymes. Part 4.[1] Catalytic Reactions of Alcohols, Aldehydes and Thiols

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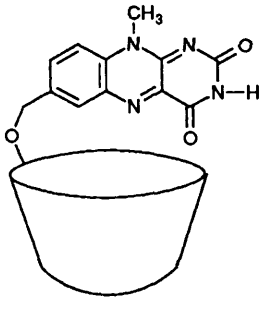
The catalytic reactions of models of flavoenzymes in which flavin is covalently attached to the catalytically important secondary side of cyclodextrin 2-[(7α-*O*-10-methyl-7-isoalloxazino)methyl]-α-cyclodextrin and 2-[(7α-*O*-10-methyl-7-isoalloxazino)methyl]-β-cyclodextrin are reported.

Flavoenzymes contain a versatile coenzyme riboflavin (Rfl) which enables them to catalyse a wide variety of electron-transfer reactions by either a single electron[2] or a simultaneous two-electron transfer mechanisms.[3–5] In the process of catalysing electron transfer reactions, the flavin molecule itself is reduced as it accepts electrons from a substrate and is re-oxidized as it transfers electrons to an acceptor.[6] The elucidation of mechanisms of flavin mediated oxidation–reduction reactions in simple chemical systems is of prime importance to the understanding and appreciation of mechanisms of catalysis by flavoenzymes.[7] Physical organic studies of reactions catalysed by riboflavin and its model compounds with substrates have proved invaluable to the understanding of enzymatic processes.[7–9] An understanding of the differences in mechanisms of reactions catalysed by enzymes and various models of the cofactor are important in explicitly defining the role of the apoenzyme.[7] The protein structure around the flavin clearly controls its versatility by introducing specificity to the catalytic step and by controlling the kinetics and thermodynamics of the electron-transfer processes.[6] Several investigations of flavoenzymes which catalyse the oxidation of thiols (or dithiols) to disulfides[10–13] using models have been reported.[14–17] The oxidation of alcohols by oxidized flavin derivatives[8,18–21] and the reduction of aldehydes by their reduced form[8,19,22,23] have been studied extensively also. But all these investigations were limited to the prosthetic group (flavins), in the absence of binding sites. In an attempt to overcome this shortcoming we designed a system which contains both a catalytic site (a flavin derivative) as well as a binding site (cyclodextrin). We recently published the synthesis[24] and structural properties[1] of three systems, 2-[(7α-*O*-10-methyl-7-isoalloxazino)methyl]-α-cyclodextrin, 2-flαCD (**1**), 2-[(7α-*O*-10-methyl-7-isoalloxazino)methyl]-β-cyclodextrin, 2-flβCD (**2**) and 6-(10-*N*-isoalloxazinomethyl)-β-cyclodextrin, 6-flβCD (**3**), which are well suited for such investigations. We now report the catalytic and mechanistic studies of these artificial redox enzymes to provide an insight into the mechanisms of real flavoenzymes.



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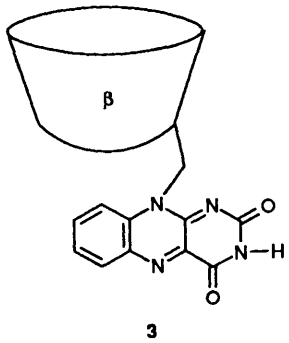

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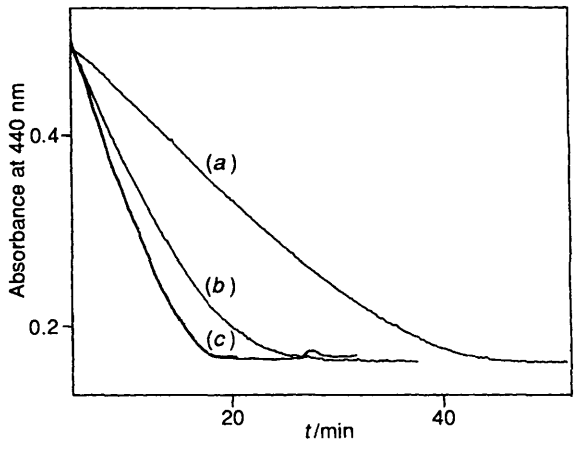


Fig. 1 Decrease in the absorbance of 2-flβCD at 440 nm during the oxidation of benzoin. [2-flβCD] = 5.00 × 10^{-5}, [BZH] = 2.0 × 10^{-3} mol dm^{-3}, T = 25 °C. pH (*a*) 10.0, (*b*) 11.0, (*c*) 11.5.

Results and Discussion

Oxidation of Alcohols by Flavins.—Oxidation of benzoin (BZH) to benzil. The reactions were carried out under anaerobic conditions and characterized by the bleaching of the Fl_{ox} absorption (at 440 nm) accompanying its conversion into the corresponding 1,5-dihydroflavin anion (1,5-FlH^-). Admittance of O_2 at the completion of the reaction immediately restores the characteristic Fl_{ox} spectra indicating that the reduction of flavocyclodextrins is fully reversible. The kinetics of the reaction were investigated in the alkaline pH range with a 40-fold excess of benzoin to make these reactions follow pseudo-first-order kinetics. An examination of the plots of absorption *vs.* time for this reaction (Fig. 1) reveals that the rate of disappearance of 2-flβCD (**2**) is independent of its concentration, to at least 70% completion of the reaction. The reaction is therefore initially zero order in [2-flβCD] changing to first order in [2-flβCD] as the concentration of 2-flβCD (**2**) is depleted. Thus, the data does not fit a pseudo-first-order equation indicating that this reaction does not occur by a direct attack of benzoin with 2-flβCD (**2**). This is consistent with the observation of Bruice[19] in the oxidation of benzoin by lumiflavin-3-acetate. The initial rate of reduction of 2-flβCD (**2**) by benzoin increases with an increase in pH (Fig. 1) and a similar effect is observed when the total carbonate–hydrogen carbonate concentration is increased at constant pH (Fig. 2). These results are similar to the flavin-mediated dehydrogenation of dimethyl *trans*-dihydrophthalate[25] and the oxidation of

