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Carbethoxylation and pH Profiles of Kinetic Parameters of 20β-Hydroxysteroid Dehydrogenase from *Streptomyces hydrogenans*

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20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* is inactivated by diethylpyrocarbonate. It was shown by spectrophotometric studies that two histidine residues per molecule of the enzyme are necessary for enzyme activity. The involvement of histidine residues in the enzyme activity is also indicated by the pH dependence of kinetic parameters for NADH, which shows an inflection point at pH 6.4. The carbethoxyhistidine residues in the modified enzyme are slowly hydrolyzed and the enzyme activity is recovered, but this reactivation is suppressed by addition of NADH. The inactivation of the enzyme by diethylpyrocarbonate is largely prevented by reduced coenzymes, though the steroid substrates alone have no protective effects. It is presumed from kinetic data that carbethoxylation of 20β-hydroxysteroid dehydrogenase does not affect the affinity of the enzyme for NADH. These findings suggest that some histidine residues participate in the enzyme activity and that these histidine residues are located near but not at the coenzyme binding site of the enzyme.

Keywords——20β-hydroxysteroid dehydrogenase; pK value; pH-profile of kinetic parameter; diethylpyrocarbonate; carbethoxyhistidine residue; inactivation by diethylpyrocarbonate; chemical modification of enzyme

20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* has been widely used for enzymatic analyses of some 20-oxo steroids,[1] and also as a model protein for studies of the interaction between proteins and steroids.[2] We have undertaken studies to examine thoroughly the active site of the enzyme and the interactions of the enzyme with steroid and coenzyme. We previously reported on the basic mechanism involved in the interaction between the enzyme and 20-oxo steroids[3] or coenzyme.[4] In the present work, we have studied the pH-dependence of kinetic parameters and the effect of modification with diethylpyrocarbonate on the activity of 20β-hydroxysteroid dehydrogenase in order to obtain information on the functional amino acid residues at the active site of the enzyme. It was confirmed that some histidine residues are involved in the active site of the enzyme and that these histidine residues are located near the coenzyme binding site.

Experimental

Materials——Cortisone was purchased from E. Merck AG. 20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* was obtained in a crystalline form from Boehringer Mannheim GmbH, and was further purified by affinity chromatography with Blue-Sepharose 6B. The purified enzyme preparation gave one protein band on polyacrylamide gel electrophoresis. All nucleotides used were purchased from Sigma Chemicals Co.

Assay of 20β-Hydroxysteroid Dehydrogenase Activity——The enzyme activity was determined at 25°C by measuring the decrease in absorption of NADH at 340 nm under the conditions described in the previous paper.[5]

Determination of Kinetic Parameters——The effect of pH on the enzyme activity was determined in 0.1 M monobasic potassium phosphate–0.05 M sodium borate buffer of various pH's. The kinetic parameters, K_m, V_{max}, and K_i, were obtained from Lineweaver-Burk[6] and Dixon[7] plots.

Treatment of the Enzyme with Diethylpyrocarbonate——Diethylpyrocarbonate was freshly diluted with cold ethanol for each experiment. Carbethoxylation was carried out at 20°C by incubating 20β-hydroxysteroid dehydrogenase with an appropriately diluted reagent in 0.1 M sodium phosphate buffer (pH 6.4).

The final concentration of ethanol in the reaction mixture was adjusted so as not to exceed 2%. The extent of inactivation was determined by measuring the residual enzyme activity under the standard assay conditions of an aliquot removed from the reaction mixture.

Results and Discussion

Effect of pH on the Kinetic Parameters

The kinetic parameters, K_m and V_{max}, of 20β-hydroxysteroid dehydrogenase using NADH as a variable substrate and cortisone as a fixed substrate were determined at various pH's from 5.8 to 8.2. The results are shown in Fig. 1. An integral slop plot according to Dixon's theory,[8] based on the pK_m–pH profile, gave a bend with concave side upwards at pH 6.4 and a bend with concave side downwards at pH 7.3. From the log V_{max} and log V_{max}/K_m–pH profiles, curves with concave side upwards at pH 7.3 and 6.4, respectively, were obtained.

