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Interaction of Nicotinamide Adenine Dinucleotide with 20β-Hydroxysteroid Dehydrogenase from *Streptomyces hydrogenans*

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Some information about the binding of coenzyme to 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* was obtained from the coenzyme specificity and from inhibition studies using various nucleotides. Nicotinamide adenine dinucleotide (NAD) and its analogs served as coenzymes of the enzyme, but NADP did not. When NAD was modified in the adenine ring, the apparent K_m values increased considerably and these values were very high as compared with those of the NAD analogs modified in the nicotinamide moiety. Adenosine 5′-diphosphate (ADP)-ribose, ADP and 5′-AMP were strong competitive inhibitors with respect to coenzyme. ATP and adenosine moderately inhibited the enzyme activity. IMP, IDP, IDP-ribose, 1,N^6-etheno-adenosine 5′-monophosphate, 2′-AMP and 3′-AMP were very poor inhibitors. The N^6-amino group of the adenine moiety may be essential for the binding of the coenzyme to the enzyme, and the phosphate group at the 5′-position of ribose of the adenosine moiety may also play an important role in the binding process. The presence of a phosphate group at the 2′- or 3′-position of ribose of the adenosine moiety resulted in a significant decrease in the binding capacity of adenine nucleotides to the enzyme. 2′-Deoxy-5′-AMP and 3′-deoxy-5′-AMP were very poor inhibitors relative to 5′-AMP, indicating that there may be an interaction between the 2′- or 3′-hydroxyl group and the coenzyme binding site. Nicotinamide and NMN showed only slight inhibitory action compared with adenine nucleotides. It was suggested that the adenosine moiety of the coenzyme may have an important role in the binding interaction with the enzyme.

Keywords——20β-hydroxysteroid dehydrogenase; coenzyme specificity; coenzyme binding; K_m and V_{max} for coenzyme; inhibition by nucleotide

We have investigated the basic mechanisms involved in the catalytic reaction of 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans*, which transfers hydrogen from NADH to various 20-oxosteroids to give the 20β-hydroxysteroids. Possible features of the interactions between 20-oxosteroids and the enzyme have already been described in our previous papers[1] and by other workers.[2] For instance, it was suggested that the coenzyme may be situated near C-16 of the steroid molecule in the steroid-coenzyme-enzyme ternary complex during the catalytic process.[1a] However, very little information is available about the interaction between the coenzyme and 20β-hydroxysteroid dehydrogenase. In the present study, the structural requirements of the coenzyme for binding interaction with the enzyme were studied by using coenzyme analogs and various related compounds.

Experimental

Materials——Crystallized 20β-hydroxysteroid dehydrogenase [EC 1.1.1.53] from *Streptomyces hydrogenans* was purchased from Boehringer Mannheim GmbH. The purity of the enzyme was examined as described previously.[1a] Cortisone was obtained from E. Merck AG, and 20β-hydroxycortisone and 20β-hydroxyprogesterone were obtained from Sigma Chemicals. Progesterone was a standard substance of the National Institute of Hygienic Sciences, Tokyo. Nicotinamide adenine dinucleotide (NAD), NADH, their analogs and all nucleotides were purchased from Sigma Chemicals.

Enzyme Assay——The standard assay of 20β-hydroxysteroid dehydrogenase was carried out according to the method described previously.[3] When NADH analogs were used as coenzymes, the reactions were

carried out according to the standard enzyme assay except that the oxidation rate was followed in terms of the decrease in absorbance at the absorption maximum of each analog, as described below. The reaction mixture contained 460 μM cortisone or 165 μM progesterone and 150 μM reduced coenzyme analog. The reverse reactions of this enzyme were assayed with 500 μM NAD or its analogs and 125 μM 20β-hydroxyprogesterone in 0.1 M Tris buffer (pH 8.7) containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 10% methanol at 25°C. The reaction rates were followed in terms of the increase in absorbance at the absorption maximum for each reduced coenzyme. The λ_{max} and corresponding millimolar extinction coefficient values for each reduced coenzyme[4] are: NADH, 6.22 at 340 nm; reduced 3-acetylpyridine adenine dinucleotide (AP-ADH), 9.1 at 363 nm; reduced pyridine-3-aldehyde adenine dinucleotide, 9.3 at 358 nm; reduced nicotinamide hypoxanthine dinucleotide (deamino-NADH), 6.2 at 340 nm; reduced thionicotinamide adenine dinucleotide, 11.3 at 395 nm; reduced 3-acetylpyridine hypoxanthine dinucleotide, 9.0 at 365 nm; reduced nicotinamide 1,N^6-ethenoadenine dinucleotide, 6.83 at 335 nm. To determine the Michaelis constants of coenzymes, their concentrations were varied, while cortisone, progesterone or 20β-hydroxyprogesterone as fixed substrates were maintained at 460, 165 or 125 μM, respectively. Linear regressions of the reciprocal of the initial reaction rate against the reciprocal of the coenzyme concentration were calculated by using the weighting procedure of Wilkinson.[5]

