Essential histidine residue in 3-ketosteroid-Δ1-dehydrogenase

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Essential Histidine Residue in 3-Ketosteroid-Δ^1-Dehydrogenase

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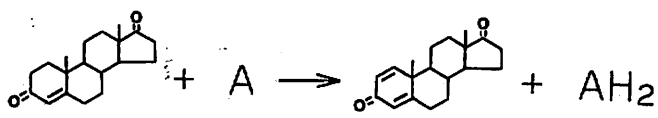
The variation with pH of kinetic parameters was examined for 3-ketosteroid-Δ^1-dehydrogenase from *Nocardia corallina*. The V_{max}/K_m profile for 4-androstenedione indicates that activity is lost upon protonation of a cationic acid-type group with a pK value of 7.7. The enzyme was inactivated by diethylpyrocarbonate at pH 7.4 and the inactivation was substantially prevented by androstadienedione. Analyses of reactivation with neutral hydroxylamine, pH variation, and spectral changes of the inactivated enzyme revealed that the inactivation arises from modification of a histidine residue. Studies with [^{14}C]diethylpyrocarbonate provided support for the idea that the 1-2 essential histidine residues are essential for the catalytic activity of the enzyme. Dye-sensitized photooxidation led to 50% inactivation of the enzyme with the decomposition of two histidine residues. This inactivation was also prevented by androstadienedione. Dancyl chloride caused a loss of the enzyme activity. Modifiers of glutamic acid, aspartic acid, cysteine, and lysine did not affect the enzyme activity. Butanedione and phenylglyoxal in the presence of *borate* rapidly inactivated the enzyme, indicating that arginine residues also have a crucial function in the active site. The data described support the previously proposed mechanism of β-oxidation of 3-ketosteroid.

The flavoenzyme 3-ketosteroid-Δ^1-dehydrogenase catalyzes the desaturation of the alicyclic A-ring of 3-ketosteroid as shown in Scheme 1.



4-androstenedione androstadienedione

Scheme 1

The enzyme is a monomeric molecule with a size of 60,500 per FAD (*1*) and its spectral properties have been clarified in detail (*2, 3*). In the proposed reaction mechanism, the enzyme abstracts 2β-hydrogen from the substrate steroid as a proton and transfers 1α-hydrogen to the FAD molecule as a hydride ion (*3, 4*). This model is supported by evidence obtained in a study of the transhydrogenation between 3-keto-4-ene-steroid and 3-keto-1,4-diene-steroid catalyzed by the dehydrogenase (*3*). A similar finding has been reported for other flavoenzyme dehydrogenases and oxidases, including general acyl CoA dehydrogenase (*5, 6*), D-amino acid oxidase (*7*), and lactate oxidase (*8*). The reaction model suggests that a nucleophilic residue and an electrophilic residue respectively in the active site of the enzyme function in the abstraction of 2β-hydrogen and the interaction of C_3-carbonyl oxygen of substrate steroid. Identification of these amino acid residues is essential for understanding the reaction mechanism of 3-ketosteroid-Δ^1-dehydrogenase. Chemical modification is one of the methods often used for this purpose.

In the present study, we investigated pK_a values of amino acid residues of 3-ketosteroid-Δ^1-dehydrogenase concerned in the dehydrogenation of 3-ketosteroid using pH-kinetic analysis and obtained a candidate for the residue functioning as a nucleophile. Chemical modification studies of the enzyme with diethylpyrocarbonate and dye-sensitized photooxidation demonstrated that 3-ketosteroid-Δ^1-dehydrogenase has 1 or 2 essential histidine residues for the catalytic site. These residues are nucleophilic and probably act in proton abstraction in the early stage of the dehydrogenation of 3-ketosteroid.

EXPERIMENTAL PROCEDURES

Materials—3-Ketosteroid-Δ^1-dehydrogenase from *Nocardia corallina* was purified as described earlier (*1*). The enzyme has a high turnover number of over 10^5 mol/min/mol.

