β-Cyclodextrin–Nicotinamide as a Model for NADH Dependent Enzymes

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The newly synthesized β-cyclodextrin–nicotinamides (1) and (2) have a nicotinamide group on the secondary hydroxy-side of β-cyclodextrin, in an axial configuration for (1) and equatorial for (2). The corresponding reduced forms (3) and (4) can reduce ninhydrin with large rate enhancements (40- and 60-fold, respectively) relative to NADH. They also show enzyme-like saturation kinetics in the reduction of ninhydrin, which indicates that the reaction involves complex formation.

CYCLODEXTRINS (CD), often called cycloamyloses, can be regarded as enzyme models from several of their characteristics: *e.g.* the formation of inclusion complexes with various compounds and the acceleration of many reactions.[1] The acceleration of a reaction by a CD was first observed by Cramer *et al.*,[2] and such accelerations were studied in more detail by Bender and his co-workers; these studies were the first to indicate that a CD could serve as an enzyme model.[3] Although CDs exhibit rate enhancement, stereospecificity, enantiomeric specificity, *etc.* in organic reactions, their catalytic activities are not high enough for them to act as true enzymes. To improve their catalytic activity, especially as models of hydrolytic enzymes, many attempts have been made to modify CDs.

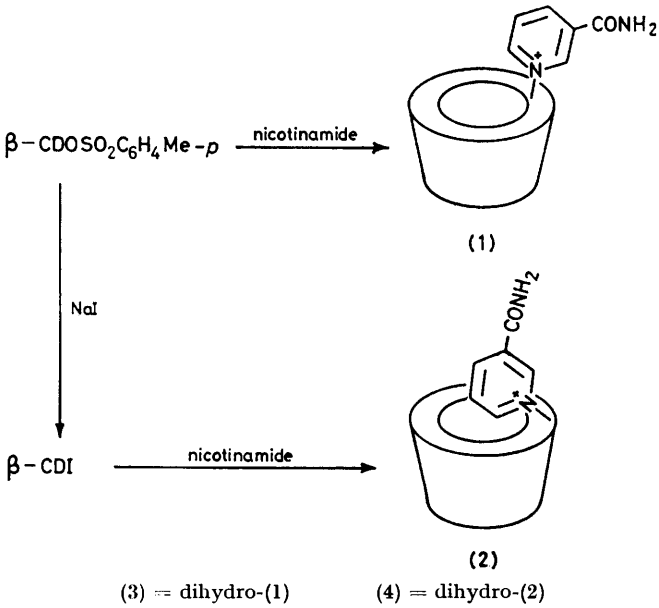
We have already reported an effective modification: the regioselective monosubstitution of α-CD by histamine.[4] α-CD–histamine accelerated the hydrolysis of *p*-nitrophenyl acetate 80 times more than α-CD itself. This result indicates the importance of modifying the secondary hydroxy-groups of CDs if they are to serve as enzyme models. The regioselective monosubstitution of a CD with a nicotinamide group on the secondary hydroxy-side has also been achieved, to give β-cyclodextrin–nicotinamide (1).[5] This was the first attempt to simulate the action of coenzyme NADH-dependent enzymes upon ternary complex formation. β-Cyclodextrin–dihydronicotinamide (3) accelerated the reduction of ninhydrin (2,2-dihydroxyindane-1,3-dione) 40 times more than NADH.

This paper describes the synthesis and types of reaction of two structurally different β-cyclodextrin–nicotinamides, each of which has a nicotinamide group on the secondary hydroxy-side of β-CD. Furthermore, the importance of an inclusion complex as an intermediate and the need for a close fit between substrates and the dihydronicotinamide unit are discussed in comparison with monomeric NADH.