Inhibition Constants——Inhibition studies were carried out with deamino-NADH as a coenzyme under the conditions of the standard enzyme assay. In all cases at least six concentrations of coenzyme and four concentrations of inhibitor were used, and each measurement was done at least twice. The Lineweaver-Burk plot[6] and Dixon plot[7] were used to determine the inhibition constant (K_i) values.

Concentrations of Enzyme, Steroids and Nucleotides——Concentrations of the enzyme and steroids were determined by the methods described in the previous paper.[1a] Nucleotides concentrations were determined from the absorbance at their absorption maxima and the corresponding molar extinction coefficient.

Results and Discussion

Coenzyme Specificity

In preliminary experiments the coenzyme specificity of 20β-hydroxysteroid dehydrogenase was examined according to the assay method described above.

The relative rates of reduction of NAD, 3-acetylpyridine adenine dinucleotide (AP-AD), pyridine-3-aldehyde adenine dinucleotide (P3A1-AD), thionicotinamide adenine dinucleotide (thio-NAD), nicotinamide hypoxanthine dinucleotide (deamino-NAD), 3-acetylpyridine hypoxanthine dinucleotide (deamino-AP-AD) and nicotinamide 1,N^6-ethenoadenine dinucleotide (ε-NAD) were found to be 100, 57, 24, 16, 6.4, 2.7 and <0.1, respectively. Under the conditions used, no reduction of NADP could be detected. On the other hand, in the presence of cortisone the relative rates of oxidation of NADH, deamino-NADH and AP-ADH were 100, 29 and <0.05, respectively, and when progesterone was used as a substrate they were 100, 51 and 0.5, respectively. The oxidation of NADPH could not be detected in the presence of these steroid substrates. It is evident that the presence of a phosphate group at the 2′-position of ribose of the adenosine moiety of NAD or NADH caused disappearance of the ability to function as a hydrogen acceptor or donor.

To obtain further information, we estimated the kinetic constants for coenzyme analogs in the presence of some representative steroid substrates such as cortisone, progesterone or their 20β-hydroxyl derivatives. However, the reduction rate of NAD or its analogs in the presence of 20β-hydroxycortisone (500 μM) was very slow, being less than 0.2% of the corresponding rate in the presence of 20β-hydroxyprogesterone (125 μM). When 500 μM NAD was used as a coenzyme, 20β-hydroxycortisone had an apparent V_{max} value of 0.88 μmol/min/mg enzyme and an apparent K_m value of 2.0 mM, whereas the apparent V_{max} and apparent K_m values of 20β-hydroxyprogesterone were 125 μmol/min/mg enzyme and 11 μM, respectively. Because of this very low velocity and affinity of 20β-hydroxycortisone, the measurement of the reaction rate of NAD or its analogs in the presence of this steroid substrate was very difficult and inconclusive results were obtained.