All reagents purchased were of suitable grade and purity for immediate use. Diethylpyrocarbonate (DEP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCD), and dicyclohexyl carbodiimide (DCCD) were from Nacalai Tesque.

N-(2-Acetamido)-2-aminoethane sulfonic acid (Aces) and 3-(cyclohexylamino)ethane sulfonic acid (Ches) were from Dohjin. Tetramethyl ammonium hydroxide, ethanolamine, and dimethyl sulfoxide were from Wako Chemicals. Dancyl chloride was from Seikagaku Kogyo, and butanedione and phenylglyoxal were from Tokyo Kasei. [^{14}C]-Diethylpyrocarbonate and tris(hydroxymethyl)aminomethane (Tris) were from Sigma. The specific activity of

Abbreviations: DEP, diethylpyrocarbonate; [^{14}C]DEP, carbonyl[^{14}C]-diethylpyrocarbonate; DCIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate; Aces, *N*-(2-acetamido)-2-aminoethane sulfonic acid; Ches, 3-(cyclohexylamino)ethane sulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DCCD, dicyclohexyl carbodiimide; EDCD, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; PCMB, *p*-chloromercuribenzoate; 4-androstenedione, androst-4-ene-3,17-dione; androstadienedione, androst-1,4-diene-3,17-dione.

[^{14}C]DEP was 13.3 μCi/μmol. Rose bengal was from Schmit GmbH. Other reagents were as described in previous papers (*1-3*).

Spectral Procedures—Routine optical absorption measurements were performed with a Hitachi model U-3400 spectrophotometer. Spectra were measured at 20-24°C, using quartz cuvettes with 2-mm or 1-cm pathlength.

pH Studies—Constant ionic strength buffers of 100 mM Aces, 52 mM Tris, and 52 mM ethanolamine, $I=0.1$ M, which cover the pH range from 6.0 to 11, were prepared according to the literature (*9*). The buffer system was adjusted to the desired pH by addition of 1 N HCl or 10% tetraethylammonium hydroxide solution. The enzyme was assayed by the DCIP method in 3.0 ml of reaction mixture consisting of 6.5-56 μM 4-androstenedione, 67 μM DCIP, 67 μM PMS, 1.2-2.4 nM enzyme, and the isoionic strength buffer, pH 6-11 (*1*). To reduce the polarity of the reaction medium, 20% dimethyl sulfoxide was added as a perturbing organic solvent (*10*). The pH was determined before the addition of the organic solvent. The K_m for 4-androstenedione and V_{max} values were determined with variable concentrations of the steroid substrate and a fixed concentration of the electron acceptor dye. The kinetic pK_a values were determined by fitting the data to Eq. 1:

$$\log (V_{max}/K_m)=\log \{C/(1+10^{pK_1-pH}+10^{pH-pK_2})\} \quad (1)$$

where C is the pH-independent value of V_{max}/K_m, and pK_1 and pK_2 are minus logarithm values of the dissociation constants of the groups on the acidic and basic side, respectively (*11*).

Modification of the Enzyme with Diethylpyrocarbonate—The concentration of stock solutions of DEP was determined by monitoring the increase in absorbance at 240 nm in the reaction with 10 mM imidazole in 50 mM potassium phosphate buffer, pH 7.4, by use of an E value of 3,200 $M^{-1}\cdot cm^{-1}$ (*12-14*). Decomposition rate of DEP in enzyme assay buffer was estimated as follows. An amount of DEP was added to the assay mixture, and 10-μl samples of the mixture were taken at intervals and added to 3 ml of 10 mM imidazole. The decomposition rate of DEP was estimated by monitoring the absorbance at 240 nm. When DEP in acetonitrile was used to treat the enzyme, the solvent concentration in the reaction mixture was kept below 5%. The enzyme (1 μM) in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, was treated by addition of 10 μl of appropriately diluted DEP solution in acetonitrile at 22°C; and 10 μl of acetonitrile was added to the control enzyme solution. Samples of 50 μl of the mixture were taken at intervals and mixed with 50 μl of 10 mM imidazole to terminate the reaction. The remaining enzyme activity was assayed by the DCIP method (*1*). The rate constants for inactivation by DEP, k_{obs}, were calculated according to Eq. 2:

