Artificial Redox Enzymes. Part 3.[1] Structure and Properties

Hongping Ye, Ding Rong, Weida Tong and Valerian T. D'Souza*
Department of Chemistry, University of Missouri-St. Louis, St. Louis MO 63121, USA

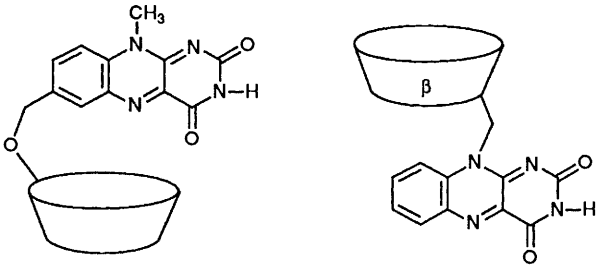
The structural and catalytic properties of models of flavoenzymes in which flavin is covalently attached to the catalytically important secondary side of 2-[(7α-*O*-10-methyl-7-isoalloxazinyl)-methyl]-α-cyclodextrin and 2-[(7α-*O*-10-methyl-7-isoalloxazinyl)methyl]-β-cyclodextrin as well as the primary side 6-(10-*N*-isoalloxazinylmethyl)-β-cyclodextrin are reported.

Although enzymes are difficult to surpass for efficiency, specificity and economy as catalysts, their complexity makes them difficult to understand and their instability restricts their utility. Artificial enzymes, which synthetically mimic the binding site and the catalytic site of enzymes, can be used to elucidate the mechanism of action of the real enzyme and to catalyse reactions under more stringent reaction conditions. We have recently reported the synthesis of artificial redox enzymes to mimic flavoenzymes which utilize cyclodextrins as binding sites and flavins as catalytic sites.[2]

Flavoenzymes consisting of a riboflavin molecule (coenzyme) bound non-covalently to a substrate binding site (apoenzyme) are important classes of enzymes which catalyse a variety of reactions including oxidation, hydroxylation, dehydrogenation, *etc.*[3] Flavopapain,[4] antibody–flavin complex,[5] flavocrown ether,[6] flavinophane[7] and flavocyclodextrin [6-(8α-*S*-riboflavo)-α-cyclodextrin)][8] are some of the other important mimics of this enzyme that have been reported in the literature. Among them, the flavocyclodextrin showed the best promise as a chemically useful model of flavoenzymes because of the versatility of its binding site (cyclodextrin)[9] and its efficiency in electron transfer and redox reactions. The limitation of this study was that only electron-transfer reactions of one such flavocyclodextrin where flavin was attached to the primary side of α-cyclodextrin were investigated.† Studies have shown that the substrates generally bind to cyclodextrins with their functional groups oriented towards its secondary side.[10] A thorough evaluation of flavocyclodextrins, especially those having flavins attached to the secondary side of cyclodextrin, would lead to a better understanding of real enzymes. We now report some of the structural and catalytic properties of models of a flavoenzyme in which flavin is covalently attached to the catalytically important secondary side of cyclodextrin 2-[(7α-*O*-10-methyl-7-isoalloxazinyl)methyl]-α-cyclodextrin (**1**) and 2-[(7α-*O*-10-methyl-7-isoalloxazinyl)methyl]-β-cyclodextrin (**2**) as well as the primary side 6-(10-*N*-isoalloxazinylmethyl)-β-cyclodextrin (**3**) (see Fig. 1).

Results and Discussion

Properties of Flavocyclodextrins.—Spectral properties. The absorption spectra of **1**–**3** have four absorption maxima with high molar extinction coefficients‡[11] indicative of $\pi \longrightarrow \pi^*$ type transitions. Flavocyclodextrins exhibit the normal bright yellow fluorescence‡[11] emission of flavins ($\lambda_{max} \sim 520$ nm) in aqueous solution.



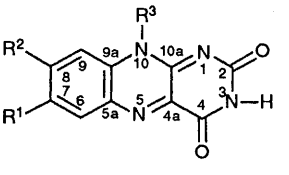
1 = α
2 = β

3

Fig. 1 Schematic drawing of artificial redox enzymes with flavin attached to (*a*) the secondary side of α- and β-cyclodextrin **1** and **2**, respectively; (*b*) the primary side of β-cyclodextrin **3**

Conformational studies of 2-flavocyclodextrins **1** *and* **2**. The conformation of the catalytic functional group covalently attached to cyclodextrins influences both the binding and the catalytic aspects of their activity. Flavocyclodextrins **1** and **2** can be envisioned to have three conformations where flavin is: (1) inside the cavity of cyclodextrin; (2) outside and above the cavity of cyclodextrin; and (3) outside and pointing away from the cavity of cyclodextrin. The computational chemistry study of **1** and **2** indicates that the plane of the flavin moiety is perpendicular to the vertical axis of cyclodextrin.[1] The flavin moiety caps the secondary side of cyclodextrin by forming hydrogen bonds between the oxygen atom of the carbonyl groups of flavin and the hydrogen atoms of the secondary hydroxy groups of cyclodextrin. This conformation may have significant consequences in the reactivity and stability of these artificial redox enzymes.

