Chemical Modification of 3α,20β-Hydroxysteroid Dehydrogenase with Diethyl Pyrocarbonate. Evidence for an Essential, Highly Reactive, Lysyl Residue

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ABSTRACT: Diethyl pyrocarbonate inactivated the tetrameric 3α,20β-hydroxysteroid dehydrogenase with second-order rate constants of 1.63 M^{-1} s^{-1} at pH 6 and 25 °C or 190 M^{-1} s^{-1} at pH 9.4 and 25 °C. The activity was slowly and partially restored by incubation with hydroxylamine (81% reactivation after 28 h with 0.1 M hydroxylamine, pH 9, 25 °C). NADH protected the enzyme against inactivation with a K_d (10 μM) very close to the K_m (7 μM) for the coenzyme. The ultraviolet difference spectrum of inactivated vs. native enzyme indicated that a single histidyl residue per enzyme subunit was modified by diethyl pyrocarbonate, with a second-order rate constant of 1.8 M^{-1} s^{-1} at pH 6 and 25 °C. The histidyl residue, however, was not essential for activity because in the presence of NADH it was modified without enzyme inactivation and modification of inactivated enzyme was rapidly reversed by hydroxylamine without concomitant reactivation. Progesterone, in the presence of NAD^+, protected the histidyl residue against modification, and this suggests that the residue is located in or near the steroid binding site of the enzyme. Diethyl pyrocarbonate also modified, with unusually high reaction rate, one lysyl residue per enzyme subunit, as demonstrated by dinitrophenylation experiments carried out on the treated enzyme. The correlation between inactivation and modification of lysyl residues at different pHs and the protection by NADH against both inactivation and modification of lysyl residues indicate that this residue is essential for activity and is located in or near the NADH binding site of the enzyme. Cysteinyl, tyrosyl, and tryptophanyl residues and the conformation and molecular size of the enzyme were not modified.

3α,20β-Hydroxysteroid dehydrogenase (17,20β,21-trihydroxysteroid:NAD^+ oxidoreductase, EC 1.1.1.53) from *Streptomyces hydrogenans* is an NAD-dependent enzyme that catalyzes the reversible reduction of 20-keto steroids (Hubener & Sahrholz, 1960; Bergmeyer et al., 1974). The specificity of the enzyme can be exploited for the analysis of steroids (Bergmeyer et al., 1974) and for the preparative-scale stereospecific reduction of the 20-keto group of several corticosteroids (Hilhorst et al., 1983; Carrea, 1984).

3α,20β-HSDH,[1] which consists of four identical subunits, has a molecular weight of 110000 (Blomquist, 1973) and is active only in the tetrameric form (Pasta et al., 1980; Carrea et al., 1984). Studies with affinity-alkylating steroids have demonstrated that there are a histidyl residue, a cysteinyl residue, and two methionyl residues in the steroid binding site of the enzyme (Sweet et al., 1978; Sweet & Samant, 1980), but the studies have not proven that these residues are essential for activity. We have no information about the nature and role of the amino acid residues in the coenzyme binding site.

In this study, DEP, a reagent "selective" for histidyl residues [for review see Miles (1977) and Lundblad and Noyes (1984)] was used to investigate the role of the histidyl residue in the steroid binding site. The results have provided evidence that the histidyl residue is not essential for activity and, more importantly, have proven that a lysyl residue essential for activity is located in or near the NADH binding site of 3α,20β-HSDH.

Experimental Procedures

Materials. Crystalline 3α,20β-HSDH, with a specific activity of 10 units/mg, was obtained from Boehringer Mannheim. Before use, the enzyme was dialyzed at 4 °C against 0.02 M potassium phosphate buffer, pH 7, and then lyophilized. The enzyme concentration was determined as previously reported (Pasta et al., 1980). NAD^+, NADH, and DTNB were from Boehringer Mannheim, and DEP was from Fluka. All other reagents and compounds were of analytical grade.

Enzyme Assay. The activity of the enzyme (20β activity) was determined spectrophotometrically in 0.05 M potassium phosphate buffer, pH 7, containing 0.1 mM NADH and 0.15 mM cortisone (17α,21-dihydroxy-4-pregnene-3,11,20-trione). In a few experiments the 3α activity was also determined with 0.15 mM dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) as a substrate.

Reaction of 3α,20β-HSDH with DEP. DEP was freshly diluted with ethanol for each experiment. The exact concentration of reagent was determined by reaction with imidazole (Dickenson & Dickinson, 1975). The enzyme (0.6 mg/mL, 5.5 μM) was incubated at 25 °C with 0.22–3.50 mM diethyl pyrocarbonate in 0.05 M potassium phosphate buffer, pH 6. The time course of inactivation was followed by measuring the residual enzyme activity in aliquots removed at different times. The activity was expressed as a percentage of the activity of the enzyme incubated similarly but without DEP. The time course of N-carbethoxylation of histidyl residues was followed in a cuvette by recording continuously the change in absorbance at 240 nm, and the number of histidyl residues modified was calculated from the difference in absorption at 240 nm between treated and untreated enzyme at the same concentration (ϵ_{240} = 3200 M^{-1} cm^{-1}; Miles, 1977). In the protection experiments, the enzyme was preincubated with coenzymes or substrates for 30 min prior to the addition of diethyl pyrocarbonate.