RESULTS AND DISCUSSION

Preparation and Structures of the β-Cyclodextrin–Nicotinamides (1) *and* (2) *and their Dihydro-derivatives* (3) *and* (4).—β-Cyclodextrin–nicotinamides (1) and (2) and their dihydro-derivatives (3) and (4) were prepared from β-cyclodextrin toluene-*p*-sulphonate (β-CDOTs) as starting material. It has been reported that tosylation of β-CD in aqueous solution provided regioselectively monotosylated β-CD.[6] Because the glucose unit of β-CD is in the *C*1 conformation,[7] the secondary hydroxy-groups are equatorial, and so the tosyl moiety attached to β-CD should be equatorial. The NADH-dependent enzyme model compounds (1) and (2) were prepared as follows.

In the synthesis of compound (1), the tosyl group was substituted by a ring nitrogen atom of nicotinamide. This reaction should proceed with inversion of configuration at the tosylated carbon atom, and thus the nicotinamide group should be axial. On the other hand, compound (2) was prepared from an intermediate β-CD iodide. The tosyl group of β-CD tosylate was replaced by I^- intermolecularly giving β-CD iodide, and the iodine atom was then substituted by a ring nitrogen of nicotinamide. Therefore compound (2) should have undergone a double inversion at the tosylated C-3 atom [6] resulting in retention of its configuration, and so the nicotinamide group of (2) should be equatorial. The structure of these compounds differs in the orientation



(3) = dihydro-(1) (4) = dihydro-(2)

of the nicotinamide group with respect to the cavity of β-CD. As illustrated in the Scheme, the nicotinamide group of (1) is located on the exterior of the cavity, while that of (2) is presumed to be partially included in the CD cavity. This was confirmed by the following spectroscopic measurements.

The u.v. spectra, in buffered solutions at pH 7.0, of (1) and its reduced form (3) are shown in Figure 1, and those of (2) and (4) are shown in Figure 2. Both (1)

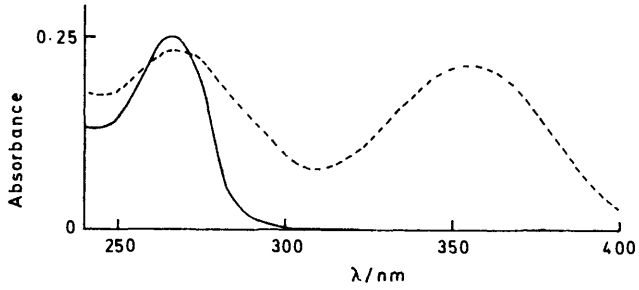


FIGURE 1 U.v. spectra of the β-CD–nicotinamides (1) (——) and (3) (– – – –)

and (2) have an absorption maximum at 267 nm, but (2) has another weak absorption maximum at 357 nm. The weak absorption at 357 nm disappeared in acidic solution (pH 4.0). Compound (2) has OH^- as the ion-pair of the pyridinium cation (see Experimental section),

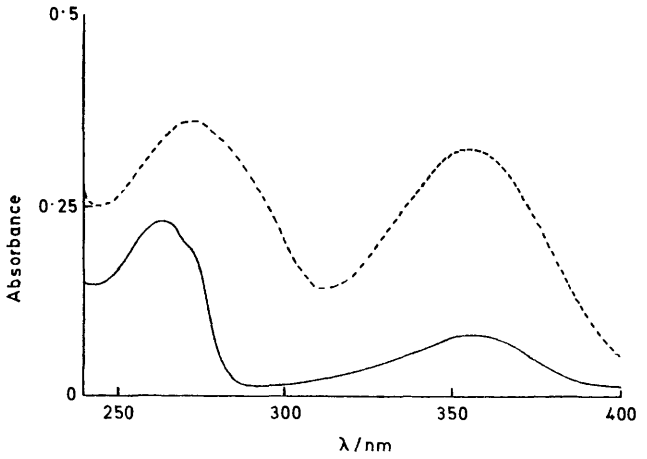


FIGURE 2 U.v. spectra of the β-CD–nicotinamides (2) (——) and (4) (– – – –)