These computational studies are further supported by the following experimental observations. The cavity of cyclodextrin is known to be non-polar.[9] If the catalytic functional group of the artificial enzyme was inside the hydrophobic cavity of cyclodextrin, its UV peak around 445 nm should be broad. However, UV spectra of artificial enzymes **1**–**3** show sharp peaks similar to the peak of riboflavin **4** and 7,10-dimethylflavin **5** in water indicating that the flavin moiety is not in the



4 $R^1 = R^2 = CH_3$, R^3 = ribityl
5 $R^1 = R^3 = CH_3$, $R^2 = H$
6 $R^1 = R^2 = R^3 = CH_3$

† Professor Tabushi passed away on March 22nd, 1987 and this work has been discontinued.

‡ Absorption spectra, fluorescence spectra and extinction coefficients for the flavins have been deposited as supplementary material [Sub. Pub. no. 56892 (4 pp)]. For details of the supplementary data deposition scheme, see 'Instructions for Authors,' *J. Chem. Soc., Perkin Trans. 2*, 1992, issue 1.

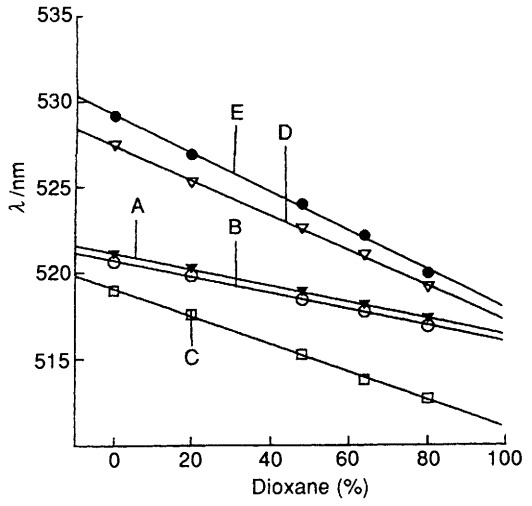


Fig. 2 Dependence of emission maxima on solvent polarity. A, **1**; B, **2**; C, **3**; D, **4** and E, **5**. [flavin] = 5.00 mol dm^{-3} at 25.0 °C in NaOH–KH_2PO_4 buffer (pH 7.0, μ = 0.08 mol dm^{-3}) and dioxane, excitation wavelength 450 nm (2 nm bandwidth).

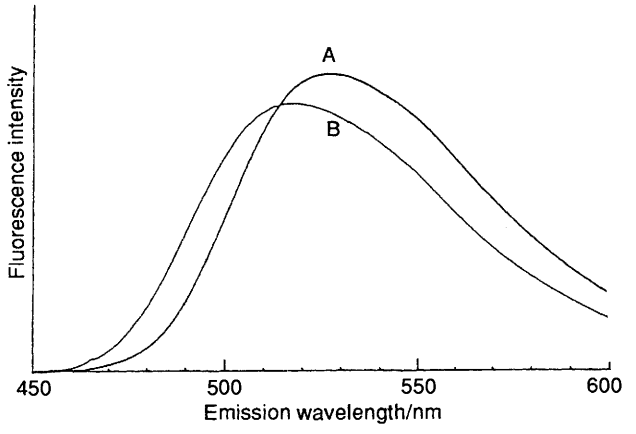


Fig. 3 Fluorescence quenching of 2-flavo-β-cyclodextrin. A, **5**; B, **2**; [flavin] = 5 × 10^{-5} mol dm^{-3}, μ = 0.08 mol dm^{-3} in pH 7.0 buffer at 25 °C and excitation wavelength 476 nm

Table 1 Rate constants for the reduction of flavins by potassium sulfite[a]

Flavins	Rate constant k_2/dm^3 mol^{-1} s^{-1}
6	0.0107[b]
4	0.016
1	0.024
2	0.026

[a] At 25.0 °C in pH 7.0 buffer, μ = 0.1 mol dm^{-3}. [b] Ref. 18, pH 7.21.

hydrophobic environment of the cavity of cyclodextrin. The blue shift in both UV and fluorescence spectra of flavins **1** and **2** compared with those of **4** and **5** can be ascribed to the interaction between the flavin moiety and the hydroxy groups of cyclodextrin. This kind of interaction may bend the isoalloxazine ring, decrease the resonance to some degree and increase the excitation energy.

Fig. 2 shows the variation in emission maxima with the change in the solvent polarity for each of the flavocyclodextrins. Polar fluorophores like flavins are sensitive to solvent polarity because the interaction between the excited fluorophore during its lifetime and surrounding polar groups lowers the energy of the excited state, which shifts the emission to longer wavelengths.[12] A shift in the emission maxima with the solvent polarity for all the flavocyclodextrins is consistent with the assertion that the flavin moiety is outside the cavity. If the flavin moiety were inside the cavity of cyclodextrin, the emission maxima of the artificial enzyme would not change when the polarity of the solvents is changed.[13] However, the degree of solvent effect is less for flavins **1** and **2** than for **4** and **5** indicating that the flavin moiety on cyclodextrin is not free. This supports the observation from the computational study that the flavin moiety of **1** and **2** is hydrogen bonded to cyclodextrin. The hydrogen bonded flavin moiety would interact less with the polar solvent and thus show a *decreased effect* of the solvent polarity.