$$\ln (V_t/V_0)=(k_{obs}/k')I_0(1-e^{-k't}) \quad (2)$$

where V_0 and V_t represent the enzyme activity at times 0 and t, respectively. I_0 is the initial concentration of DEP, k_{obs} is the second-order rate constant for the reaction of the enzyme with the modifier, and k' is the first-order rate constant for hydrolysis of the reagent (*15*). In 50 mM potassium phosphate buffer at pH 7.4 and 22°C, k' was estimated to be 9.50×10^{-2} min^{-1}. The order of the inactivation was determined from Eq. 3 (*16*).

$$\log (1{,}000/t_{1/2})=n \log[DEP]+\log k \quad (3)$$

where $t_{1/2}$ represents the half-life and n is the order of inactivation.

DEP-modification of the enzyme was monitored by differential spectrophotometry at 18°C. The sample and the reference cuvette contained 30 μM enzyme in 50 mM potassium phosphate buffer, pH 7.4. The reaction was started by addition of 30 mM DEP and was followed by measuring the spectral changes in the range of 220-300 nm. The number of histidine residues modified was estimated from the absorbance change at 242 nm.

Modification with [^{14}C]Diethylpyrocarbonate—3-Ketosteroid-Δ¹-dehydrogenase (2.2 μM) was incubated with 1.65 mM [^{14}C]DEP, 2×10^6 cpm, in 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.51 ml at 30°C. After a definite time of incubation, the reaction was terminated by the addition of 0.1 ml of 50 mM imidazole. The incubated enzyme was separated from the unreacted reagent by gel-filtration on a Sephadex G-15 column (0.8× 35 cm) and mixed with 10 ml of aqueous counting scintillant. The radioactivity was counted on an Aloka model LSC-671 liquid scintillation spectrometer.

Photooxidation in the Presence of Methylene Blue—3-Ketosteroid-Δ¹-dehydrogenase (1 μM) in 50 mM potassium phosphate buffer, pH 7.4, was irradiated in an ice-water bath in the presence or absence of 0.13 mM methylene blue at a distance of 30 cm with a 750-W tungsten light bulb. A glass filter, Tohshiba V-053 orange, was placed in front of the light source (*17*). The solution was sampled at intervals, and the residual enzyme activity was measured by the DCIP method and HPLC (*1, 3*). Rose bengal was also used in the same procedures.

Amino Acid Analysis—3-Ketosteroid-Δ¹-dehydrogenase photoinactivated in the presence of methylene blue was dialyzed against redistilled water for 3 days to remove the dye. The samples were lyophilized, dissolved in 0.5 ml of 6 N HCl, and hydrolyzed at 110°C for 24 h in an evacuated, sealed tubes. The hydrolyzed samples were treated with 100 μl of 40 mM NaOH and kept in an evacuated desiccator for 3 days to remove ammonia. Amino acid analyses were performed in a Hitachi L-8500 amino acid analyzer.