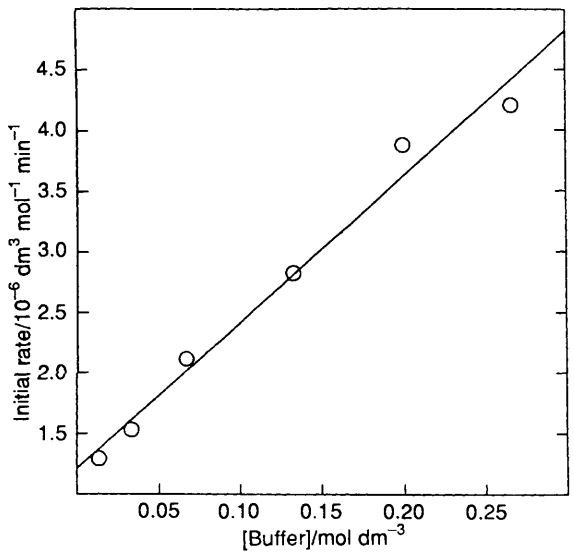


Fig. 2 Change in the initial rate of oxidation of benzoin by 2-flβCD with the concentrations of buffer. [2-flβCD] = 5.00×10^{-5}, [BZH] = 2.0×10^{-3} mol dm^{-3}, T = 25 °C.

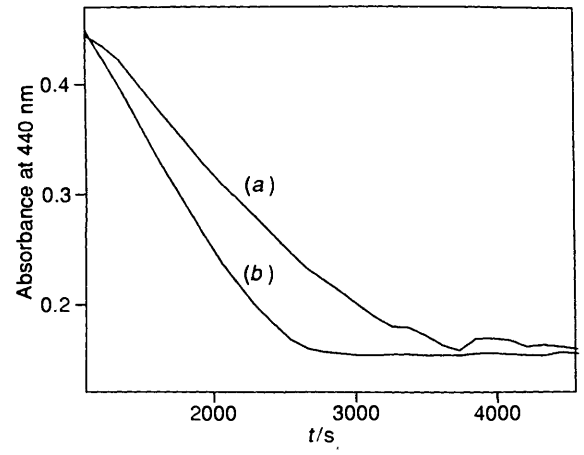


Fig. 3 Decrease in the absorbance of 440 nm during the oxidation of benzoin by (*a*) Rfl and (*b*) 2-flβCD. [Catalyst] = 5.00×10^{-5}, [BZH] = 2.00×10^{-3}, T = 25 °C.

benzoin by lumiflavin-3-acetate.[19] According to the mechanistic study of the oxidation of benzoin by lumiflavin-3-acetate, the reaction involves the addition of carbanion [formed by deprotonation of benzoin at the carbon atom (C–OH) attached to the hydroxy group] to the 4a-position of Fl_{ox} followed by a hydroxide ion catalysed elimination of benzil from the 4a-adduct.[19] It is expected that if 2-flβCD (**2**) can form a productive inclusion complex with benzoin and bring the α-carbon of the guest closer to the C(4a)-position of the flavin moiety, it should accelerate the reaction. However, a computational chemistry study done to investigate the conformation of the 2-flβCD–benzoin complex showed that the binding energy for this is +11.2 kcal mol^{-1}. These results suggest that there is virtually no binding between 2-flβCD (**2**) and benzoin. The oxidation of benzoin by 2-flβCD (**2**) is as shown in Scheme 1 and is analogous to the identical oxidation

$$\underset{\text{Benzoin}}{BZH} \underset{k_{-b}(BH)}{\overset{k_b(B)}{\rightleftharpoons}} \underset{\text{Enediolate anion}}{BZ^-} \xrightarrow{k_1(2\text{-fl}\beta\text{CD})} 2\text{-fl}\beta\text{CDH}^- + \underset{\text{Benzil}}{BZL}$$

Scheme 1

by lumiflavin-3-acetate.[19] A general base catalysed ionization of benzoin is the rate determining step at high 2-flβCD (**2**) concentration, the reaction of the benzoin carbanion (endiolate anion) with 2-flβCD (**2**) is rate determining at low 2-flβCD (**2**) concentration and no rate acceleration is predicted. The observed twofold acceleration by 2-flβCD (**2**) over Rfl (Fig. 3) can be attributed to the difference[1] in the redox potential of the two flavins.

Oxidation of other alcohols. The oxidation of other alcohols, such as benzyl alcohol, *p*-nitrobenzyl alcohol, 2-methyl-3-nitrobenzyl alcohol and mandelonitrile, by Rfl and 2-flβCD (**2**) was also investigated. The reactions were performed at pH 10.0 under anaerobic conditions by following the decrease in absorbance due to flavins at 445 nm. Plots of absorption *vs.* time for the reactions are almost identical with the decomposition of the flavins. There is virtually no reaction between these alcohols and flavins. The oxidation of *p*-nitrobenzyl alcohol by Rfl and 2-flβCD (**2**) was also tried at pH 12.5. The reaction did not proceed but the decomposition of flavins was faster than at pH 10.0.

