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REACTION OF CYCLODEXTRIN-NICOTINAMIDE AS A NADH COENZYME MODEL

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ABSTRACT. β-cyclodextrin-1,4-dihydronicotinamide can reduce cytochrome c in aqueous solution, by adding redox dyes as mediators. In the reduction of cytochrome c mediated by redox dyes, the speeds of reduction differ depending on the activities of the dyes. Inhibition using cyclohexanol occured in the presence of nile blue or methylene blue, and showed competitive inhibition. In the case of using neutral red as a mediator, the reduction was accelerated by adding cyclohexanol. Reaction rate constants of this reaction were independent of the redox potential of the redox dyes.

1. INTRODUCTION

Cyclodextrins(CDs), forming inclusion complexes with a wide variety of substrates in aqueous media[1], affect the rate of various chemical reactions and exhibit substrate selectivity, so that they are often conveniently and successfully utilized as an enzyme model[2]. Although cyclodextrins exhibit rate enhancement, stereospecificity, enantiomeric specificity, etc. in organic reactions, their catalytic activities are not high enough for them to act as a true enzyme.

To improve their catalytic activity, many modified CD derivatives were prepared, especially as models of chymotripsin-catalyzed hydrolysis. We have already reported an effective modification: the regioselective monosubstitution of α- or β-CD by histamine. α-CD-histamine accelerated the hydrolysis of p-nitrophenyl acetate 15 times more than CD itself at pH 8.0 [3]. The catalytic rate constant of α-CD-histamine is close to an actual enzyme, chymotripsin, in the hydrolysis of p-nitrophenyl acetate.

Also we have already reported syntheses of α- and β-CD-nicotinamide as models for an NADH dependent enzyme. The corresponding reduced forms were more stable in aqueous solution, and showed a large rate enhancement (15–50 times greater) in the reduction of ninhydrin, compared with monomeric NADH[4].

This paper describes the nonenzymatic reduction of cytochrome c with β-CD-1,4-dihydronicotinamide (β-CD-NAH), mediated by redox dyes as a mediator.

2. MATERIALS AND METHODS

Mono-tosylated-β-CD was prepared as described previously and recrystallized from water[5]. This compound is identical in all respects with mono-tosylated-β-CD substituted at C-6 position of glucose ring[6].

Iodination of mono-tosylated-β-CD(20g) with sodium iodide(24g) was carried out in methanol(300ml) at 70°C for 50 hours. After reprecipitation with acetone, β-CD-iodide was purified by a column of highly porous polystyrene gel(DIAION HP-20). The purity was confirmed by HPLC. A solution of β-CD-iodide(5g) and nicotinamide(5g) in DMF(50ml) was stirred for 2 days at 100°C and then diluted with acetone to precipitate the product. The precipitated crude product was dissolved in water and evaporated to dryness until no odour of DMF could be detected. The product was dissolved in water and applied to a column of HP-20. The column was eluted with water, followed by 5% aqueous methanol and 20% aqueous methanol. The 20% methanolic eluate was evaporated to dryness and the dried material was dissolved in water and applied to a column of CHP-20P. The column was eluted with water, followed by 5% aqueous methanol, and 10% aqueous methanol. The final eluate was evaporated to dryness, and the chromatographic procedure repeated until HPLC indicated that the material was pure. The yield of β-CD-NA was ca.10% based on the starting mono-tosylated-β-CD. Anal.calc.for $C_{48}H_{75}O_{35}N_2I$: C,42.17; H,5.53; N,2.05. Found: C,42.96; H,5.55; N,1.98.