Interactions between the cyclodextrin and the flavin moiety in 2-flavocyclodextrins **1** *and* **2**. Interactions between the cyclodextrin and the flavin moieties in 2-flavocyclodextrins **1** and **2** were experimentally investigated using fluorescence quenching experiments. Fig. 3 shows the fluorescence spectra of flavins **2** and **5** with the excitation wavelength 476 nm. At this wavelength, the intensities of their absorption spectra are the same. An identical fluorescence with a quantum yield of 0.26 has been reported[14] for several flavins in dilute aqueous solution at pH 7.0. The same value for quantum yield of fluorescence is obtained irrespective of the excitation wavelength[15] (260–500 nm). The intensity of the fluorescence spectrum of **2** is expected to be the same as **5** if there are no interactions between the flavin moiety and the cyclodextrin ring in **2**. However, fluorescence quenching observed for **2** (Fig. 3) indicates the presence of interactions between the flavin moiety and the cyclodextrin in this molecule. Fluorescence quenching for artificial enzymes **1** and **3** was also observed. Similar fluorescence quenching experiments have been used to show the interactions in enzyme-bound flavin.[16] Specific interactions *via* hydrogen bonding between hydrogen atoms of the hydroxy groups at the 2-position of cyclodextrins and the oxygen atom of the carbonyl groups of flavin have been suggested by the results of the computational study of flavocyclodextrins.[1]

Flavoenzymes are classified into two groups based on the involvement of the atoms C(4a) or N(5) in their mechanism of action[17] stemming from regiospecific hydrogen bonding between flavin coenzyme and apoproteins. Shinkai[18] has shown that these two groups can be distinguished by their ability to catalyse the oxidation of sulfite anion.* Intramolecular hydrogen bonding interactions between the acidic[9] hydrogen atoms of the secondary hydroxy groups of cyclodextrin (pK_a = 12.1) and N(5) of flavin in artificial enzymes **1** and **2** were examined by the method prescribed by Shinkai.[18] The rates of oxidation of sulfite anion by either **1** or **2** (Table 1) are the same as those by **4** and 7,8,10-trimethylflavin (**6**) indicating that such hydrogen bonds are unlikely. The absence of hydrogen bonds between the hydrogen atom of the secondary hydroxy group with either N(1) or N(5) atoms of the flavin moiety is also suggested by the computational studies.[1]

Reversibility of redox reactions of flavocyclodextrins. Reduced flavins can be oxidized by air with $t_{\frac{1}{2}} < 1$ s which allows turnover of these catalysts. The turnover of the flavin moiety of artificial enzymes **1–3** in redox reactions was tested by photoreduction of **1–3** with EDTA under anaerobic conditions followed by re-oxidation by air. Deoxygenated solutions of flavocyclodextrins containing EDTA were reduced using a 100 W lamp for 10 min. The solutions became colourless after this time and the UV spectra were recorded. When these solutions were exposed to air, the original spectra of flavocyclodextrins reappeared within minutes indicating that flavocyclodextrins are fully reversible in redox reactions.

* The C(4a) group oxidizes sulfite anion 10^3 times faster than the N(5) group.

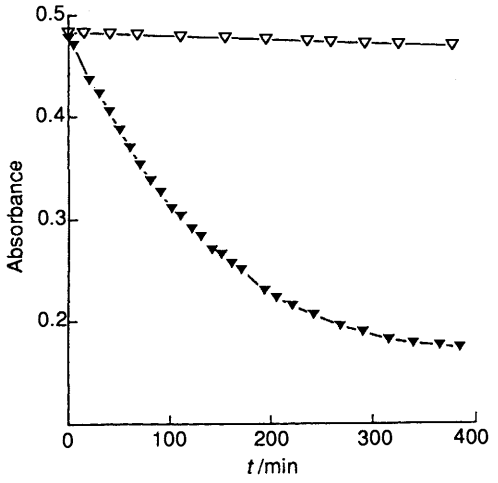


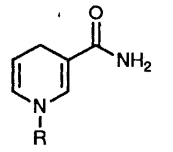
Fig. 4 Decomposition of ▽ flavocyclodextrin and ▼ riboflavin under photochemical conditions

Stability of 2-flavocyclodextrins under photochemical conditions. Flavin derivatives decompose when exposed to light. The stability of flavocyclodextrin **2** under photochemical conditions was compared with that of riboflavin in pH 7.0 buffer at room temperature. Flavins **2** and **4** were exposed to light from a slide projector with a CBA lamp. The light beam was filtered through combined $CuSO_4$–$NaNO_2$ solutions so that it transmitted light[19] at $360 < \lambda < 440$ nm. As shown in Fig. 4, flavin **2** is more stable than **4**. After irradiation for 6 h, *ca.* 85% of **4** but only 4.5% of **2** were decomposed. This property provides an advantage for the artificial enzymes over riboflavin during photochemical reactions.

Reactions of 2-Flavocyclodextrins.—In all our experiments the concentrations of dihydronicotinamides were maintained in the range 1×10^{-2}–5×10^{-4} mol dm^{-3} to circumvent[20] the formation of pre-equilibrium charge-transfer complexes[21,22] between flavins and dihydronicotinamide. In order to avoid the difficulty of maintaining anaerobic conditions, all the reactions were carried out under aerobic (turnover) conditions and monitored at 380–400 nm corresponding to the absorbance of nicotinamide. Although flavin has some absorption in this range, it does not affect the observed rate constant. The absorption of flavocyclodextrins at 440 nm due to the flavin moiety does not change during a reaction because the re-oxidation of reduced flavin by O_2 is much faster[3] than oxidation of nicotinamides and the concentration of flavin remains constant.[23,24] The second-order rate constants for the oxidation of dihydronicotinamide by flavin derivatives have been shown to be almost constant at pH < 10 and are not affected by substituting the proton at the 3-position by a methyl group.[18] In the kinetic analysis of oxidation of dihydronicotinamides by artificial flavoenzyme systems, the ionization state of –NH– at the 3-position ($pK_a = 10$) is generally not differentiated[4,8] and this practice has been followed in this study.