The pH profile of the K_i values was studied for comparison with that of the K_m values. ADP-ribose, which is the most potent competitive inhibitor among various nucleotides tested with respect to coenzyme,[4] was used in this experiment. The pK_i–pH profile of ADP-ribose seems to be very similar to the pK_m–pH profile of Fig. 1a, and a bend with the concave side upwards was found at pH 6.3 (Fig. 2). This pH value was nearly equal to that of the bend with concave side upwards in the pK_m–pH profile. Another bend with the concave side downwards was found at pH 6.9.

The coenzyme substrate, NADH, and the inhibitor, ADP-ribose, have no groups dissociable in the neutral pH region. Therefore, it is not unreasonable to consider that the bends at pH 6.4 and 6.3 in Figs. 1 and 2 are associated with the active site of the enzyme. The bends at pH 7.3 and 6.9 may reflect the pK values of the enzyme-NADH and enzyme-inhibitor complex, respectively.

The pK values of 6.4 and 6.3 are very close to that of free histidine or an imidazole group in a protein or peptide.[9] This similarity strongly

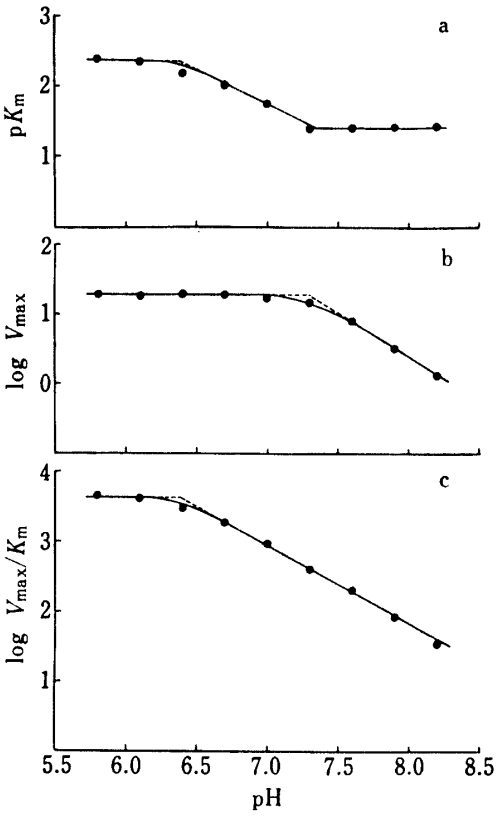

Fig. 1. Effect of pH on pK_m, log V_{max} and log V_{max}/K_m measured with NADH as a Variable Substrate

The activity of 20β-hydroxysteroid dehydrogenase was measured at seven different NADH concentrations (6.0—30 μM) and at a fixed cortisone concentration (0.46 mM) under the conditions described in the text. The data obtained were plotted according to Lineweaver and Burk to obtain the K_m and V_{max} values for NADH. These determin ations were repeated at different pH values.

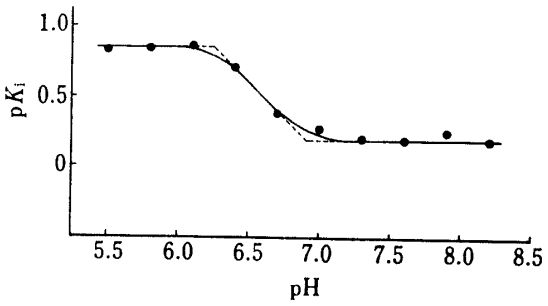

Fig. 2. Effect of pH on pK_i with ADP-ribose as an Inhibitor

The enzyme activities were measured at three different NADH con centrations (15, 35 and 80 μM) and at a fixed cortisone concentration (0.46 mM) in the presence of five different ADP-ribose concentrations (0—0.67 mM) under the conditions described in the text. The dataobtained were plotted according to Dixon to obtain the K_i value for ADP-ribose. These determinations were repeated at different pH values.

suggests the presence of a histidine residue in the active site of 20β-hydroxysteroid dehydrogenase, though the precise nature of amino acid residues participating in the active site of an enzyme cannot always be determined simply from the pK value.