and so the weak absorption at 357 nm in the pH 7.0 buffer solution probably results from addition, in part, of OH^- to the pyridinium ring (pseudo-base formation) to generate a chromophore analogous to that of (4). The spectrum of (3) has maxima at 268 and 355 nm, while that of (4) has maxima at 274 and 357 nm. The shifts in the maxima of (4) to longer wavelength than those of (3) may be attributed to the fact that the dihydropyridine group of (4) is probably in a more hydrophobic environment than that of (3). A similar phenomenon was observed for the absorption spectrum of *p*-t-butylphenol included in β-CD.[8] These results are consistent with the structural differences between (1) and (2) inferred from the synthetic routes. The fluorescence spectra of (1) and (2) were measured in buffer solutions at pH 7.0. Though (2) has a fluorescence maximum at 435 nm (Figure 3), (1) has no emission. This result also indicates that the nicotinamide group of (2) is in a more hydrophobic environment than that of (1). The ^1H n.m.r. spectra of (1) and (2) in D_2O showed multiplet peaks at δ 9.5—8.0 due to nicotinamide. Compound (1) also showed quartet peaks due to *p*-$MeC_6H_4SO_3^-$ which may form an ion-pair with the quatenary pyridinium ion, but (2) showed no other peaks except those of CD.

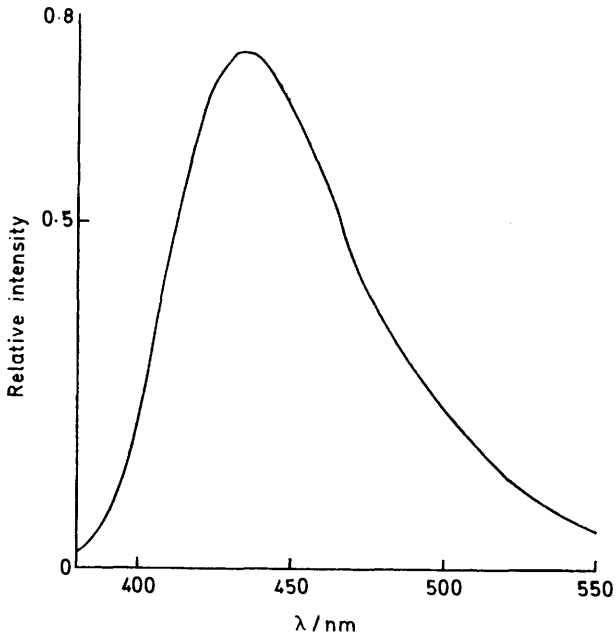


FIGURE 3 Fluorescence spectrum of β-CD–nicotinamide (2)

The half-wave reduction potentials of (1) and (2) were determined by polarography as −1.05 and −0.99 V (*vs.* standard calomel electrode), respectively, which are comparable with that of NAD^+ (−1.00 V [9]). The similarity of half-wave potentials between the model compounds and NAD^+ suggests that (1) and (2) might be effective in simulating NAD^+–NADH-dependent enzyme reactions.

High-performance liquid chromatography (h.p.l.c.) analyses using starch gel (TSK LS-170) showed a longer retention time for (1) than for (2). The difference in retention times can probably be explained by the electrostatic interaction between the positively charged nicotinamide group and the hydroxy-anions of the starch gel. Since the positively charged nicotinamide group of (2) may be partially included within the β-CD cavity, (2) would interact less strongly than (1) with the gel.

Reduction of Ninhydrin with (3), (4), *or NADH.*—To simulate dehydrogenase catalysis, systems comprising (3), (4), or NADH as the hydrogen donor and ninhydrin (half-wave reduction potential −0.95 V) as the hydrogen acceptor were studied in aqueous media (pH 7.0) at

25.0 °C, and the reduction rates were followed spectrometrically. The disappearance of the dihydronicotinamide unit of (3), (4), or NADH was followed at 350 nm by a conventional method. Compounds (3) and (4) were stable in aqueous solution (pH 7.0) at room temperature, their self-decomposition was negligible. The ^{1}H n.m.r. spectra of (3) and (4) showed no peaks in the δ 9.5—8.0 region before the reaction, but when the reactions were complete, multiplets at δ 9.5—8.0 due to nicotinamide were observed, indicating that (3) and (4) were completely oxidized to (1) and (2), respectively.