Oxidation reactions of 2-flavocyclodextrins that follow second-order kinetics. Substrates **7**, 1-(*n*-hexyl)-1,4-dihydronicotinamide (**8**) and reduced nicotinamide adenine dinucleotide (NADH) were oxidized by the artificial enzyme **2**. Plots of initial velocities (v) *vs.* the concentrations of these substrates give straight lines and their double reciprocal plots give a zero Y intercept indicating that these reactions are pseudo-first-order reactions. Table 2 gives the second-order rate constants calculated from the first-order rate constants for these reactions.

The pseudo-first-order kinetics observed for the oxidation of substrates **7** and **8** by 2-flavocyclodextrins **1** and **2** rather than



7 R = C_6H_5-CH_2-
8 R = $CH_3[CH_2]_4$-CH_2-
9 R = $C_{10}H_7$-CH_2-

the saturation kinetics followed by real enzymes has two possible explanations. Firstly, according to Tabushi, the dissociation constant[8] for the complex of 6-(8α-*S*-riboflavo)-α-cyclodextrin with **7** is 9.5×10^4 mol dm^{-3}. The concentrations used in these experiments are in the optimum range if the binding constants of the complexes of **7** with 6-(8α-*S*-riboflavo)-α-cyclodextrin and flavocyclodextrin (**1**) are assumed to be similar. In this case, **1** oxidizes the free **7** while the bound substrate remains unreacted. It is important to note that the difference between 6-(8α-*S*-riboflavo)-α-cyclodextrin and 2-flavocyclodextrins is the position and the orientation of the flavin moiety with respect to the binding site, cyclodextrin. The absence of enzyme type reaction (saturation) kinetics for 2-flavocyclodextrins with these substrates can be rationalized by suggesting that the dihydronicotinamide part of the bound substrate is not oriented towards the flavin moiety of the artificial enzyme. Secondly, to obtain reliable values of K_m and k_{cat}, the concentration of the substrate should be in the range[25] 0.3–2 K_{diss}. The reported K_{diss} of the complex between the phenyl ring and α-cyclodextrin[10] is 2×10^{-2} mol dm^{-3}. Assuming the K_{diss} of substrate **7** to be the same, the concentrations of **7** required to obtain interpretable results should be between 6×10^{-3} and 4×10^{-2} mol dm^{-3}. The solubility of **7** dictates that the concentrations of substrate used in these experiments be in the range 3×10^{-4}–1×10^{-3} mol dm^{-3}. Thus, only the initial portion of saturation kinetics, which is linear,[25] could be experimentally observed and substrate saturation could not be reached.

The results that pseudo-first-order kinetics rather than saturation kinetics were observed for the oxidation of NADH by **1** and **2** are consistent with the fact that the substituent on nicotinamide of NADH is too bulky to sit inside the cavity of β-cyclodextrin. The difference in second-order rate constants between riboflavin and 2-flavocyclodextrins **1** and **2** in the oxidation of each of the substrates can be attributed to the difference in their structures. The rate constants presented in Table 2 suggest that the redox potentials of flavins **1** and **2** are similar[26,23] and are higher than **3**–**5**.

Oxidation reactions of 2-flavocyclodextrins that follow saturation kinetics. The oxidation of 1-(1-naphthyl)methyldihydronicotinamide (**9**) by the artificial enzyme **2** was carried out under anaerobic conditions and monitored by observing the decrease of absorption of flavin at 440 nm. The decrease in the absorption due to flavin followed first-order kinetics. In contrast to the oxidation of substrates **7**, **8** and NADH by **2**, the first-order rate constants (k_1) for this reaction followed saturation kinetics with increasing concentration of **9** and gave an excellent fit to Michaelis–Menton kinetics indicating a pre-equilibrium binding before the reaction. K_{diss} and k_{cat} for this data calculated by Michaelis–Menton kinetics using ENZFITTER (non-linear regression) are given in Table 3. These results are in contrast with the results obtained in the oxidation of **9** with flavins **1** and **4**. These flavins follow second-order kinetics and do not exhibit saturation kinetics. The second-order rate constants for oxidation of **9** by **1** and **4** are also reported in Table 3.

The rate constants for the oxidation of substrate **9** catalysed by flavocyclodextrin **1** is higher than riboflavin **4** indicating that their redox potentials and chemical reactivities are not similar.

Table 2 The second-order rate constants for oxidation of dihydronicotinamides by flavins[a]

Flavins/substrates	NADM[b]/dm^3 mol^{-1} s^{-1}	**7**[c]/dm^3 mol^{-1} s^{-1}	**8**[c]/10^{-1} dm^3 mol^{-1} s^{-1}
1	4.2 ± 0.0	45 ± 1	24 ± 0
2	5.8 ± 0.1	56 ± 1	25 ± 1
3	1.0 ± 0.0	22 ± 0	14 ± 0
4	0.69 ± 0.02	22 ± 1	12 ± 0
5	1.7 ± 0.0	29 ± 0	17 ± 0

[a] k_2 at 25.0 °C in pH 7.0 buffer (μ = 0.1 mol dm^{-3}). [b] NaOH–KH_2PO_4 buffer. [c] NaOH–KH_2PO_4 buffer containing 10% methanol.