Survey of Chemical Modification Reagents—Inactivation of 3-ketosteroid-Δ¹-dehydrogenase by chemical modification was explored by addition of a large excess of a reagent to the enzyme solution and incubation at 22°C. Solutions were sampled at intervals and assayed for the activity over a 2-h period. The inactivation of the dehydrogenase by modification of glutamic acid or aspartic acid residues was investigated by addition of either 10 mM EDCD or 10 mM DCCD to a 1-μM enzyme solution in 50 mM potassium phosphate buffer, pH 6.0 to 7.0, and incubation for 2 h (*18, 19*). Cysteine residues were modified by addition of 2.5 mM *p*-chloromercuribenzoate (PCMB) to a 1-μM enzyme solution in 30 mM Tris-HCl buffer, pH 7.4, and incubation for 2 h. Iodoacetate (50 mM) was added to a 1-μM enzyme solution in 50 mM potassium phosphate buffer, pH 7.4, and the mixture was incubated for 0-2 h. Inactivation by modification of arginine residues was studied by incubation of the enzyme with various concentrations of butanedione or phenylglyoxal in 500 mM borate buffer, pH 9.0, for 0-90 min (*20, 21*). Dancyl chloride (100 μM) was added to an enzyme solution (0.5 μM) in 1 ml of

50 mM potassium phosphate buffer, pH 7.0, and the mixture was incubated at 22°C for 0-2 h (*22*). Samples were taken for determination of the remaining enzyme activity. Lysine residues were modified with 0.4 mM pyridoxal phosphate in 30 mM Tris-HCl buffer, pH 7.4, and also with 0.4 mM succinic anhydride in 50 mM Na-Ches buffer, pH 9.0, for 0-2 h.

RESULTS

Effect of pH on 3-Ketosteroid-Δ^1-Dehydrogenase—The effects of pH on the enzyme activity were examined in a constant ionic strength buffer of the cationic acid type in the range from pH 6.0 to 11. The V_{max}/K_m profile shows a bell-shaped curve with a maximum value in the pH range from 8 to 9 (Fig. 1, curve A). At lower pH, the curve decreases with a limiting slope of +1, and at higher pH it decreases with a limiting slope of −1. The data from the pH-dependence of V_{max}/K_m were fitted to Eq. 1. From the fit, a pK value of 7.7 was obtained on the low-pH side of the profile and 9.4 on the high-pH side. The pK values of V_{max}/K_m profile were also measured in the presence of 20% dimethyl sulfoxide. The addition of dimethyl sulfoxide caused no significant change of the ionization on the low side of the pH profile, but on the high side, pK was shifted from 9.4 to 10.5 (Fig. 1, curve B). These data suggest that the ionization group of the enzyme has a cationic property on the acidic side (*10*).

*Inactivation of 3-Ketosteroid-Δ^1-Dehydrogenase by Di*ethylpyrocarbonate—DEP was used to modify the essential amino acid residues of the active site of 3-ketosteroid-Δ^1-dehydrogenase. The inactivation of the enzyme proceeded at a very high rate at 22°C and the results of the

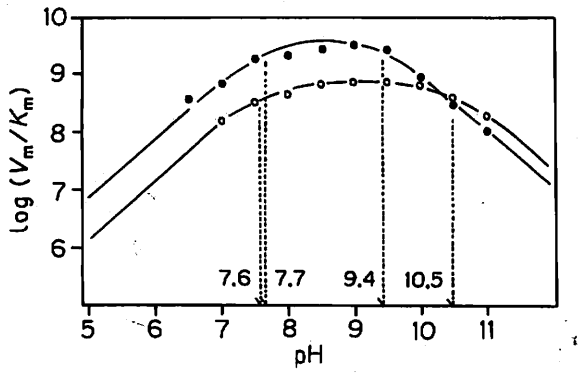


Fig. 1. **Effect of pH on V_{max}/K_m of 3-ketosteroid-Δ^1-dehydrogenase.** ●, with a constant ionic strength buffer system of Aces/Tris/ethanolamine (I=0.1 M), and ○, in the presence of 20% dimethyl sulfoxide in the buffer system. Other experimental conditions are described in "MATERIALS AND METHODS."