Plots of the pseudo-first-order rate constants $k_{obs.}$ for reactions of (3) and (4) *versus* ninhydrin concentration, with ninhydrin in excess, are shown in Figure 4. These

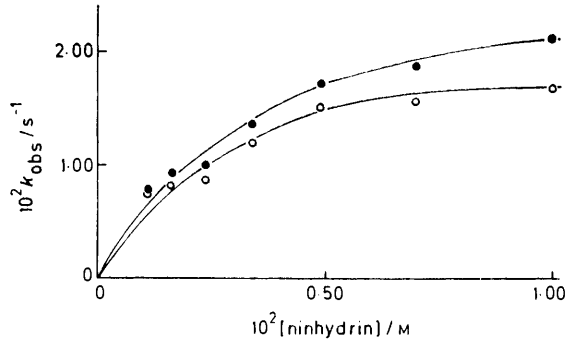


FIGURE 4 Reduction of ninhydrin with (3) (○) or (4) (●)

show increasing curvature with increasing ninhydrin concentration, and such saturation behaviour in the presence of (3) or (4) may be regarded as a manifestation of complex formation between (3) or (4) and ninhydrin [equation (1)] (see also Experimental section).

$$S + C \overset{K_d}{\rightleftharpoons} CS \xrightarrow{k} P + C' \qquad (1)$$

Thus β-cyclodextrin–nicotinamide (C) and ninhydrin (S) reversibly form an aggregate of the complex (CS) to produce the oxidized form of (1) or (2) (C′) and product (P′) with rate constants k. Since these reactions are of the Michaelis–Menten type (enzyme-like), rate constants k and dissociation constants K_d can be evaluated from Eadie–Hofstee plots (Table). The K_d values in the

Kinetic parameters for the reduction of ninhydrin with β-CD–dihydronicotinamide (1), (2), and NADH

Compound	$10^2/s^{-1}$ [a]	$10^5 K_d$/M [a]	k_2/l mol^{-1} s^{-1} [b]
(1)	2.0	2.1	12.6
(2)	2.7	3.0	18.5
NADH			0.31

[a] Determined from an Eadie plot (pH 7.0, 25.0 °C). [b] Determined from half-lives (pH 7.0, 25.0 °C).

Table indicate that (3) binds a substrate more tightly than (4), smaller K_d values suggesting a tighter association. However, the rate constants in the Table show that (4) is more reactive than (3). The results in the Table can be explained as follows.

Since the dihydronicotinamide group of (4) is directed towards the inside of the CD cavity, it may prevent the CD unit from forming an inclusion complex with a substrate. In contrast, the dihydronicotinamide group of (3) is located on the outside of the CD cavity, and so may not prevent complex formation. In considering proximities of the reaction site and the substrate in these inclusion complexes, the dihydronicotinamide group of (4) may be closer to the substrate than that of (3), which may cause the difference in the reaction rates. This is an example of the way in which small structural differences may have a large effect on reactivities.[10]

NADH analogues such as (3) and (4) with binding sites may be taken as representing the active site of natural enzymes, and so it would be expected that they would show higher reactivities than coenzyme NADH alone. To confirm this assumption, the redox reactions of (3), (4), or NADH with an equimolar amount of ninhydrin were studied in buffer solutions (pH 7.0) at 25.0 °C. The second-order rate constants k_2 evaluate from the half-lives are shown in the Table, and these indicate a large rate enhancement (40- and 60-fold, respectively) for (3) and (4) compared with monomeric NADH. This result clearly shows how effectively enzymes may react with substrates upon complex formation. Under equimolar conditions (4) is more reactive than (3), which corresponds to the results of the experiments already described with the substrate in excess.