Table 3 Rate constants for the oxidation of dihydronicotinamide[a] **9** by flavins **1**, **2** and **4**

Flavins	K_{diss}[b]/10^{-3} mol dm^{-3}	k_{cat}[b]/10^{-2} s^{-1}	k_2/dm^3 mol^{-1} s^{-1}	k_{cat}/K_{diss}/dm^3 mol^{-1} s^{-1}	$(k_{cat}/K_{diss})/k_2$[c]
2	3.7 ± 0.4	2.8 ± 0.2		7.7	2.4
1			3.3 ± 0.1		
4			1.3 ± 0.0		

[a] At 25 + 0.1 °C in pH 7.0, 50% aqueous methanol, μ = 0.4 mol dm^{-3}. [b] Calculated by Michaelis–Menton equation using non-linear regression. [c] k_2 is the second-order rate constants for reaction with **1**.

Therefore, acceleration of a redox reaction of a substrate by a flavocyclodextrin in comparison to riboflavin cannot indicate the presence of an enzyme type reaction scheme. However, the presence of an enzyme type reaction scheme may be suggested by an acceleration of a redox reaction of a substrate by **2** in comparison with **1**. The ratio of two second-order rate constants obtained for the oxidation of **9** by **2** in saturation kinetics (k_{cat}/K_{diss}) and by **1** in second-order kinetics (k_2) can be used to discuss the efficiency of the artificial enzyme system. The 2.4-fold acceleration gained by the artificial enzyme **2** compared to flavin **1** (which has the same redox potential but does not bind **9**) for the oxidation of **9** can be attributed to the binding which brings the substrate into proximity with the flavin.

Conclusions

These investigations support the structure of the flavocyclodextrin that was obtained in the computational chemistry study.[1] These artificial enzymes can catalyse reactions for only those substrates that bind to the cyclodextrin cavity with the correct orientation. Thus, although substrates **7** and **8** are known to bind to the cyclodextrin cavity[7] their oxidation is not catalysed by these artificial redox enzymes and second-order kinetics are observed. Substrate **9** does not bind to the flavocyclodextrin **1** and likewise follows second-order kinetics. Substrates **9** binds to the artificial enzyme **2** with the correct spatial orientation and its oxidation is catalysed by the artificial enzyme as indicated by the saturation kinetics. It is important to emphasize that the 2.4-fold acceleration observed in this reaction is insignificant compared to the rate accelerations obtained in enzyme-catalysed reactions.

Experimental

Materials.—Flavins **1**, **2**, **3** and **5** were synthesized and purified in our laboratory,[2] and stored as solids at −20 °C. NADH (95%) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Riboflavin (98%), nicotinamide, 1-bromohexane and 1-bromomethylnaphthalene were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Buffers were made up with glass distilled water employing reagent grade salts and base. 1-Benzyl-1,4-dihydronicotinamide[27] (**7**) and 1-(*n*-hexyl)-1,4-dihydronicotinamide[28] (**8**) were synthesized according to reported procedures and had identical physical properties. Coupling constant values *J* are given in Hz.

1-(1-*Naphthyl*)*methyl*-1,4-*dihydronicotinamide* (**9**). Nicotinamide (3.0 g, 25 mmol) was added to a solution of 1-bromomethylnaphthalene (5.4 g, 24 mmol; 98%) in ethanol (30 cm^3) and refluxed for 24 h. The reaction mixture was kept in a refrigerator for 3 days at 4 °C, filtered, washed with ethyl alcohol and dried under reduced pressure at room temperature to provide 6.5 g of crude product, presumably the hydrobromide salt of 1-(1-naphthyl)methylnicotinamide, as a white solid, δ_H(300 MHz; [$^2H_6$]DMSO) 9.60 (1 H, s), 9.20 (1 H, d, *J* 6.1), 9.00 (1 H, d, *J* 6.1), 8.64 (1 H, s), 8.03–8.30 (4 H, m), 7.66–7.55 (3 H, m) and 6.49 (2 H, s, *N*-CH_2).

To a solution of the crude compound obtained above (2.0 g) in water (30 cm^3) was added K_2CO_3 (1.38 g, 9.99 mmol) and $Na_2S_2O_3$ (2.4 g, 15.2 mmol) and the mixture was stirred for 30 min at room temperature to give an orange, oily compound. The reaction mixture was then kept in a freezer at −20 °C overnight. The orange compound, which separated out when the ice melted, was washed with ice–water to provide 1.0 g of crude **9**. The crude product was then recrystallized from ethanol–water (15:25) to provide 1.0 g (50% total yield from 1-bromomethylnaphthalene) of **9** as a light yellow crystal: δ_H(300 MHz; [$^2H_6$]DMSO) 8.04–7.88 (3 H, m), 7.63–7.45 (4 H, m), 7.06 (1 H, s), 6.01 (1 H, s), 6.02 (1 H, d, *J* 5.4), 4.81 (2 H, s), 4.66 (2 H, hept), 3.38 (2 H, s) and 3.00 (1 H, s); δ_C(300 MHz; [$^2H_6$]DMSO) 169.09, 137.96, 133.89, 133.40, 130.67, 129.86, 128.64, 127.96, 126.43, 126.03, 125.52, 125.30, 123.28, 101.77, 101.43, 53.67 and 22.46.