The apparent K_m and V_{max} values for NADH, NAD and their analogs, estimated in the presence of an appropriate steroid substrate, are shown in Table I. Modification of the adenine moiety of NAD or NADH caused marked increase in the apparent K_m values (about 50—180 fold), whereas on modification of the nicotinamide moiety, the increases in the K_m values were

Table I. Kinetic Constants of 20β-Hydroxysteroid Dehydrogenase for Coenzyme Analogs

Coenzyme analog	Co-substrate	Apparent K_m (mM)	Apparent V_{max} (μmol/min/mg)
NADH	Cortisone	0.0041	19.1
	Progesterone	0.0039	23.6
AP–ADH	Cortisone	0.028	0.0079
	Progesterone	0.012	0.124
Deamino–NADH	Cortisone	0.35	18.0
	Progesterone	0.17	25.6
NAD	20β-Hydroxyprogesterone	0.045	157
AP–AD	20β-Hydroxyprogesterone	0.88	214
P3Al–AD	20β-Hydroxyprogesterone	0.080	38.2
Thio–NAD	20β-Hydroxyprogesterone	0.10	31.4
Deamino-AP–AD	20β-Hydroxyprogesterone	6.4	49.0
Deamino–NAD	20β-Hydroxyprogesterone	4.5	86.0
ε-NAD	20β-Hydroxyprogesterone	8.0	1.55

The enzyme activities were assayed as described in Experimental except that various concentrations of pyridine nucleotides were used. The initial reaction was determined at 10 concentrations of coenzyme, and each measurement was done at least twice.

Table II. Inhibition of 20β-Hydroxysteroid Dehydrogenase by Coenzyme Analogs and Related Compounds

Compound	Inhibition (%)
Strong inhibitors	
ADP-ribose	82
Thio–NAD	81
P3Al–AD	72
NAD	68
ADP	63
5′-AMP	62
AP–AD	58
NA–AD	47
Moderate inhibitors	
ATP	37
3′-Deoxy-5′-AMP	26
Adenosine	19
N^6-Methyl-AMP	18
2′-Deoxy-5′-AMP	10
Weak inhibitors	
3′-AMP	4
2′-AMP	<1
ε-AMP	<1
5′-IMP	3
IDP	2
IDP-ribose	3
Nicotinamide	<1
NMN	<1
GTP	4
UTP	<1
CTP	<1
NADP	<1
ε-NAD	<1

The enzyme activities were assayed in terms of the rate of oxidation of deamino-NADH according to the method described in Experimental. All compounds tested were added to the assay mixture to give a final concentration of 0.67 mM. Inhibition was expressed as a percentage obtained by comparison with the uninhibited control.

relatively small (to about 2—20 fold). These results suggested that the adenosine moiety rather than the nicotinamide moiety mainly contributed to the coenzyme binding to the enzyme.

Inhibition of 20β-Hydroxysteroid Dehydrogenase by Various Nucleotides

To get further insight into the structural requirement for coenzyme binding, various compounds structurally related to the coenzyme molecule were tested for ability to inhibit the enzymatic reaction. The results are summarized in Table II.

All compounds that possess a 5′-AMP or ADP moiety in the molecule effectively inhibited the enzyme reaction. The addition of a γ-phosphate group (ATP) or the removal of 5′-phosphate (adenosine) caused a decrease in the inhibitory effect. Although a 5′-phosphate group seemed to be important for the interaction of adenine nucleotides, the presence of a 2′- or 3′-phosphate group caused the disappearance of the effect (2′-AMP, 3′-AMP, NADP). This may be due to steric hindrance by and/or the chemical nature of the phosphate group at the 2′- or 3-position of the adenosine moiety. However, the finding that 2′- or 3′-deoxy-5′-AMP had relatively small inhibitory effect may imply some contribution of an unoccupied 2′- or 3′-hydroxyl group itself to the binding interaction. The adenine ring itself may also be an important contributor to the interaction with the enzyme, since modification of the adenine ring or replacement with other purine or pyrimidine bases caused marked decrease or disappearance of the inhibitory effect. On the other hand, nicotinamide and NMN hardly inhibited the enzyme activity at the concentration tested.