Treatment of the Enzyme with Diethylpyrocarbonate

Since the presence of a histidine residue in the active site of the enzyme was suggested by the effect of pH on the kinetic parameters, we examined whether a histidine residue is involved in the catalytic action of the enzyme, using diethylpyrocarbonate as a histidine-modifying reagent. 20β-Hydroxysteroid dehydrogenase was rapidly inactivated by a relatively low concentration of diethylpyrocarbonate at pH 6.4 (Fig. 3a). The difference spectrum between the diethylpyrocarbonate-treated and native enzymes showed a peak with an absorption maximum at 242 nm, characteristic of the N-carbethoxyhistidine residue in protein[10] (Fig. 3b). The loss of the enzyme activity was linearly related to the increase in absorbance at 242 nm. It was found from the numbers of N-carbethoxyhistidine residues calculated by assuming a difference molar absorbance of 3200 $M^{-1} \cdot cm^{-1}$ at 242 nm[10] that the modification of two histidine residues per molecule of the enzyme is required for complete inactivation of the enzyme (Fig. 3c).

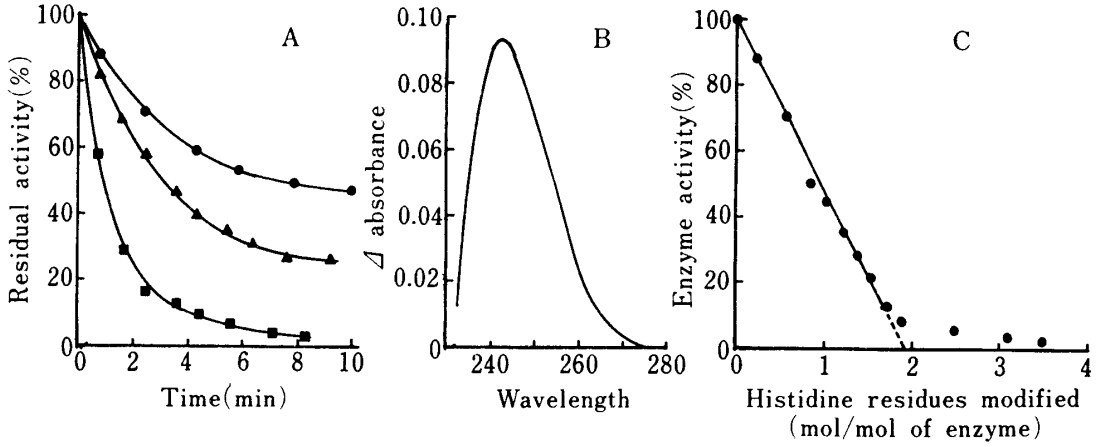

Fig. 3. Effect of Diethylpyrocarbonate on the Enzyme Activity, Spectrum and Histidine Content of 20β-Hydroxysteroid Dehydrogenase

A: Effect of diethylprocarbonate on the enzyme activity as a function of time. The enzyme (325 μg) was incubated with 0.2 mM, ●; 0.4 mM, ▲; or 1.5 mM, ■ diethylpyrocarbonate in 1 ml of 0.1 M phosphate buffer (pH 6.4) at 20°C, and an aliquot of the mixture was assayed for enzyme activity periodically. B: Difference spectrum between carbethoxylated 20β-hydroxysteroid dehydrogenase and native enzyme. The enzyme (1.02 mg) was incubated with 1 mM diethylpyrocarbonate in 1 ml of 0.1 M phosphate buffer (pH 6.4) for 15 min at 20°C and the difference spectrum was recorded at intervals between 230 nm and 280 nm. C: Relationship between enzyme activity and the number of carbethoxyhistidine residues. An aliquot of the treated enzyme was removed immediately after the difference absorbance at 242 nm had been recorded, and assayed for enzyme activity.