Spectral Measurements.—Absorption spectra of flavins were obtained at 25 ± 0.1 °C on a Cary 2200 spectrophotometer (Varian Instrument Co., CA) equipped with a thermostatted cell holder connected to a water circulator. Fluorescence spectra were acquired on a SLM 4800C spectrofluorometer (SLM Instruments, Inc., Urbana, IL) with a thermostatted cell holder maintained at 25.0 ± 0.1 °C using a water circulator. The pH of all the buffers were verified using Accumet pH meter 925 (Fisher Scientific) and the ionic strength was adjusted by the addition of KCl. Stock solutions of flavins **1**–**4** (3.00 × 10^{-4} mol dm^{-3}) were prepared by dissolving the required amount of flavins in distilled water. The stock solutions of flavin **5** were made by dissolving the required amount of **5** in distilled water and dioxane separately as required. All the flavin solutions were stored in a refrigerator at 4 °C.

Flavin solutions (5.00 × 10^{-5} mol dm^{-3}) in 0%, 20%, 48%, 64% and 80% of dioxane for the acquisitions of absorption and fluorescence spectra were prepared by placing flavin stock

solution (0.42 cm^3) in five cuvettes and then adding the required amount of phosphate buffer (pH 7, μ = 0.08 mol dm^{-3}) and dioxane to make the final volume up to 2.50 cm^3.

Reversibility Test of Flavocyclodextrin.—A stock solution of **1** (0.420 cm^3; 3.00 × 10^5 mol dm^{-3}) was mixed with NaOH–KH_2PO_4 buffer (2.08 cm^3) (pH 7.0, μ = 0.1 mol dm^{-3}, containing 0.001 mol dm^{-3} of EDTA) in a UV cuvette sealed with a rubber septum. An absorption spectrum was recorded for this solution. Deoxygenation of the solution was accomplished by passing vanadous-ion scrubbed argon through the solution for 45 min.[29,30] Photoreduction of the solution of **1** to the 1,4-dihydro form was achieved by using a 100 W lamp placed at a distance of 10 cm from the cuvette. An absorption spectra of this reduced sample was recorded. The solution was then exposed to air for 5 min and another absorption spectrum was recorded. All the spectra were taken at 25.0 ± 0.1 °C.

The Stability Test of Flavins under Photochemical Conditions.—The sample solution was made by placing flavocyclodextrin **2** stock solution (0.430 cm^3; 3.00 × 10^{-4} mol dm^{-3}) in a glass cuvette. A stirring bar and NaOH–KH_2PO_4 buffer (2.08 cm^3) (pH 7.0, μ = 0.12 mol dm^{-3}) were added to this cuvette to make the concentration of **2** and the ionic strength of the solution up to 5.00 × 10^{-5} mol dm^{-3} and 0.1 mol dm^{-3}, respectively. The cuvette containing the sample solution was kept in the cell holder circulated with 25.0 °C water and was stirred during the photolysis.

A slide projector equipped with a CBA lamp placed at a distance of 40 cm from the sample was used as the light source. The light beam was made to pass through two filter solutions placed between the lamp and the sample. The first filter solution consisted of 17.6 g $CuSO_45H_2O$ and 1.303 dm^3 of 29% NH_4OH diluted to 4 dm^3 with distilled water. The second filter solution consisted of 300 g of $NaNO_2$ in 4 dm^3 of distilled water. These two solutions were placed in two 10 cm wide glass jars in tandem. The decomposition of **2** was followed by monitoring the adsorption at 434 nm. The same procedure was used for photolysis of flavin **4**, but its decomposition was monitored observing the decrease in absorption at 445 nm.

Kinetic Measurements.—General methods. Kinetic measurements for the oxidation of dihydronicotinamides by flavins under turnover conditions of flavins at 25 °C were monitored by observing the decrease of the absorption of dihydronicotinamides on a Cary 2200 Spectrophotometer. The reaction mixtures were gently bubbled with oxygen and were stirred to keep the molecular oxygen in excess for aerobic reactions. The data were collected electronically and the rate constants were calculated by using Lab Calc (Galactic Industries Corp.). The initial rates (v) were determined from the change in the absorption within completion of 2–5% of the reaction and were used to calculate first-order rate constants. Michaelis–Menton rate constants were calculated from initial rates using ENZFITTER.[31] The ionic strength of the phosphate buffer (pH 7) was adjusted to 0.2 mol dm^{-3} by addition of KCl. The stock solutions of substrates **7**–**9** in methanol were prepared daily and stored in a freezer at *ca.* −20 °C. The stock solution of NADH in distilled water was prepared daily and stored in a refrigerator at 4 °C. The stock solution of Na_2SO_3 was prepared by dissolving 1.2861 g of Na_2SO_3 in 10.00 cm^3 of distilled water. The concentration of SO_3^{2-} was calculated to be 1.00 mol dm^{-3}. The reference solutions used for the UV measurements for all the reactions were the same solvents as used for the reactions.