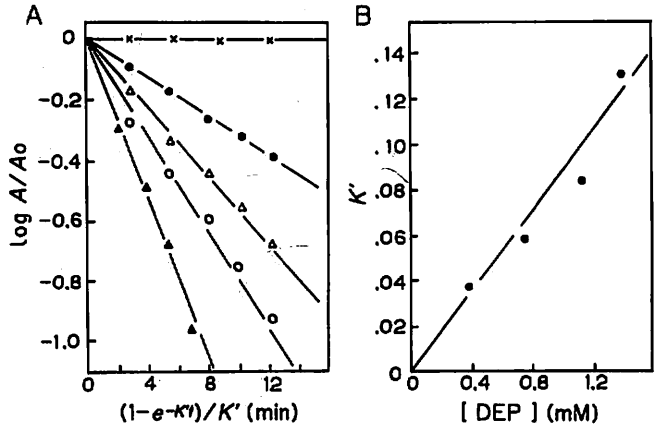


Fig. 2. **Inactivation of 3-ketosteroid-Δ^1-dehydrogenase by diethylpyrocarbonate.** A: Pseudo-first-order plots of the fractions of residual activity, A/A_0, *versus* the incubation time $(1-e^{-k't})/k'$. A_0 is the initial activity and A is the activity at time t. Enzyme (1 μM) in 50 mM potassium phosphate buffer, pH 7.4, was incubated at 20°C with 0 (×), 0.38 (●), 0.76 (△), 1.14 (○), and 1.52 mM (▲) of DEP, respectively. Other conditions are described in "MATERIALS AND METHODS." B: Secondary plot of the pseudo-first-order rate constant against DEP-concentration. Second-order rate constant of DEP-inactivation of the enzyme was calculated from the slope of the line as 81.9 $min^{-1} \cdot M^{-1}$.

TABLE I. **Effects of androstadienedione on the inactivation of 3-ketosteroid-Δ^1-dehydrogenase with DEP.** A mixture containing 0.96 μM enzyme, 0.76 mM DEP, and androstadienedione in 50 mM potassium phosphate buffer, pH 7.4, was incubated at 22°C for 30 min, and the residual activity was assayed by the DCIP method.

Androstadienedione (μM)	Activity remaining (%)
0	17.7
100	44.3
200	54.2
300	55.8
400	59.6
500	63.3

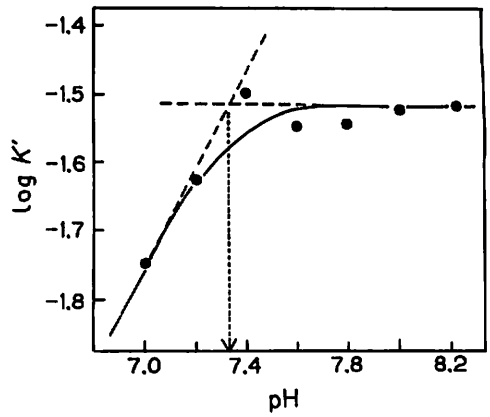


Fig. 3. **Effect of pH on the pseudo-first-order rate constant of DEP-inactivation of 3-ketosteroid-Δ^1-dehydrogenase.** The incubation mixture contained 1 μM enzyme and 0.76 mM DEP in 3.0 ml of 50 mM potassium phosphate buffer of the indicated pH. The mixture was sampled and the residual activity was assayed at definite times during the 15-min incubation. The curve was obtained by fitting the data to Eq. 4.

TABLE II. **Modification of 3-ketosteroid-Δ^1-dehydrogenase with [^{14}C]DEP.** Modification experiments were carried out as described in "MATERIALS AND METHODS."