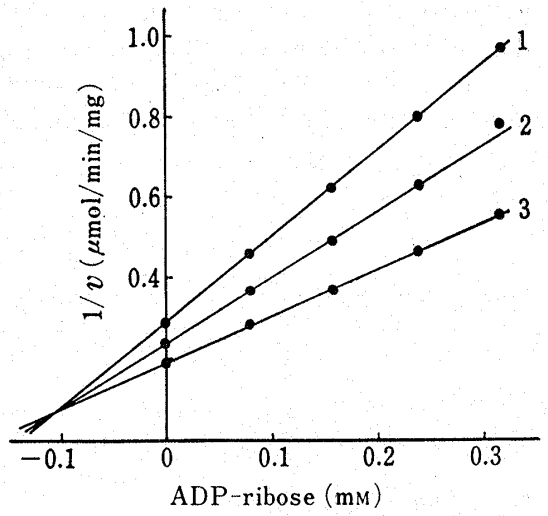

Fig. 1. Dixon Plots of Inhibition of 20β-Hydroxysteroid Dehydrogenase by ADP-Ribose

The enzyme activities were assayed as described in Experimental except that various concentrations of coenzyme were used. The concentrations of deamino-NADH were: 1, 90 μM; 2, 110 μM; 3, 150 μM.

Table III. Inhibitor Constants for Various Compounds structurally related to the Coenzyme

Compound	K_i (mM) Lineweaver–Burk plots	K_i (mM) Dixon plots
Adenosine	1.9	2.4
5′-AMP	0.34	0.34
ADP	0.33	0.33
ADP-ribose	0.14	0.11
ATP	0.99	0.94
2′-Deoxy-5′-AMP	3.0	3.4
5′-IMP	27	30
Nicotinamide	24	26
NMN	34	31
NAD	0.23	0.24
AP–AD	0.39	0.37

Inhibitor constants were calculated from kinetic studies carried out under the conditions described in Experimental.

Kinetic analyses were carried out for 11 compounds with various inhibitory actions. Typical data with ADP-ribose as the inhibitor are shown Fig. 1. The kinetic pattern of the inhibition by ADP-ribose was competitive when coenzyme was used as the variable substrate, and noncompetitive when steroid was used as the variable substrate. All other compounds tested were also competitive inhibitors with respect to coenzyme. These results strongly suggest that these compounds bind to the coenzyme binding site of the enzyme. The inhibitor constants (K_i) for these compounds were calculated from two types of procedure, *i.e.*, Lineweaver-Burk and Dixon plots (Table III). Since the K_i values for 5′-AMP and ADP

were lower than that for adenosine, it was suggested that the presence of a phosphate group at the 5-position of ribose of adenosine enhanced the binding of adenine nucleotide to the enzyme. However, because the K_i values for 5′-AMP and ADP were similar, the presence of a monophosphate or diphosphate group at the 5′-position of adenosine may have no pronounced effect on its binding to the enzyme. The K_i value for ADP-ribose was about one-third of that for ADP. This suggested that the ribosyl group attached to the ADP moiety causes ADP-ribose to bind more strongly than ADP to the enzyme, and further that this ribosyl group plays an important part in the binding of ADP-ribose. However, the interaction of this ribosyl group with the enzyme appeared to require the simultaneous presence of an adenosine moiety, since NMN had very little binding affinity to the enzyme. The K_i values for 2′-deoxy-5′-AMP and 5′-IMP were about 10- and 100-fold higher, respectively, than that for 5′-AMP. It was inferred from this result that the 2′-hydroxyl group and N^6-amino group in adenosine moiety both participate significantly in the binding to the enzyme. However, since the K_i value for 5′-IMP was 10-fold higher than that for 2′-deoxy-5′-AMP, the N^6-amino group rather than the 2′-hydroxyl group appeared to be of prime importance in the binding of the adenosine moiety to the enzyme. The presence of the N^6-amino group of the adenosine moiety in NAD may be essential for the binding of the coenzyme to the enzyme. The degree of participation of the 2′-hydroxyl group in the binding may be nearly equal to that of the 5′-phosphate group, because the K_i value for 2′-deoxy-5′-AMP was similar to that for adenosine.

The present studies on coenzyme specificity and inhibition of the enzyme by several nucleotides revealed that several parts of the molecule are involved in the binding interaction of coenzyme with 20β-hydroxysteroid dehydrogenase. The adenosine moiety and α-phosphate group at the 5′-position of ribose of the adenosine moiety appeared to contribute to the overall binding of the coenzyme. The binding of the adenosine moiety of the coenzyme to the enzyme may occur through interactions involving the N^6-amino, 2′-hydroxyl and 3′-hydroxyl groups. The nicotinamide moiety may also participate in the binding interaction, but its contribution appears to be small.

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