Reactions carried out under anaerobic conditions were monitored by observing the decrease in the absorption of flavin at 440 nm on a Cary 2200 spectrophotometer. Deoxygenation of the reaction mixtures was accomplished by passing vanadous-ion scrubbed argon through the reactants separately for 45 min.[29,30] UV cuvettes were sealed with rubber septums for anaerobic reactions.

Reaction of Na_2SO_3 *with flavocyclodextrin* **2**. Na_2SO_3 stock solutions (0.200, 0.500 and 0.800 cm^3 of 1.00 mol dm^{-3}) were placed separately in three UV cuvettes and diluted to 0.830 cm^3 with distilled water. To each cuvette containing the diluted Na_2SO_3 solution and a stirring bar, was added the stock solution of NaOH–KH_2PO_4 buffer (1.250 cm^3) (pH 7.0, μ = 0.2 mol dm^{-3}). 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 stock solution of **2** (0.420 cm^3 3.00 × 10^{-4} mol dm^{-3}) was then added quickly to the cuvette and the reaction was monitored immediately at 440 nm. The concentration of **2** in the reaction mixture was 5.00 × 10^{-5} mol dm^{-3}. The initial concentrations of Na_2SO_3 were 0.0800, 0.200 and 0.320 mol dm^{-3}. The reactions were followed for more than seven half-lives and were found to fit first-order kinetics.

Reaction of NADH with flavins. NADH stock solutions (0.350, 0.500, 0.700, 0.850 and 1.000 cm^3 of 8.00 × 10^{-3} mol dm^{-3} in water) were placed separately in five curvettes and diluted to 1.090 cm^3 with distilled water. To each cuvette containing the diluted NADH solution and a stirring bar was added stock solution of NaOH–KH_2PO_4 buffer (1.200 cm^3; pH 7.0, μ = 0.2 mol dm^{-3}). The mixtures were allowed to equilibrate with stirring in a cell holder for 15 min at 25.0 ± 0.1 °C. Stock solution of flavin (0.210 cm^3; 3.00 × 10^{-4} mol dm^{-3}) was delivered quickly to the open, stirring cuvettes. The oxidation of NADH was monitored immediately at 380 nm. The calculated ionic strength (μ) of the reaction mixture was 0.1 mol dm^{-3}. The concentration of flavin was 2.50 × 10^{-5} mol dm^{-3}. The initial concentrations of NADH were 1.12, 1.60, 2.24, 2.72 and 3.20 mmol dm^{-3}. The same procedure was used for the oxidation of NADH by flavins **1**–**5**.

Reaction of dihydronicotinamides **7** *and* **8** *with flavins.* Methanolic stock solution (0.050, 0.100, 0.150, 0.200 and 0.250 cm^3 of 2.00 × 10^{-2} mol dm^{-3}) of dihydronicotinamides **7** or **8** were placed separately in five UV cuvettes and diluted to 0.250 cm^3 with methanol. To each cuvette containing the diluted **7** or **8** solution and a stirring bar was added distilled water (0.840 cm^3) and stock solution of NaOH–KH_2PO_4 buffer (1.20 cm^3) (pH 7.0, μ = 0.2 mol dm^{-3}). The mixtures were allowed to equilibrate with stirring in a cell holder for 15 min at 25.0 ± 0.1 °C. Flavin stock solution (0.210 cm^3; 3.00 × 10^{-4} mol dm^{-3}) was added quickly to the cuvettes. The reaction was monitored (at 400 and 405 nm for **7** and **8**, respectively) immediately. The concentration of flavin was 2.50 × 10^{-5} mol dm^{-3}. The initial concentrations of the substrates were 0.400, 0.800, 1.20, 1.60 and 2.00 mol dm^{-3}. The calculated ionic strength was 0.1 mol dm^{-3}. One of the reactions was monitored for more than seven half-lives and was found to follow first-order kinetics. The same procedure was utilized for the oxidation of substrates **7** and **8** by flavins **1**–**5**.

Reaction of dihydronicotinamide (**9**) *with flavocyclodextrin* (**2**). Methanol aliquots (0.890, 0.800, 0.680, 0.530 and 0.000 cm^3) were placed separately in five UV cuvettes. A stirring bar, stock solution of **2** (0.210 cm^3; 3.00 × 10^{-4} mol dm^{-3}) and stock solution of NaOH–KH_2PO_4 buffer (1.040 cm^3) (pH 7.0, μ = 0.24 mol dm^{-3}) were added to each cell containing methanol. The cuvettes were sealed with rubber septums and argon was allowed to gently bubble through the solution 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 10 cm^3 round bottom flask, sealed with a rubber septum and containing a solution of **9** in methanol (8.00 × 10^{-3} mol dm^{-3}), was deoxygenated by bubbling argon through the solution. Portions of this solution (0.360, 0.450, 0.570, 0.720 and 1.250 cm^3) were transferred by syringe into

each of the five cuvettes containing flavin separately. The decrease in absorbance due to flavin at 440 nm was recorded immediately. The initial concentrations of **2** was 2.50×10^{-5} mol dm^{-3} and of **9** were 1.15, 1.44, 1.82, 2.30 and 4.00 mol dm^{-3}. The calculated ionic strength of reaction mixture was 0.10 mol dm^{-3}.