Effect of pH on the Inactivation of the Enzyme by Diethylpyrocarbonate

The rate of inactivation by diethylpyrocarbonate increased with increase in pH up to pH 7.0. Figure 4 shows a plot of the logarithm of apparent first order rate constant (k) at various pH values against pH. The log k *versus* pH plot gave a curve with two asymptotes whose slopes are +1 and 0 at low and high pH values, respectively. This indicates that only the unprotonated form of the residue can react with diethylpyrocarbonate, and a simple pH dependence also suggests the involvement of a single residue in the inactivation. The intersection of two asymptotes determines the pK value of the ionizing residue, and a value of 6.2 was obtained. This value is similar to the pK of free histidine or an imidazole group in a protein[9] and the pK values obtained from Fig. 1 and 2. Thus, the result obtained from Fig. 4 is consistent with the view that a histidine residue is present in the active site of 20β-hydroxy-

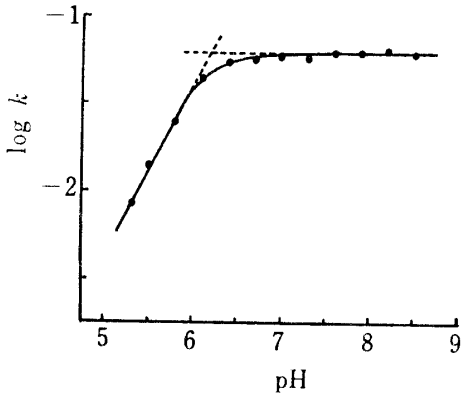

Fig. 4. Effect of pH on Inactivation of the Enzyme by Diethylpyrocarbonate

The enzyme (250 μg) was incubated with 0.4 mM diethylpyrocarbonate in 0.5 ml of 0.1 M phosphate buffer at the pH values indicated. At various times, aliquots were removed for measurements of enzyme activity. The apparent first order rate constants (k) were determined from the pseudo-first order kinetic plots.

steroid dehydrogenese and that the inactivation is due to modification of the histidine residue.

Effect of Coenzyme and Substrate on Inactivation of the Enzyme by Diethylpyrocarbonate

In order to determine whether the coenzyme or substrate of 20β-hydroxysteroid dehydrogenase can protect the enzyme from inactivation, the enzyme was incubated with diethylpyrocarbonate in the presence of these compounds. The inactivation was significantly retarded with NADH and slightly retarded with NAD^+. This difference is considered to be due to a difference in the conformation of the enzyme induced by the binding of these coenzymes and a difference in the affinity of the coenzymes for the enzyme. It is known that the affinity of oxidized coenzyme for the enzyme is very much lower than that of reduced coenzyme.[4] The substrates, cortisone and progesterone, had virtually no effect on the inactivation when they were added alone. It has been shown that the reaction mechanism of 20β-hydroxysteroid dehydrogenase is essentially an ordered Bi Bi mechanism, in which the enzyme first binds the coenzyme and then the steroid.[2a] Therefore, it is considered that the lack of the effect of these substrates arises from their inability to bind to the enzyme in the absence of the coenzyme.

Table I. Effect of Coenzyme and Substrate on Inactivation of 20β-Hydroxysteroid Dehydrogenase by Diethylpyrocarbonate

Compounds added	Concentration (mM)	Residual activity (%)
None	—	9
NADH	0.10	61
	0.46	88
	1.0	96
NAD	2.0	13
	8.0	27
AP-ADH[a]	1.0	87
AP-AD[b]	1.0	10
Progesterone	0.93	12
Cortisone	2.5	8
20β-Hydroxyprogesterone	0.45	8
16β-Methylprogesterone	0.45	8
16β-Methylprogesterone + NADH	0.45 0.10	59

The enzyme (350 μg) in 1 ml of 0.1 M phosphate buffer (pH 6.4) was preincubated with the compounds at the concentrations indicated and then diethylpyrocarbonate (1 mM) was added. Incubation was carried out for 15 min at 22°C and an aliquot of the mixture was assayed for enzyme activity. The residual activity represents the percentage of activity relative to that of the uninhibited control.
a) Reduced 3-acetylpyridine adenine dinucleotide.
b) Oxidized 3-acetylpyridine adenine dinucleotide.

Reactivation and Kinetics of Diethylpyrocarbonate-treated Enzyme

Figure 5 shows the reactivation of diethylpyrocarbonate-treated enzyme in various solutions at 20 °C. Even if hydroxylamine was absent, over a period of days the modified

enzyme gradually regained activity. This reactivation is presumably due to the hydrolysis of the *N*-carbethoxyhistidine residues. This shows that no irreversible structural or conformational changes in the enzyme were caused by the carbethoxylation of histidine residues. Restoration of enzyme activity in the absence of hydroxylamine has been observed in pig heart lactate dehydrogenase[11] and yeast alcohol dehydrogenase.[12] The reactivation rate of modified 20β-hydroxysteroid dehydrogenase in the absence of hydroxylamine is much slower than that observed for lactate dehydrogenase.