Enzyme–coenzyme tight binding is known to be the first step of dehydrogenase catalysis,[11] and the enzyme-catalysed reactions take place within ternary (enzyme–coenzyme–substrate) complexes. Many 1,4-dihydronicotinic acid derivatives have been employed to study model reactions of NAD(P)H in the dependent enzymes. For examples, Ohno and his co-workers reported stereoselective reductions by a model NAD(P)H in the presence of magnesium ions.[12] However, none of those model compounds contained a binding site which is essential to the true enzyme. The present work shows that the β-cyclodextrin–nicotinamides (1) or (2), which have a CD unit as a chiral binding site, may be used for the investigation of NADH-dependent enzymes such as dehydrogenase, transhydrogenase *etc.*

EXPERIMENTAL

β-NADH was obtained from Tokyo Kasei Kogyo Co., Ltd. Nicotinamide and ninhydrin were obtained from Yoneyama Yakuhin Kogyo Co., Ltd. β-CD tosylate (toluene-*p*-sulphonate) was prepared as described previously [6] and recrystallized from water. Absorption spectra were recorded with a Hitachi model 100-60 spectrophotometer, using 3 ml quartz absorption cells with a 1 cm light path. Fluorescence spectra were obtained with a Hitachi MPF-4 fluorescence spectrophotometer, and n.m.r. spectra with a Hitachi R-40 high resolution spectrometer, for *ca.* 10% solutions in D_2O, with sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate as internal standard. Polarograms were measured with a Yanaco P8-D d.c. polarograph in pH 7.0 buffer solutions (0.1M-KH_2PO_4–0.2M-NaOH–0.1M-KCl) and referred to the standard calomel

electrode. High-performance liquid chromatography was performed with a Waters ALC 202 instrument using Toyo Soda LS-170 gel (ϕ 2.5 × 50 cm) with 10% aqueous acetonitrile as eluant. T.l.c. was performed on silica gel with n-butanol–dimethylformamide–water (2 : 1 : 1) as eluant and iodine vapour and fluorescence spots were used to detect the β-CD and its derivatives.

Preparation of β-Cyclodextrin–Nicotinamide (1).—A solution of β-CD tosylate (5.0 g, 3.9 mmol) and nicotinamide (5.0 g, 41 mmol) in dimethylformamide (DMF) (80 ml) was stirred for 5 days at 110 °C. The mixture was then diluted with acetone, and the precipitated crude product was filtered off and dried *in vacuo*. Chromatographic separation was carried out on columns of highly porous polystyrene gel HP-20 and HP-20AG by the following procedure.

The dried product was dissolved in water (1 l) and applied to a column (ϕ 3 × 70 cm) of highly porous polystyrene gel DIAION HP-20. The column was eluted with water (1 l), 10% aqueous methanol (1 l), and then 18% aqueous methanol (4 l). The final eluate was evaporated to dryness, and then the dried material was dissolved in water and again applied to a column (ϕ 1.5 × 30 cm) of HP-20AG. The column was eluted with water, followed by 5% aqueous methanol (1 l) and 10% aqueous methanol (1 l). The final eluate was evaporated to dryness. The procedure was repeated until t.l.c. indicated that the material was pure (R_f 0.05); the yield of the *β-cyclodextrin–nicotinamide tosylate* (1) was *ca.* 10% based on the starting β-CD tosylate; $\lambda_{max.}$ 267 nm (ε 2 500) (Found: C, 46.1; H, 5.7; N, 2.1; S, 2.5. $C_{55}H_{82}N_2O_{38}S,3H_2O$ requires C, 46.2; H, 5.9; N, 2.0; S, 2.2%).