Incubation time (min)	Residual activity (%)	Number of modified residues (mol per mol enzyme)
3	50.1	1.29
6	28.5	1.62
9	17.2	2.16
12	10.1	5.24

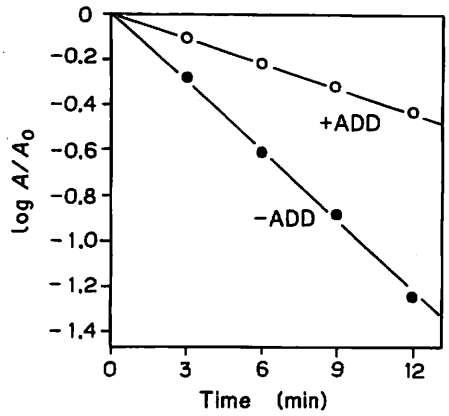


Fig. 4. **Inactivation of 3-ketosteroid-Δ¹-dehydrogenase by methylene blue-sensitized photooxidation.** In the absence (●) and presence (○) of 10 μM androstadienedione. Other experimental conditions are described in "MATERIALS AND METHODS."

TABLE III. **Dye-sensitized photooxidation of 3-ketosteroid-Δ¹-dehydrogenase.** Experimental conditions for photooxidation and amino acid analyses are described in "MATERIALS AND METHODS."

Residual activity (%)	Number of histidine residues modified (mol per mol enzyme)
100	0
63	1.6
44	1.7
25	3.1
0	4.9

experiments at various concentrations of DEP are presented as a plot of log V_t/V_0 *versus* time $(1-e^{-k't})/k'$ (Fig. 2A). The plots indicate that the inactivation follows approximately first-order kinetics at all DEP concentrations used. The linearity of this plot shows that no reversible complex is formed between the enzyme and DEP (*23*). Plots of the observed first-order rate constant, k_{obs}, *versus* the concentration of DEP gave a straight line (Fig. 2B), from the slope of which the second-order rate constant for the inactivation of 3-ketosteroid-Δ¹-dehydrogenase at pH 7.4 and 22°C was calculated to be 81.9 $M^{-1}\cdot min^{-1}$. A plot of the logarithm of $1{,}000/t_{1/2}$ *versus* the logarithm of the concentration of DEP from the data of Fig. 2A gave a straight line with a slope of 1.04 (plot not shown). This value is the reaction order of DEP, suggesting that the modification of a single residue of histidine results in the inactivation of 3-ketosteroid-Δ¹-dehydrogenase. Androstadienedione is the dehydrogenation product of 3-ketosteroid-Δ¹-dehydrogenase and its binding to the enzyme causes perturbation of the visible spectrum (*2*). The steroid markedly protected the enzyme against DEP-inactivation (Table I). When DEP-inactivated 3-ketosteroid-Δ¹-dehydrogenase was treated with 1 M hydroxylamine in 50 mM potassium phosphate buffer, pH 7.4, at 22°C, the enzyme was reactivated to about 60% of the initial activity after 30 min, after which reactivation continued at a slower rate.

Modification of amino acid residues in protein with DEP is dependent on pH for the reaction of unprotonated residues. Inactivation of 3-ketosteroid-Δ¹-dehydrogenase was examined between pH 7.0 and 8.2. Pseudo-first-order rate constants were determined and plotted *versus* the

TABLE IV. **Summary of effects of chemical modification reagents on 3-ketosteroid-Δ¹-dehydrogenase.** Experimental conditions are described in "MATERIALS AND METHODS."

Targeted residue	Reagent	pH	Inactivation rate
His	DEP	7.4	81.9 $min^{-1}\cdot M^{-1}$ [a]
His	Methylene blue photooxidation	7.4	9.5×10^{-2} min^{-1} [b]
His	Dancyl chloride	7.4	2.5×10^{-1} min^{-1} [b]
His, Cys	Iodoacetate	7.4	0
Arg	Butanedione	9.0	1.2 $min^{-1}\cdot M^{-1}$ [a]
Arg	Phenylglyoxal	9.0	0.2 $min^{-1}\cdot M^{-1}$ [a]
Cys	PCMB	7.4	0
Glu, Asp	DCCD	6.0	0
Glu, Asp	EDCD	6.0	0
Lys	Pyridoxal phosphate	7.4	0
Lys	Succinic anhydride	9.0	0

[a]Inactivation rate constant. [b]Apparent inactivation rate constant.