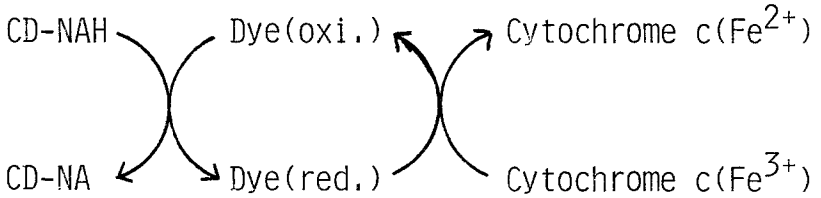
Reduction at the C-4 position of the nicotinamide moiety in β-CD-NA was carried out according to the general procedure of Hynes and Todd[7], using hydrosulphite as the reducing reagent, and giving β-CD-NAH. The reacting solution was applied to a column of CHP-20P, and eluted with water followed by 10% aqueous methanol to remove unchanged β-CD-NA, and then 40% aqueous methanol. The final eluate was evaporated to remove methanol, and the concentrated solution was freeze-dried, to give purified β-CD-NAH in ca.40% yield. β-CD-NAH was checked spectrometrically at 358nm, and the purity was confirmed by HPLC(Toyo Soda, Toyosoda TSK Ods gel, ϕ5 X 500mm, solvent; acetonitrile-water system).

Rates of reduction were followed spectrometrically using a Hitachi model 220A spectrophotometer. The increase of reduced cytochrome c was followed at 550nm. All rates were determined using 3ml quartz cells with a 1cm light path, under anaerobic conditions. The reaction were carried out in pH 7.0(±0.01) phosphate buffer solutions at 25°C, and initiated by addition of β-CD-NAH dissolved in DMF. All reagent were purchased from commercial suppliers and were used without further purification.

3. RESULTS AND DISCUSSION

To simulate dehydrogenase catalysis, system comprising β-CD-NAH as the hydrogen donor and cytochrome c as the hydrogen acceptor was studied in aqueous media. Since no direct reduction of cytochrome c occured by β-CD-NAH, the use of redox as a mediator promoted the reduction as shown in Scheme 1. Four kinds of dyes[nile blue(NB), methylene blue(MB), neutral red(NR), and phenosafranine(PS)] having good solubility were used in this experiment.

Scheme 1.



A typical reduction of cytochrome c with β-CD-NAH, as mediated by various redox dyes, is given in Figure 1. The reduction rate was obtained spectrometrically by measuring the intensity of the absorption band at 550nm, under anaerobic condition. This result shows that NB as a mediator accelerates the reduction of cytochrome c more significantly than other dyes, whereas there was scarcely alteration of reduction rate in the case of using PS as a mediator.

The inhibition experiments were carried out in order to investigate the relationships between rate enhancements and properties of dyes. The change of reactivity by adding cyclohexanol as inhibitor in the presence of NB, MB, or PS is shown in Figure 2. The reduction mediated by NB or MB was largely depressed in the presence of cyclohexanol. In the case of PS, there was no inhibition effect. On the other hand, Figure 3 shows that reduction was greatly accelerated by adding cyclohexanol in the presence of NR. Cyclohexanol acts as a catalyst in this case, not inhibitor.

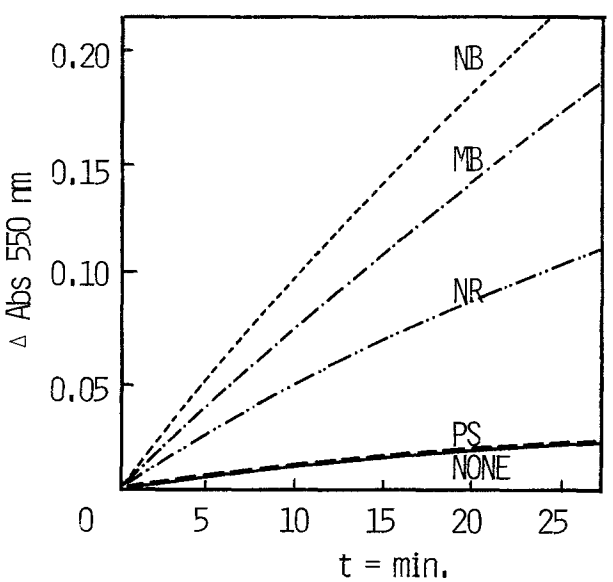


Figure 1. Effects of various dyes on the reduction of cytochrome c with β-CD-dihydronicotinamide.
[β-CD-NAH] = 2.0 X 10^{-4}M [cytochrome c] = 3.6 X 10^{-5}M
[dye] = 5.0 X 10^{-6}M