Since the rate determining step of the oxidation of benzoin by flavins is the formation of the enediolate anions[19] [*e.g.* BZ^-], electron withdrawing groups connected to C–OH will stabilize the enediolate anions and accelerate the oxidation of alcohols. Considering this mechanism and observed experimental results, it is possible that the benzene ring connected to the carbonyl group of benzoin is more important than the electron withdrawing group attached to C–OH. The basis of this deduction is that although –CN in mandelonitrile is a stronger electron withdrawing group than –CO– in benzoin, the latter is oxidized by flavins while former apparently is not. The resonance system in the benzoin endiolate anion makes it more stable than normal carbanions (like that of mandelonitrile). In the oxidation of methanol by lumiflavin-3-acetate investigated by Bruice[7] using 6.12 mol dm^{-3} methanol in pH 9.65 buffer and monitoring at 443 nm by the disappearance of the flavin molecule for 8.5 days, only 8.2×10^{-6} mol dm^{-3} of formaldehyde was formed. So the oxidation of alcohols by flavins is very slow. In their case, *N*(3)-acetate made the flavin stable and the oxidation of methanol was faster than decomposition of the flavin molecule.

Reduction of Carbonyl Compounds by Dihydroflavins.—The reduction of aldehydes by dihydroflavins was found to be very slow. These reactions were monitored by observing the increase in the absorbance at 440 nm due to the oxidation of dihydroflavins. Plots of absorbance at 440 nm *vs.* time were almost identical with and without substrates. In these experiments, 1,5-dihydroflavins were obtained by the photoreduction (with EDTA) of flavins.[26] EDTA is oxidized to produce glyoxylic acid and formaldehyde[27] when a flavin molecule is reduced under these conditions. When a solution of the substrate is added to the above reaction mixture, the 1,5-dihydroflavin reduces both HCHO and the substrate. In the case of benzaldehyde, *p*-chlorobenzaldehyde, phenylacetaldehyde, 2,4,6-trimethoxybenzaldehyde, cinnamaldehyde, *m*- and *p*-cyanobenzaldehyde and *p*-chloroacetophenone, the reduction by a dihydroflavin was found to be slower than the reduction of HCHO. On the other hand, the reduction of terephthalaldehyde by dihydroflavins was found to be faster than the reduction of HCHO by dihydroflavins. The reduction of terephthalaldehyde by 2-flβCDH$_2$ was compared to its reduction by RflH$_2$ as shown in Fig. 4. These curves fit first order kinetics and rate constants calculated for these curves are given in Table 1. Rate constants for reactions without substrate were subtracted from those with substrates and the corrected rate constants, $k'_{correct}$, are given in Table 1. The ratio of the corrected rate constants for the reaction with 2-flβCDH$_2$ to that with RflH$_2$ is 4. It is known that the redox potential of 2-flβCD (**2**) is slightly higher than Rfl based on the investigation of oxidations of

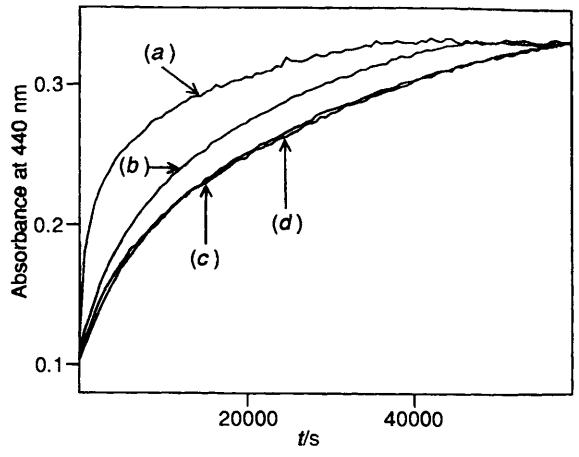


Fig. 4 Increase in the absorbance of 440 nm during the oxidation of 2-flβCDH$_2$ and RflH$_2$ by terephthalaldehyde; (*a*) 2-flβCDH$_2$ in the presence of terephthalaldehyde; (*b*) RflH$_2$ in the presence of terephthalaldehyde; (*c*) 2-flβCDH$_2$ in the absence of terephthalaldehyde; (*d*) RflH$_2$ in the absence of terephthalaldehyde. [Dihydroflavin] = 5.0 × 10^{-5}, [terephthalaldehyde] = 2.0 × 10^{-3} mol dm^{-3}, T = 25 °C.

Table 1 Rate constants for the reduction of terephthalaldehyde by dihydroflavins[a]

	2-flβCDH$_2$		RflH$_2$	
	with TP	without TP	with TP	without TP
k'/10^{-5} s^{-1}	10.79	4.13	6.23	4.56
$k'_{correct}$/10^{-5} s^{-1}		6.66		1.67

[a] [TP] = 2.0 × 10^{-3}, [FlH$_2$] = 5.0 × 10^{-5} mol dm^{-3}, pH 7.0, μ = 0.68 mol dm^{-3}, 25 °C. $k'_{correct}$ (2-flβCDH$_2$)/$k'_{correct}$ (RflH$_2$) = 4.0.

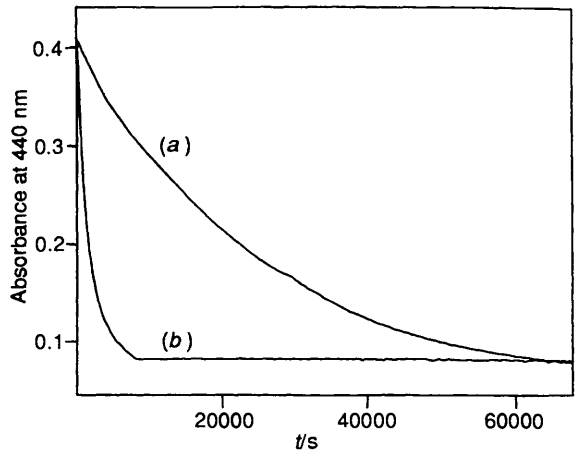


Fig. 5 Decrease in the absorbance of 440 nm during the oxidation of phenylmethanethiol catalysed by (*a*) Rfl and (*b*) 2-flβCD. [Catalyst] = 5.0 × 10^{-5}, [substrate] 4.0 × 10^{-3} mol dm^{-3}, T = 25 °C.

dihydronicotinamides[1] by these flavin derivatives. Therefore, the reduction of an aldehyde by 2-flβCDH$_2$ should be slower than that by RflH$_2$ in the absence of any binding between the aldehyde and cyclodextrin moiety of 2-flβCDH$_2$. The fourfold increase in rate gained by 2-flβCDH$_2$ over RflH$_2$ can be attributed to complex formation, which lowers the activation energy of the reaction. A computational chemistry study shows that the binding energy of the 2-flβCDH$_2$–terephthalaldehyde complex is −26.51 kcal mol^{-1}. It is likely that the oxidation occurs by means of complex formation because the binding energy is negative and the distances of reacting atoms are reasonable.