reaction pH (Fig. 3). In the figure, the closed circles represent the experimental data, and the *solid* line is a theoretical curve represented by Eq. 4:

$$k_{obs}=(k_{obs\ max})/(1+[H^+]/K_a) \quad (4)$$

where K_a is the dissociation constant of a reacting residue and ($k_{obs\ max}$) represents the pseudo-first-order rate constant of the unprotonated reacting residue (*24*). From the fitted curve, the apparent pK_a value and $k_{obs\ max}$ were calculated to be 7.3 and 0.39 min^{-1}, respectively. The apparent pK_a value is similar to the pK_1 value obtained by pH-studies (Fig. 1), and the results suggest strongly that the inactivation of 3-ketosteroid-Δ¹-dehydrogenase is due to the modification of a histidine residue in the active site. The difference spectrum between the native enzyme (30 μM) and the enzyme treated for 30 min with 30 mM DEP showed a large absorption peak at 242 nm (data not shown). The reaction proceeded in biphasic form: an initial rapid phase ($t_{1/2}$ of less than 20 s), and a subsequent slow phase ($t_{1/2}$ of about 2.7 min). The number of histidine residues reacting in 30 min was estimated to be about 4 residues per mol enzyme, and one further residue was modified in the slow phase. No spectral change was observed in the region of 260-290 nm, in which range a DEP-modified tyrosine residue would show as a decrease in absorbance (*13*).

The stoichiometry of chemical modification of the enzyme with DEP was studied using [^{14}C]DEP. The data shown in Table II indicate that the modification proceeds in a time-dependent manner, and about 80% inactivation occurs with the modification of 2 mol of histidine residues per mol enzyme.

Photoinactivation of 3-Ketosteroid-Δ¹-Dehydrogenase—3-Ketosteroid-Δ¹-dehydrogenase in 50 mM potassium phosphate buffer, pH 7.4, was inactivated rapidly by irradiation at 4°C in the presence of 0.13 mM methylene blue (Fig. 4). Androstadienedione showed a protective effect against the photoinactivation. The inactivation also followed pseudo-first-order kinetics and the observed rate constant (k') was 9.50×10^{-2} min^{-1}. In the presence of androstadienedione, the value decreased to 3.65×10^{-2} min^{-1}. Rose bengal-sensitized photoinactivation was also examined, but no significant loss of the enzyme activity was observed because an orange glass filter was used to protect flavin of the enzyme from decomposition. Dye-sensitized irradiation is known to decompose histidine, tyrosine,

tryptophan, methionine, and cysteine residues (*25*). Under the experimental conditions employed, histidine residues are most modifiable. The 3-ketosteroid-Δ^1-dehydrogenase contains 10 histidine residues per mol enzyme (*1*). Amino acid analyses of the enzyme preparations photooxidized in the presence of methylene blue showed that most of the enzyme activity disappeared by modification of three or more histidine residues (Table III). The result is similar to that obtained in the modification experiment with [^{14}C]-DEP shown in Table II. It appears that the essential histidine residues of the enzyme molecule are not photooxidized preferentially, but rather that other histidine residues are also oxidized under the experimental conditions used. Amino acid analyses showed no significant loss of tyrosine or methionine residues during the photooxidation under these conditions.

Inactivation with Other Modification Reagents—Several chemical modification reagents which might inactivate 3-ketosteroid-Δ^1-dehydrogenase were surveyed and the results are summarized in Table IV. Dancyl chloride affected the activity of 3-ketosteroid-Δ^1-dehydrogenase in a time- and also a concentration-dependent manner. The k_{obs} of the inactivation with 200 μM dancyl chloride was 2.5×10^{-1} min^{-1} in 50 mM potassium phosphate buffer, pH 7.4. This inactivation was prevented by the addition of 100 μM androstadienedione and more than 90% of the initial activity remained after the 40-min incubation. The enzyme inactivated with dancyl chloride recovered more than 70% of its activity on addition of 1 M hydroxylamine. No significant loss of activity was observed with PCMB, iodoacetate, EDCD, DCCD, pyridoxal phosphate, or succinic anhydride. The enzyme was inactivated completely by 1 mM butanedione and also 1 mM phenylglyoxal. This inactivation was largely prevented by addition of 1 mM androstadienedione.