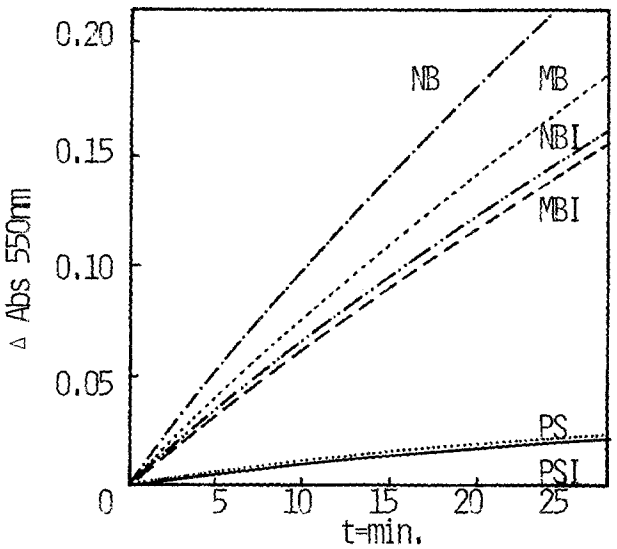


Figure 2. Effects of added cyclohexanol on the reduction of cytochrome c with β-CD-dihydronicotinamide.

[β-CD-NAH] = 2.0 X 10^{-4}M [cytochrome c] = 3.6 X 10^{-5}M

[dyes] = 5.0 X 10^{-6}M [cyclohexanol] = 1.0 X 10^{-3}M

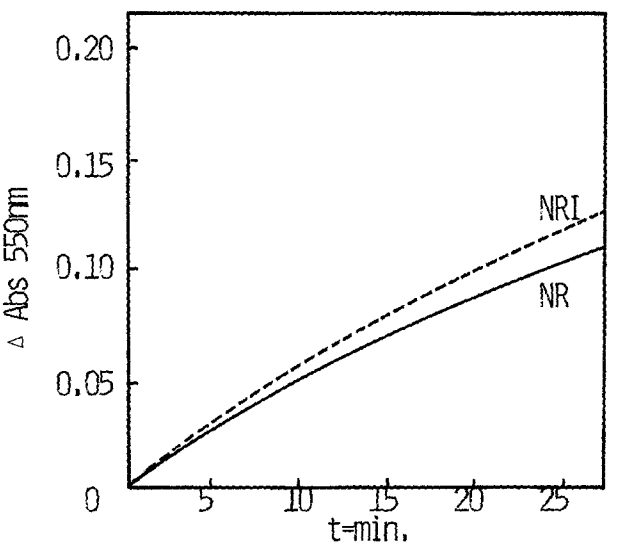


Figure 3. Effects of added cyclohexanol on the reduction of cytochrome c with β-CD-dihydronicotinamide.

[β-CD-NAH] = 2.0 X 10^{-4}M [cytochrome c] = 3.6 X 10^{-5}M

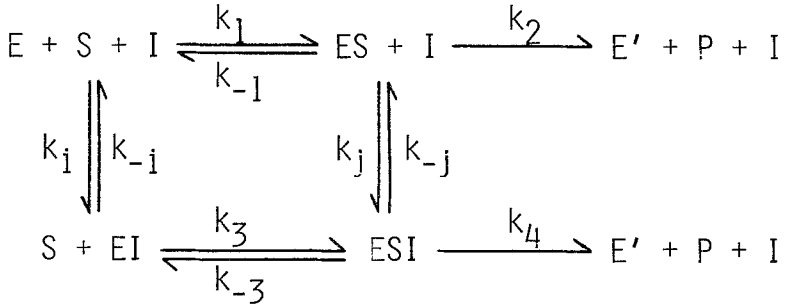
[dyes] = 5.0 X 10^{-6}M [cyclohexanol] = 1.0 X 10^{-3}M

Since these reductions are of the Michaelis-Menten type(coenzyme-like), catalytic rate constants according to Lineweaver-Burk plots which are alteration of first order rate constants depending on the concentration changes of dyes, were calculated and summarized in Table 1. Where, k-cat, Km, and k-cat/Km mean reduction rate constant, dissociation constant, and second order rate constant. NB as a mediator has a larger value of k-cat/Km and a smaller value of Km than the other dyes. These results indicate that NB is a better mediator in the reduction of cytochrome c with β-CD-NAH and is included into the cavity of β-CD-NAH easily. In the case of NR, Table 1 clearly shows an acceleration effect by adding cyclohexanol. This result can be explained by assuming Scheme 2.