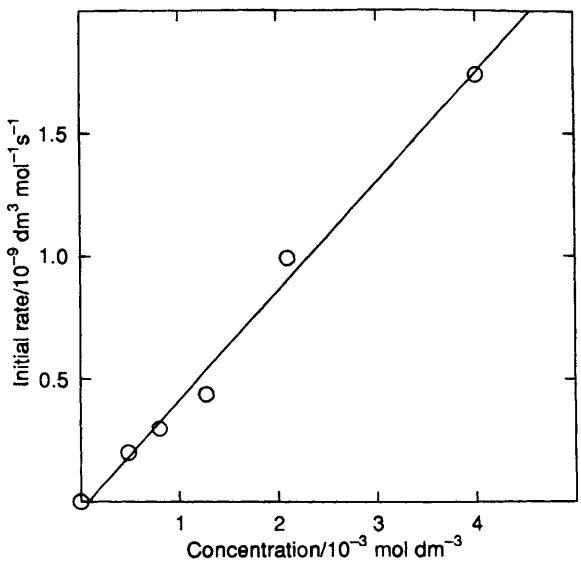


Fig. 6 Change in initial rates of oxidation of phenylmethanethiol with changes in its concentrations. [Rfl] = 5.0 × 10^{-5} mol dm^{-3}, T = 25 °C.

Oxidation of Thiols.—The oxidation of thiols 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 mol dm^{-3} (adjusted with KCl) at 25.0 °C. A slow decrease in the absorbance of 2-flβCD (**2**) at 440 nm is observed when a solution of 2-flβCD (**2**) was mixed with an 80-fold excess of phenylmethanethiol under anaerobic conditions and after several hours, a spectrum characteristic of reduced flavin remained. The spectrum of 2-flβCD (**2**) can be completely restored by re-oxidation of the reaction mixture with air. The decomposition of flavins is so slow that it can be ignored in comparison with the oxidation of thiols by flavins. The absorbance of 2-flβCD (**2**) and Rfl as a function of time gave good first order plots under anaerobic conditions in reactions with excess of phenylmethane- and substituted phenylmethanethiols, but the reaction with 2-flβCD (**2**) was faster than that with Rfl (Fig. 5). In reactions with Rfl as a catalyst, when the concentration of thiols (still in excess) was varied, a plot of the initial rates *vs.* the concentrations of the substrate gave a straight line showing the reaction to be overall second order (Fig. 6). Second order rate constants thus obtained from this plot are shown in Table 2. In contrast to Rfl, similar plots for reactions of 2-flαCD (**1**) and 2-flβCD (**2**) with phenylmethanethiol showed saturation kinetics. The double reciprocal plot of initial rates *vs.* concentrations of phenylbenzenethiol for the oxidation of phenylbenzenethiol by 2-flβCD (**2**) is given in Fig. 7. The K_{diss} and k_{cat} calculated from the plot are listed in Table 2.

The oxidations of benzenethiol, phenylethanethiol and cyclohexanethiol by Rfl and flavocyclodextrins were also investigated in a similar manner. The oxidations by Rfl or by flavocyclodextrins were either very slow, or did not proceed at all because the plots of absorbance *vs.* time for these reactions were almost indistinguishable from the decomposition of flavins.

In Table 2 which gives all results for the oxidation of phenylbenzenethiol by flavins, first of all, the oxidation of phenylmethane- and substituted phenylmethane-thiols by 2-flavocyclodextrins (**1** and **2**) shows saturation kinetics, *i.e.*, reactions proceed by the complex formation between 2-flavocyclodextrins (**1** and **2**) and thiols. In contrast to 2-flavocyclodextrins (**1** and **2**), 6-flavo-β-cyclodextrin (6-flβCD, **3**) gives second order kinetics in the oxidation of *p*-chlorophenylmethanethiol (entry 5 in Table 2), which is similar

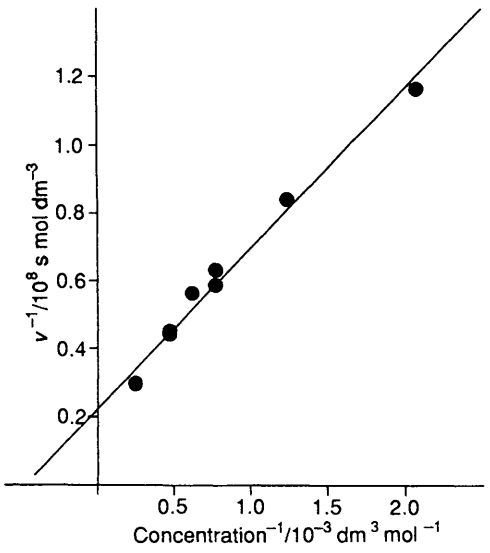


Fig. 7 Double reciprocal plot of initial rate of oxidation of phenylmethanethiol *vs.* substrate concentration in the reaction catalysed by 2-flβCD. [2-flβCD] = 5.0×10^{-5} mol dm^{-3}, T = 25 °C.

to the oxidation of thiols by Rfl. A computational chemistry study[28] of the conformations of the inclusion complexes between phenylmethanethiol and 2-flβCD (**2**) 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^{-1}. The preferred conformation brings the thiol group of the substrate close to the catalytic functional group of 2-flavocyclodextrins (**1** and **2**). In the case of 6-flβCD (**3**), the complex formation brings the thiol group of the substrate away from the catalytic functional group. Therefore, the flavin moiety of 6-flβCD (**3**) only reacts with unbound *p*-chlorophenylmethanethiol.