The reactivation of modified enzyme was suppressed by NADH. The restoration of the enzyme activity caused by hydroxylamine was also suppressed by addition of NADH. This finding suggests that NADH protects the carbethoxyhistidine residues from hydrolysis.

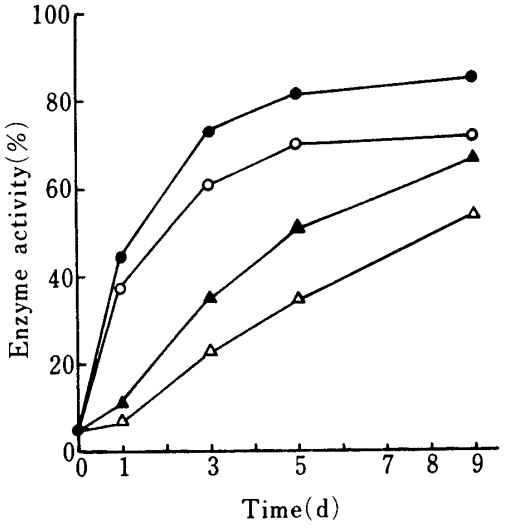

Fig. 5. Reactivation of Diethylpyrocarbonate-treated Enzyme

The enzyme (780 μg) was incubated with 1 mM diethylpyrocarbonate in 0.8 ml of 0.1 M phosphate buffer (pH 6.4) for 30 min at 20°C. Then, the mixture was adjusted to pH 7.0 with 0.1 N sodium hydroxide and to 2 ml with 0.1 M phosphate buffer (pH 7.0). Four aliquots were taken, and hydroxylamine and/or NADH were added to each aliquot.
○, No hydroxylamine and/or NADH added.
●, Hydroxylamine (100 mM) added.
△, NADH (0.45 mM) added.
▲, Hydroxylamine (100 mM) and NADH (0.45 mM) added.

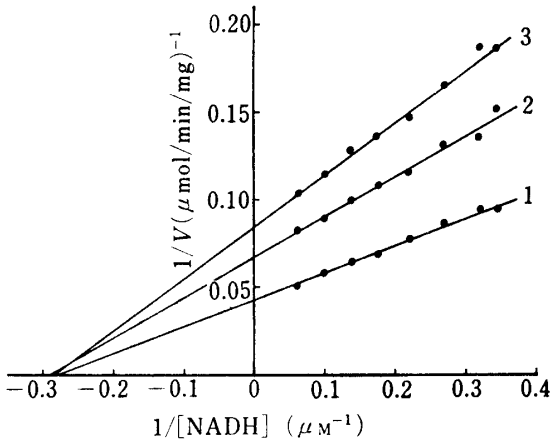

Fig. 6. Lineweaver–Burk plots for Native and Diethylpyrocarbonate-treated Enzymes

The enzyme (700 μg) was inactivated in 1 ml of 0.1 mM phosphate buffer (pH 6.4) by incubation with 0.15 mM or 0.3 mM diethylpyrocarbonate for 20 min. 1), Native enzyme; 2), enzyme inactivated to 63% of the original activity; 3), enzyme inactivated to 46% of the original activity.

To obtain further information on the nature of the inactivation by diethylpyrocarbonate, the kinetics of the modified enzyme were examined and compared with those of the native enzyme. The results obtained from a Lineweaver–Burk plot[6] indicated that the modification of the enzyme by diethylpyrocarbonate caused a decrease in the V_{max} value of the enzyme with no significant change in the K_m value for NADH (Fig. 6).

Therefore, it was considered that the region containing modified histidine residues may be masked by bound NADH and the suppression of reactivation of the modified enzyme by NADH may be due to the obstruction of hydrolysis of the carbethoxyhistidine residues by the bound NADH.

From the results described in this paper, it is suggested that some histidine residues participate in the activity of 20β-hydroxysteroid dehydrogenase and that these histidine residues are located near but not at the coenzyme binding site.

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