DISCUSSION

3-Ketosteroid-Δ^1-dehydrogenase catalyzes trans-axial β-oxidation of C_1 and C_2-positions of 3-ketosteroid. 2β-Hydrogen of the substrate is abstracted as a proton by a nucleophile by a mechanism similar to that for acyl-CoA dehydrogenase (*3, 5*). Analyses of pH-dependence of the kinetic parameters and chemical modification gave, on the acidic side, pK values of 7.7 and 7.3, respectively. These values are in fairly good agreement with the pK_a of a histidine residue in protein. That the value is not perturbed by organic solvents indicates that the functional group protonated when enzymic activity is lost is of the cationic acid type (*10*). These facts suggest that a histidine residue is the most likely to be the nucleophilic functional group of 3-ketosteroid-Δ^1-dehydrogenase.

Diethylpyrocarbonate has been used widely to investigate histidine residue functioning in the active sites of enzymes (*14, 22, 26*). It is a highly selective agent modifying residues in protein under definite conditions, but its reactions with tyrosine, lysine, and cysteine have been also reported (*27, 28*). 3-Ketosteroid-Δ^1-dehydrogenase from *N. corallina* was rapidly inactivated by this reagent under the specified conditions. The protection by androstadienedione against DEP-inactivation and the reactivation of the enzyme by neutral hydroxylamine indicate strongly that the inactivation by DEP is due to the modification of histidine residue(s). The order of the DEP-modification reaction, $n=1.04$, suggests that one essential histidine residue is present in the active site of the enzyme molecule. The experimental results of incorporation of [^{14}C]DEP, methylene blue-sensitized photooxidation, and modification with dancyl chloride also support the inference that one or two histidine residues are crucial to the enzyme activity.

The specificity of modification reagents is important in obtaining information about the functional group(s) in the catalytic site of an enzyme. Since most reagents generally used do not have a strictly limited reactivity toward a single kind of amino acid residue, the results of several analytical procedures using more than one modifying reagent must be combined in order to draw any conclusion. The results summarized in Table IV show that glutamic acid, aspartic acid, cysteine, and lysine residues can be ruled out as functional groups, and that arginine and histidine residues are important for the enzyme reaction. We have also studied in detail the arginine residues of the 3-ketosteroid-Δ^1-dehydrogenase and will describe the results in a separate paper.

Lactate oxidase, glycolate oxidase, and L-lactate dehydrogenase (b_2), which catalyze the abstraction of α-hydrogen from carbonic acids, have a conservative histidine residue, which withdraws α-hydrogen from their respective substrates as a proton and produces a carbanion intermediate (*29*). A histidine residue of D-amino acid oxidase has been shown to function in the proton abstraction of α-hydrogen from the substrate (*7, 22*). Studies on acyl-CoA dehydrogenase with 2-octynoyl-CoA revealed that a glutamyl residue (Glu^{376}) is the base which abstracts α-hydrogen from the substrate (*30*). In contrast, studies with Glu^{376}-Gln mutant of human medium chain acyl-CoA dehydrogenase showed that the glutamyl residue is an important, but not essential, residue for catalysis of the reaction (*31*). Our study with carbodiimide suggested that acidic amino acid residues of the 3-ketosteroid-Δ^1-dehydrogenase do not play an important role in the elimination of 1α,2β-hydrogen of the substrate.

Further study is required to determine which histidine residue of 3-ketosteroid-Δ^1-dehydrogenase is the essential one acting as a nucleophile in the dehydrogenation of 3-ketosteroid.

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