Entries 1, 4 and 9 in Table 2 give rate constants of the oxidation of phenylmethanethiol, *p*-chlorophenylmethanethiol and *m*-chlorophenylmethanethiol by 2-flβCD (**2**). The stability of the complexes formed between these substrates and 2-flβCD (**2**) is directly related to the reaction rate. The more stable the complex (small K_{diss}), the higher the rate acceleration factor. These examples show the significance of binding in enzyme-like reactions.

The oxidation of phenylmethanethiol by 2-flβCD (**2**) (entry 1) is faster than by 2-flαCD (**1**) (entry 2). The difference in the rate is caused mainly by the difference of catalytic rate constant k_{cat} because dissociation constants of these systems are similar. Model studies for the oxidation of thiols to disulfides by oxidized flavins suggest that thiolate anions add to the C(4a)-position of oxidized flavins, producing an adduct (**4**). The reaction of a second molecule of thiol with the adduct (**4**) gives the disulfide and reduced flavin[14,15] (Scheme 2). It has been shown that the rate-determining step in the oxidation of thiols by flavins is the formation of the C(4a) adduct (**4**).[2,14] 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. The reaction rates for the oxidation of different phenylmethanethiols by 2-flβCD (**2**) can be explained by the $d_{S-C(4a)}$ distance. Computational chemistry studies[28] performed to determine the relationship between the experimental catalytic rates and the $d_{S-C(4a)}$ distance indicate that the shorter the distance $d_{S-C(4a)}$, the faster the oxidation of the thiols by the artificial enzyme. This indicates that the distance between the two reactive atoms in productive binding dictates the rate of enzyme catalysed reaction. Because the cavity size of α-cyclodextrin and β-cyclodextrin differs, the $d_{S-C(4a)}$ will be different in the complexes of phenylmethanethiol with 2-flαCD (**1**) and 2-flβCD (**2**). This difference is then transferred to the reaction rate in the case of phenylmethanethiol (entries 1 and 2). For the same reason, the oxidation of naphthalene-1-thiol by 2-flβCD (**2**) shows second order kinetics. Although naphthalene-1-thiol can form a complex with β-cyclodextrin,[29] the $d_{S-C(4a)}$ of the complex with 2-flβCD (**2**) is too long to undergo reaction. The artificial enzyme 2-flβCD (**2**) reacts with only the unbound naphthalene-1-thiol and the reaction follows the second order kinetics. These results are in contrast to oxidation of dihydronicotinamides by 2-flβCD (**2**) in which the only substrate that follows saturation kinetics is 1-(1-naphthyl)methyldihydronicotinamide, whereas others that contain a phenyl ring give second order kinetics.[1] These results clearly emphasize that it is not binding, but the orientation of binding and the distance between the two reacting atoms in this complex that is important in enzyme catalysed reactions.

The results shown above provide evidence that the thiol 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 indicate that oxidation of thiols by the artificial enzyme proceeds *via* formation of an enzyme–substrate complex. This reaction path allows the artificial enzyme to catalyse the reaction 53 times faster than riboflavin in the case of phenylmethanethiol.

Conclusions

Among the three types of chemical transformation, oxidation of alcohols, reduction of carbonyls and oxidation of thiols, the last one is effectively catalysed by our artificial enzymes. This leads to the conclusion that in these reactions both binding and chemical reactivities should be suitable for an enzyme-like activity. From the oxidation of thiols, it can be concluded that although binding is necessary, effective catalysis in these systems is produced only by the orientation of binding in which the two reacting atoms are precisely aligned for the chemical transformation.

Experimental

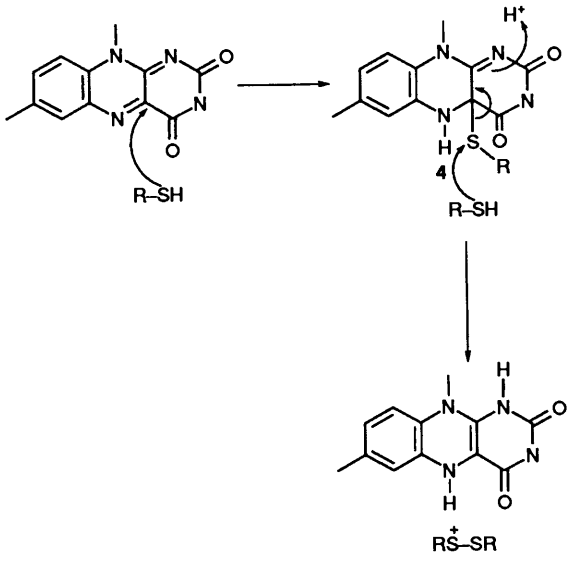
Benzoin (analysis grade) was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Benzyl alcohol (99%), *p*-nitrobenzyl alcohol (99%), 2-methyl-3-nitrobenzyl alcohol (99%), mandelonitrile (tech.), benzenethiol (99+ %), 2-phenylethanethiol (99%), cyclohexanethiol (97%), phenylmethanethiol (99%), *p*-chlorophenylmethanethiol (98%), *o*-chlorophenylmethanethiol (98%), terephthalaldehyde (99%), benzaldehyde (99%), *p*-nitrobenzaldehyde (99.5%), *p*-chlorobenzaldehyde (97%), phenylacetaldehyde, 2,4,6-trimethoxybenzaldehyde (98%), (*E*)-cinnamaldehyde (99%), *m*- and *p*-cyanobenzaldehyde (99%) p-chloroacetophenone (97%) and riboflavin (98%) were purchased from Aldrich Chem. Co. (Milwaukee, WI). All chemicals were used without further purification. 2-FlαCD (**1**), 2-flβCD (**2**) and 6-flβCD (**3**) were synthesized and purified in our laboratory.[24] Buffers were made up with glass distilled water employing reagent grade salts and base.