Table 1. Rate constants for the reduction of cytochrome c by β-CD-dihydronicotinamide.

Mediator	Cyclohexanol	Km(10^{-5}M)	k_{cat}($10^{-4}S^{-1}$)	k_{cat}/Km($M^{-1}S^{-1}$)
NB	None	1.4	1.6	12
	Added	2.2	1.4	6.0
MB	None	8.2	4.6	5.7
	Added	6.3	3.6	5.6
NR	None	6.3	2.4	3.9
	Added	2.9	2.4	8.4

Scheme 2.



E(β-CD-NAH) and S(dyes) reversibly form an aggregate of ES(complex) to produce E'(β-CD-NA) and P(product). When I(cyclohexanol) is concerned in the reaction, it is considered to form complex EI and ESI with ES.

Overall reaction rate : $k_{cat} = (1-X)k_2 + Xk_4$

Where, X means partial ratio of reaction rate occuring by adding cyclohexanol, and depends on the concentration of

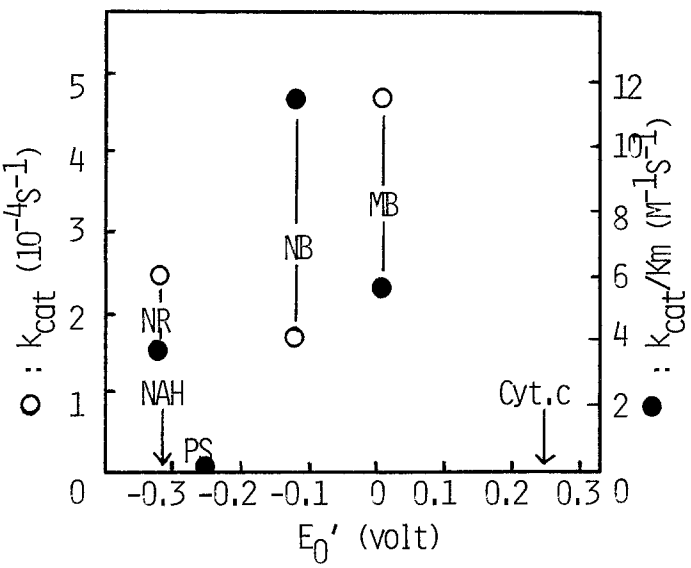


Figure 4. Relations of catalytic rate constants and redox potential in the reduction of cytochrome c mediated by redox dyes with β-CD-dihydronicotinamide.

cyclohexanol. Therefore, $X = [I] / (k_{-j}/k_j + [I])$

without cyclohexanol, $X = 0$ $k_{cat} = k_2$.

When cyclohexanol was added, $X = 0$ $k_{cat}(obs) = k_2$

Therefore, $k_2 = k_4$.

This assumption indicates that β-CD-NAH forms complex of identical structure with dyes in the absence or presence of cyclohexanol.

The relations of catalytic rate constants with redox potential of dyes are given in Figure 4. The reduction of cytochrome c was accelerated even in the presence of NR which has higher redox potential than β-CD-NAH, while the reduction mediated by PS has extraordinarily lower activity than that expected from its redox potential. These results make clear that the reduction mediated by redox dyes in the presence of β-CD-NAH depends upon the other elements, not redox potential.

4. CONCLUSION

The most important step in the reduction of cytochrome c mediated by various redox dyes was ascertained to be the complex formation between β-CD-NAH and each dye. When there was sufficient complex formation between β-CD-NAH and a mediator then the reduction of cytochrome c could be enhanced greatly.

That is, these nonenzymatic cytochrome c reduction systems can be

regarded as an artificial respiratory electron transport chain *in vitro*.

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5. ACKNOWLEDGMENT

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