Reaction of dihydronicotinamide (**9**) *with flavins* **1** *and* **4**. Methanolic solutions of **9** (0.450, 0.550, 0.700, 0.900 and 1.250 cm^3); 5.50×10^{-3} mol dm^{-3}) were placed separately in five UV cuvettes and diluted to 1.25 cm^3 with methanol. To each cell containing the solution of **9** and a stirring bar was added stock solution of $NaOH–KH_2PO_4$ buffer (1.04 cm^3) (pH 7.0, μ = 0.24). The mixtures in the cuvettes were allowed to equilibrate with stirring in a cell holder for 15 min at 25.0 ± 0.1 °C. Flavin stock solution (0.210 cm^3; 3.00×10^{-4} mol dm^{-3}) was added quickly to the cuvette. The decrease in the absorption of **9** at 400 nm was recorded immediately. The concentration of flavin was 2.50×10^{-5} mol dm^{-3} and of **9**, 0.990, 1.21, 1.54, 1.98 and 2.75 mmol dm^{-3}. The calculated ionic strength was 0.10 mol dm^{-3}.

Acknowledgements

The authors gratefully acknowledge the financial support from Mallinckrodt Speciality Chemicals Company, the Missouri Research Assistance Act, the Petroleum Research Fund and the University of Missouri-St. Louis.

References

1 Part 2, T. Weida, H. Ye, D. Rong and V. T. D'Souza, *J. Comput. Chem.*, 1992, **13**, 614.
2 D. Rong, H. Ye, T. R. Boehlow and V. T. D'Souza, *J. Org. Chem.*, 1992, **57**, 163–167.
3 C. Walsh, *Enzymatic Reaction Mechanisms*, W. H. Freeman, San Francisco, 1979, ch. 10–12.
4 H. L. Levine and E. T. Kaiser, *J. Am. Chem. Soc.*, 1978, **100**, 7670.
5 P. G. Schultz, *Acc. Chem. Res.*, 1989, **22**, 287.
6 S. Shinkai, Y. Ishikawa, H. Shinkai, T. Tsuno, H. Makishima, K. Ueda and O. Manabe, *J. Am. Chem. Soc.*, 1984, **106**, 1801.
7 E. M. Seward, R. B. Hopkins, W. Sauerer, S.-W. Tam and F. Diederich, *J. Am. Chem. Soc.*, 1990, **112**, 1783.
8 I. Tabushi and M. Kodera, *J. Am. Chem. Soc.*, 1987, **109**, 4734.
9 M. L. Bender and M. Komiyama, *Cyclodextrin Chemistry*, Springer-Verlag, New York, 1978.
10 R. L. VanEtten, J. F. Sebastian, G. A. Clowes and M. L. Bender, *J. Am. Chem. Soc.*, 1967, **89**, 3242.
11 This paper, Supplementary material.
12 J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, 1983, p. 5.
13 A. J. Visser and F. Müller, *Helv. Chim. Acta*, 1979, **62**, 593.
14 G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, 1957, **53**, 646.
15 J. K. Eweg, F. Müller, A. J. W. G. Visser, C. Veeger, D. Bebelaar and D. W. van Voorst, *Photochem. Photobiol.*, 1979, **30**, 463.
16 M. A. Slifkin, *Charge Transfer Interactions of Biomolecules*, Academic Press, New York, 1971, p. 132.
17 V. Massey and P. Hemmerich, *Biochem. Soc. Trans.*, 1980, **8**, 246.
18 S. Shinkai, N. Honda, Y. Ishikawa and O. Manabe, *J. Am. Chem. Soc.*, 1985, **107**, 6286.
19 J. G. Calvert and J. N. Pitts, *Photochemistry*, John Wiley & Sons, New York, 1966, p. 737.
20 G. Blankenhorn, *Biochemistry*, 75, **14**, 3172.
21 D. J. Porter, G. Blankenhaorn and L. L. Ingram, *Biochem, Biophys. Res. Commun.*, 1973, **52**, 447.
22 T. C. Bruice, L. Main, S. Smith and P. Y. Bruice, *J. Am. Chem. Soc.*, 1971, **93**, 7328.
23 I. M. Gascoigne and G. K. Radda, *Biochim. Biophys. Acta*, 1967, **131**, 498.
24 C. H. Suelter and D. E. Metzler, *Biochem. Biophys. Acta*, 1960, **44**, 23.
25 I. H. Segel, *Enzyme Kinetics*, John Wiley & Sons, New York, 1975.
26 T. C. Bruice, T. W. Chan, J. P. Taulane, I. Yokoe, D. L. Elliott, R. F. Williams and M. Novak, *J. Am. Chem. Soc.*, 1977, **99**, 6713.
27 D. Mauzerall and F. H. Westheimer, *J. Am. Chem. Soc.*, 1955, **77**, 2261.
28 J. T. Slama, C. Radziejewski, S. Oruganti and E. T. Kaiser, *J. Am. Chem. Soc.*, 1984, **106**, 6778.
29 L. Meitis and T. Meites, *Scientific Commun.*, 1948, **20**, 985.
30 T. C. Bruice and J. P. Taulane, *J. Am. Chem. Soc.*, 1976, **98**, 7769.
31 R. J. Leatherbarrow, *A Non-linear Regression Data Analysis Program for the IBM PC*, Elsevier-Biosoft, UK, 1987.

Paper 2/01217K
Received 6*th March* 1992
Accepted 18*th June* 1992