m-Chlorophenylmethanethiol was synthesized according to the reported procedure.[30,31] However, the detailed procedure was not given in the literature: it was synthesized as follows. A mixture of 25 g (98%; 0.152 mol) of *m*-chlorobenzyl chloride, 11.6 g (0.152 mol) of thiourea and 70 cm^3 of 95% ethanol was refluxed for 4 h. The mixture was allowed to stand at room temperature overnight and 33 g of crystals were collected by filtration. A mixture of 20 g of the above crystals, 9 g (0.225 mol) NaOH and 100 cm^3 water were refluxed for 3 h. The

Table 2 Rate constants for the oxidation of phenylmethanethiol and substituted phenylmethanethiols by flavins[a]

Entry	Substrate	Flavin	$K_{diss}/10^{-3}$ mol dm^{-3}	$k_{cat}/10^{-3}$ s^{-1}	$k_{cat}K_{diss}$/mol dm^{-3} s^{-1}	$k_2/10^{-2}$ mol dm^{-3} s^{-1}	$(k_{cat}/K_{diss})/k_2$[c]
1	Phenylmethanethiol[b]	2-flβCD	1.89 ± 0.23	1.11 ± 0.13	0.587		53
2	Phenylmethanethiol[b]	2-flαCD	2.38 ± 0.09	0.585 ± 0.022	0.246		22
3	Phenylmethanethiol[b]	Rfl				1.11 ± 0.06	
4	*p*-Chlorophenylmethanethiol	2-flβCD	2.91 ± 0.17	1.20 ± 0.07	0.412		21
5	*p*-Chlorophenylmethanethiol	6-flβCD				11.8 ± 0.8	
6	*p*-Chlorophenylmethanethiol	Rfl				1.93 ± 0.1	
7	*o*-Chlorophenylmethanethiol	2-flβCD	1.53 ± 0.11	0.178 ± 0.013	0.116		4
8	*o*-Chlorophenylmethanethiol	Rfl				2.82 ± 0.12	
9	*m*-Chlorophenylmethanethiol	2-flβCD	8.85 ± 1.02	3.53 ± 0.40	0.400		16
10	*m*-Chlorophenylmethanethiol	Rfl				2.45	
11	Naphthalene-1-thiol	2-flβCD				13.2 ± 1.8	

[a] The kinetics measurements were carried out in $NaHCO_3$–NaOH buffer (pH 10.0) containing 30% methanol (v/v) at 25.0 ± 0.1 °C at calculated ionic strength of 0.24 mol dm^{-3}. [b] $NaHCO_3$–NaOH buffer containing 10% (v/v) methanol. [c] k_2 are second-order rate constants for the oxidation for thiols by Rfl.



Scheme 2

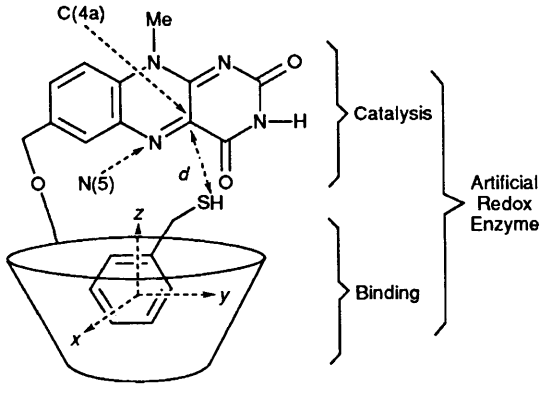


Fig. 8 Schematic of the productive complex of 2-flβCD and phenylmethanethiol; *d* indicates the distance between the two reacting atoms

mixture was cooled to room temperature and the organic and aqueous layers were separated. To the aqueous layer were added 44 cm^3 of dilute sulfuric acid (4 cm^3 concentrated sulfuric acid with 40 cm^3 water) and it was then extracted with 35 cm^3 of diethyl ether. The extract was combined with the organic layer, dried (sodium sulfate) and evaporated. Distillation of the remaining liquid gave 9.0 g *m*-chlorophenylmethanethiol (b.p., 128 °C/*ca.* 20 mmHg); $\delta_H(CDCl_3)$ 7.29 (1 H, m), 7.19 (2 H, m), 3.67 (2 H, d, *J* 7.8) and 1.76 (1 H, t, *J* 7.7); $\delta_C(CDCl_3)$ 28.34, 126.10, 127.07, 128.08, 129.79, 134.18 and 142.94; *m/z* 158 and 160. Naphthalene-1-thiol was made using a similar procedure.

General Kinetic Measurements.—All kinetic studies were carried out at 25.0 ± 0.1 °C under anaerobic conditions by means of a Cary 2200 spectrophotometer (Varian Instrument Co., CA) equipped with a stirrer and thermostatted cell holder connected to a water circulator. The measurement of pH values was performed using a Accumet pH Meter 925 and MicroProbe combined Ag/AgCl-glass electrode (Fisher Scientific). Deoxygenation was accomplished by bubbling vanadous-ion scrubbed argon through the reaction mixtures for 45 min.[19,32] UV cuvettes were sealed with rubber septa. The data were collected and the rate constants were calculated by using Lab Calc (Galactic Industries Corp.).