Preparation of β-Cyclodextrin–Nicotinamide (2).—A solution of β-CD tosylate (25 g, 20 mmol) and sodium iodide (7.5 g, 50 mmol) in DMF (250 ml) was stirred for 5 h at 100 °C, and then diluted with acetone to precipitate the product. The β-CD iodide was filtered off and dissolved in DMF again. Nicotinamide (25 g, 205 mmol) was added, and the solution was stirred for 2 days at 100 °C. Acetone was then added, and the precipitated product filtered off. The crude product was dissolved in water and evaporated to dryness until no odour of DMF could be detected. The chromatographic procedures were similar to those for (1). The product was dissolved in water (1 l) and applied to a column of HP-20. The column was eluted with water, followed by 10% aqueous methanol (2 l) and 18% aqueous methanol. Each methanolic eluate was evaporated to dryness, and the combined products were dissolved in water and applied to a column of HP-20AG. The column was eluted with water (1 l), followed by 5% aqueous methanol (1 l), and 10% aqueous methanol (1 l). The final eluate was evaporated to dryness, and the chromatographic procedure repeated until t.l.c. indicated that the material was pure (R_f 0.08). The yield of (2) was *ca.* 10% based on the starting β-CD tosylate; $\lambda_{max.}$ 267 (ε 2 200) and 357 (700) nm; fluorescence maximum 435 nm. Before elemental analysis, the product was further purified by silica gel chromatography. An aqueous solution was applied to a column of silica gel (ϕ 4 × 18 cm), and the column eluted with 85% aqueous methanol followed by 0.5% aqueous sodium carbonate. The sodium carbonate eluate was applied to an HP-20AG column, and eluted with water, followed by 10% aqueous methanol. The methanolic eluate was collected and evaporated to dryness to give the *β-cyclodextrin–nicotinamide* (2) (Found: C, 45.8; H, 5.95; N, 2.1. $C_{48}H_{76}N_2O_{36}$ requires C, 45.9; H, 6.1; N, 2.2%).

Hydrogenation of (1) *and* (2).—Reduction at the C-4 position of the nicotinamide moiety in (1) or (2) was carried out according to the general procedure of Haynes and Todd,[13] using hydrosulphite as the reducing reagent, and giving the cyclodextrin–dihydronicotinamides (3) or (4). The solutions were applied to HP-20AG columns, and eluted with water followed by 10% aqueous methanol to remove unchanged (1) or (2), and then 40% aqueous methanol. The eluates were evaporated to remove methanol on a water bath at ≤30 °C, and the concentrated solutions were freeze-dried, to give the purified (3) or (4) in *ca.* 50% yields; (3): $\lambda_{max.}$ 268 (ε 2 300) and 355 (2 100); (4): $\lambda_{max.}$ 274 (ε 3 600) and 357 (3 200) nm.

Reaction Kinetics.—Rates of reduction were followed spectrometrically using a Hitachi model 100-60 spectrophotometer. The disappearance of dihydronicotinamide at 25.0 °C was followed at 350 nm. All rates were determined using 3 ml quartz cells with a 1 cm light path. The reactions were initiated by addition of 15 μl of aqueous solutions of the β-CD–dihydronicotinamides (3) or (4). Pseudo-first-order rate constants were evaluated from Guggenheim plots.[14] Second-order rate constants were evaluated from the half-lives.

Determination of Maximum Rate Constants and Dissociation Constants.—The rate constants k for fully complexed ninhydrin and the complex dissociation constants K_d were determined using equation (1), where S is the ninhydrin which can be complexed with the β-CD–dihydronicotinamide C and reduced to give the product P with a rate constant k, and C′ is the β-nicotinamide (1) or (2). The well known Eadie [15] expression for the treatment of enzyme kinetic data [equation (2)] shows that k can be obtained

$$k_{obs.} = k - k_{obs.} K_d/[S] \qquad (2)$$

from the intercept of Eadie plots and K_d from the slope. The rate constants were obtained using initial β-CD–dihydronicotinamide concentrations of 10^{-4}M and seven concentrations of ninhydrin varying from 10^{-3} to 10^{-2}M. The slopes, intercepts, and correlation coefficients were calculated by a computerized least-squares treatment.

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