Oxidation of Alcohols by Flavins.—Oxidation of benzoin (BZH) by flavins. Na_2HPO_4–NaOH buffer (pH 11.5) was made by mixing 50 cm^3 of Na_2HPO_4 solution (0.5 mol dm^{-3}) and 11.1 cm^3 of 0.1 mol dm^{-3} NaOH and diluted to 100 cm^3 with distilled water. Na_2HPO_4–NaOH buffer (pH 11.0) was prepared by mixing 50 cm^3 of 0.5 mol dm^{-3} Na_2HPO_4 solution and 4.1 cm^3 of 0.1 mol dm^{-3} NaOH and diluted to 100 cm^3 with distilled water.[33] $NaHCO_3$–NaOH buffer (0.5 mol dm^{-3}, pH 10.0) was made by dissolving 4.2 g $NaHCO_3$ in about 85 cm^3 of distilled water, adjusting pH to 10 with 50% NaOH solution, then adding water to 100.0 cm^3. The pH values for all buffers were checked after the final dilution. The reaction was followed under anaerobic conditions in UV cuvettes at 440 nm. For a typical run the stock solution of flavin (0.420 cm^3, 3.00 × 10^{-4} mol dm^{-3}) was mixed with 1.330 cm^3 of appropriate buffer in a UV cuvette. The solution was then deoxygenated with a stream of argon scrubbed of traces of O_2 by means of a vanadous ion trap. The reaction was initiated by transferring 0.750 cm^3 of deoxygenated benzoin stock solution (6.65 × 10^{-3} mol dm^{-3} in CH_3CN) by syringe to the sealed cuvette and was monitored by the decrease of absorption due to flavin at 440 nm. For a typical reaction, the concentrations of benzoin and flavin were 2.00 × 10^{-3} and 5.00 × 10^{-5} mol dm^{-3}, respectively. The buffer concentration was in the range of 0.01–0.27 mol dm^{-3}.

Oxidation of substituted benzyl alcohols. The oxidation of benzyl alcohol, 2-methyl-3-nitrobenzyl alcohol and mandelonitrile were performed under anaerobic conditions in 10%, 30% and 30% acetonitrile aqueous buffer respectively. For a typical run, the stock solution of the flavin derivative **1** or **3** (3.00 × 10^{-4} mol dm^{-3}; 0.420 cm^3) was mixed with 1.830 cm^3 of 0.0125 mol dm^{-3} $NaHCO_3$–NaOH buffer (pH 10.0, μ = 1.36 mol dm^{-3} with KCl) in a UV cuvette. The solution was then deoxygenated with argon scrubbed of traces of O_2 by means of a vanadous ion trap. The mixture was allowed to equilibrate for 15 min at 25 °C. The reaction was initiated by transferring 0.250 cm^3 of deoxygenated benzyl alcohol stock solution (0.20 mol dm^{-3} in distilled water) by syringe into the sealed cuvette and was monitored by the decrease of absorption due to flavin at 445 nm. The initial concentrations of benzyl alcohol and flavin were 2.00 × 10^{-2} and 5.00 × 10^{-5} mol dm^{-3}, respectively. The calculated ionic strength and the buffer concentration were 1.0 and 0.009 mol dm^{-3}, respectively.

Reduction of Carbonyl Compounds by 1,5-Dihydroflavins.—The reactions were carried out under anaerobic conditions in 30% methanol. Typically, the stock solution of flavin (0.420 cm^3; 3.00 × 10^{-4} mol dm^{-3}) was mixed with 0.080 cm^3 of distilled water and 1.250 cm^3 of 0.10 mol dm^{-3} KH_2PO_4–NaOH buffer (pH 7.0, μ = 1.36 mol dm^{-3} with KCl, containing 1.0 × 10^{-3} mol dm^{-3} EDTA) in a UV cuvette. The solution was then deoxygenated by bubbling with argon scrubbed of traces of O_2 by means of a vanadous ion trap. Photoreduction (by EDTA) of the flavin to the 1,5-dihydro form was achieved using a 100 W tungsten lamp placed at a distance of 10 cm from the cuvette. The cuvette was protected from temperature changes by a water-cooled jacket and the photolysis was continued for 15 min to ensure complete reduction. The spectrum of the reduced material was recorded after the irradiation was stopped. Before initiation of the reaction the 1,5-dihydroflavin was monitored at 445 nm for a minimum period of 20 min at 25 °C. The absorbance remained constant during the time, indicating the absence of oxygen in the cuvette. The reaction was initiated by transferring 0.750 cm^3 of deoxygenated teraphthaldehyde stock solution (6.67 × 10^{-3} mol dm^{-3} in MeOH) by syringe to the sealed cuvette and was monitored by the increase of absorption due to flavin at 440 nm. The initial concentrations of terephthalaldehyde and flavin were 2.00 × 10^{-3} and 5.00 × 10^{-5} mol dm^{-3}, respectively. The calculated ionic strength and the buffer concentration were 0.68 and 0.05

mol dm^{-3}, respectively. The reduction of terephthalaldehyde by $RflH_2$ and 2-flβCDH_2 was investigated. Blank experiments (reduction of HCHO by $RflH_2$ and 2-flβCDH_2) were done using the same procedure with 0.750 cm^3 of MeOH instead of 0.750 cm^3 of terephthalaldehyde solution.

The same procedure was utilized for the reduction of benzaldehyde, *p*-chlorobenzaldehyde, phenylacetaldehyde, 2,4,6-trimethoxybenzaldehyde, cinnamaldehyde, *m*- and *p*-cyanobenzaldehyde and *p*-chloroacetophenone. The concentrations of flavins and EDTA were 5.00×10^{-5} and 0.001 mol dm^{-3}, respectively.

Oxidation of Thiols by Flavins.—In a typical run, a stirring bar, the stock solution of flavin (0.420 cm^3; 3.00×10^{-4} mol dm^{-3} in distilled water) and the stock solution of sodium hydrogen carbonate buffer (1.830 cm^3; 0.25 mol dm^{-3}; pH 10.0; μ = 0.33 mol dm^{-3}) were added to each of the five UV cuvettes containing methanol (0.220, 0.200, 0.150, 0.120 and 0.000 cm^3). The cuvettes were sealed with rubber septa and argon was allowed to pass through the mixtures for 45 min to deoxygenate the system. The mixtures in the cuvettes were allowed to equilibrate with stirring in a cell holder for 15 min at 25.0 ± 0.1 °C. A round bottomed flask (10 cm^3) sealed with a rubber septum containing methanolic phenylmethanethiol solution (4.00×10^{-2} mol dm^{-3}) was deoxygenated by bubbling with argon and then 0.030, 0.050, 0.100, 0.130 and 0.250 cm^3 of this solution were transferred by syringe into each of these five cuvettes separately. The decrease in absorption due to flavins at 440 nm was recorded immediately. The initial concentration of flavins and the calculated ionic strength of reaction mixture were 5.00×10^{-5} and 0.24 mol dm^{-3}, respectively. The initial concentrations of phenylmethanethiol were 0.480, 0.800, 1.60, 2.10 and 4.00 mmol dm^{-3}. For Rfl and 2-flβCD (**2**), reactions with phenylmethanethiol (4.00 mmol dm^{-3}) (Fig. 5) were monitored for more than seven half-lives and were found to fit first order kinetics.

Oxidation of *o*-, *m*- and *p*-chlorophenylmethane-, phenylethane-, cyclohexane- and napthalene-1-thiols and benzenethiol were carried out in a similar manner. The stock solutions of flavin, buffer, thiols and methanol were changed so that the final volume, the initial concentration of flavin and the ionic strength remained the same as this run, but the concentration of the substrate varied.

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References

1 H. Ye, D. Rong, W. Tong and V. T. D'Souza, *J. Chem. Soc., Perkin Trans. 2*, 1992, 2071.
2 T. C. Bruice, *Prog. Bioorg. Chem.*, ed., E. T. Kaiser and F. J. Kezdy, Wiley-Interscience, New York, 1976, p. 1.
3 G. A. Hamilton, *Prog. Bioorg. Chem.*, 1971, **1**, 83.
4 T. C. Bruice, L. Main, S. Smith and P. Y. Bruice, *J. Am. Chem. Soc.*, 1971, **93**, 7327.
5 C. Kemal and T. C. Bruice, *J. Am. Chem. Soc.*, 1976, **98**, 3955.
6 M. T. Stankovich, in *Chemistry and Biochemistry of Flavoenzymes*, ed., F. Müller, CRC Press, Boston, 1991, vol. 1, p. 402.
7 R. F. Williams, S. S. Shinkai and T. C. Bruice, *J. Am. Chem. Soc.*, 1977, **99**, 921.
8 R. F. Williams and T. C. Bruice, *J. Am. Chem. Soc.*, 1976, **98**, 7752.
9 T. C. Bruice and Y. Yano, *J. Am. Chem. Soc.*, 1975, **97**, 5263.
10 C. H. Williams, B. D. Burleigh, Jr., S. Ronchi, L. D. Arscott and E. T. Jones, in *Flavins and Flavoproteins*, ed., H. Kamin, University Park Press, Baltimore, 1971, p. 295.
11 V. Massey and C. H. Williams, Jr., *J. Biol. Chem.*, 1965, **240**, 4470.
12 G. Zanetti and C. H. Williams, Jr., *J. Biol. Chem.*, 1967, **242**, 5232.
13 S. Ronchi and C. H. Williams, Jr., *J. Biol. Chem.*, 1972, **247**, 2083.
14 I. Yokoe and T. C. Bruice, *J. Am. Chem. Soc.*, 1975, **97**, 450.
15 E. L. Loechler and T. C. Hollocher, *J. Am. Chem. Soc.*, 1975, **97**, 3235.
16 I. M. Gascoigne and G. K. Radda, *Biochim. Biophys. Acta*, 1967, **131**, 498.
17 E. L. Loechler and T. C. Hollocher, *J. Am. Chem. Soc.*, 1980, **102**, 7312, 7322, 7328.
18 D. O. Carr and D. E. Metzler, *Biochim. Biophys. Acta* 1970, **205**, 63.
19 T. C. Bruice and J. P. Taulane, *J. Am. Chem. Soc.*, 1976, **98**, 7769.
20 R. C. Kerber, G. W. Urry and N. Kornblum, *J. Am. Chem. Soc.*, 1965, **87**, 4520.
21 G. A. Russell and W. C. Dannen, *J. Am. Chem. Soc.*, 1966, **88**, 5663.
22 J. Ciabattoni and G. A. Berchtold, *J. Org. Chem.*, 1966, **31**, 1336.
23 P. S. Rao and E. Hayon, *J. Am. Chem. Soc.*, 1974, **96**, 1287.
24 D. Rong, H. Ye, T. R. Boehlow and V. T. D'Souza, *J. Org. Chem.*, 1992, **57**, 163.
25 L. Main, G. J. Kasperek and T. C. Bruice, *Biochemistry*, 1972, **11**, 3991.
26 R. F. Williams and T. C. Bruice, *J. Am. Chem. Soc.*, 1976, **98**, 7752.
27 R. Traber, H. E. A. Kramer and P. Hemmerich, *Biochemistry*, 1982, **21**, 1687.
28 T. Weida, H. Ye, D. Rong and V. T. D'Souza, *J. Comput. Chem.*, 1992, **13**, 614.
29 K. H. Harata and H. Uedaira, *Bull. Chem. Soc. Jpn.*, 1975, **48**, 375.
30 J. G. Lombardino and E. H. Wiseman, *J. Med. Chem.*, 1970, **13**, 206.
31 G. G. Urquhart, J. W. Gates, Jr. and R. Connor, *Organic Syntheses*, Coll. Vol. III, ed. E. C. Horning, Wiley, New York, 1955, p. 363.
32 L. Meitis and T. Meitis, *Scientific Commun.*, 1948, **20**, 985.
33 R. G. Bates and V. E. Bower, *Anal. Chem.*, 1956, **28**, 1322.

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