Reaction with Hydroxylamine. The inactivated enzyme was incubated at 25 °C with 0.1 M hydroxylamine at pH 7–9. Untreated enzyme was similarly incubated. The time course

[1] Abbreviations: 3α,20β-HSDH, 3α,20β-hydroxysteroid dehydrogenase; DEP, diethyl pyrocarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDNB, 1-fluoro-2,4-dinitrobenzene.

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of reactivation was followed by measuring the enzyme activity in aliquots removed at different times. The time course of removal of the carbethoxyl group from *N*-carbethoxyhistidyl residues was followed by recording continuously the change in absorbance at 240 nm.

Dinitrophenylation. Samples of the enzyme (0.6 mg) were treated with 1 mM DEP under various conditions. At set times, 2 mM histidine was added to block unreacted DEP, and the samples were dialyzed for 8 h in the cold against distilled water and then lyophilized. The samples were dissolved in 0.75 mL of dimethylformamide–water mixture (3:1) titrated to pH 10.3 with triethylamine and reacted with 45 mg of FDNB for 2 h at 25 °C in the dark. The samples were lyophilized, extracted 5 times with ethyl acetate to remove unreacted FDNB, dried under vacuum, and hydrolyzed with 6 M HCl for 18 h at 110 °C before amino acid analysis. The number of lysine residues per enzyme subunit was calculated relative to the amount of arginine, assuming 10 arginines per subunit (Edwards & Orr, 1978).

Determination of Sulfhydryl Residues. The DEP-treated enzyme was dialyzed overnight against 0.05 M potassium phosphate buffer, pH 7, to remove the excess of reagent. The sulfhydryl content was assayed with DTNB (Ellman, 1959) in 0.05 M potassium phosphate buffer, pH 7, and 6 M urea. Untreated enzyme was processed similarly.

Spectroscopic Studies. The intrinsic protein fluorescence of 3α,20β-hydroxysteroid dehydrogenase, excited at 280 nm, was measured in a Jasco FP-550 spectrofluorometer at 25 °C. Circular dichroism measurements were made with a Jasco 500A spectropolarimeter at 25 °C.

Results

Inactivation of 3α,20β-HSDH by DEP. Incubation of 3α,20β-HSDH with DEP in 0.05 M potassium phosphate buffer at pH 6 and 25 °C resulted in a time-dependent loss of enzyme activity. The activity remaining, corrected for decomposition of DEP in a buffered solution, is described by

$$\ln (A/A_0) = -(k/k')I_0(1 - e^{-k't})$$

in which the percent of activity remaining is A/A_0 at time t, I_0 is the initial concentration of DEP, k is the second-order rate constant for reaction of the enzyme with the reagent, and k' is the pseudo-first-order rate constant of hydrolysis of DEP (Gomi & Fujioka, 1983). In 0.05 M potassium phosphate buffer at pH 6 and 25 °C, the value of k' was 0.44×10^{-3} s^{-1} (half-time 1560 s). The data obtained from inactivation experiments of the enzyme at various concentrations of DEP gave straight lines in a plot of the logarithm of remaining activity against $(1 - e^{-k't})/k'$ (Figure 1). A plot of pseudo-first-order rate constants for inactivation (k_{obsd}) against diethyl pyrocarbonate concentration was linear (inset of Figure 1), indicating that no reversible complex between the enzyme and the reagent was formed (Church et al., 1985). The second-order rate constant for inactivation (k) at pH 6 and 25 °C was calculated to be 1.63 M^{-1} s^{-1} from the data of the inset of Figure 1. A double-logarithmic plot of the half-times of inactivation against reagent concentration (Levy et al., 1963) gave a reaction order (stoichiometry) of 1.04, which is compatible with one essential residue per catalytic unit.

The pH dependence of the inactivation of 3α,20β-HSDH by DEP was studied over the range of pH 6.0–9.4. The plot of the logarithm of the second-order rate constants for inactivation (k) against pH is shown in Figure 2, with the plots of the logarithm of the second-order rate constants for reaction of DEP with N^{α}-acetylhistidine and *N*-acetyltyrosine. The rate of inactivation of the enzyme as a function of pH differed markedly from the rate of reaction of *N*-acetyltyrosine and was similar to that of N^{α}-acetylhistidine only in the pH range 6–6.8. It was not possible to reliably determine the rate constant of inactivation of the enzyme at pH higher than 9.4 because of the instability of DEP and because of the extremely

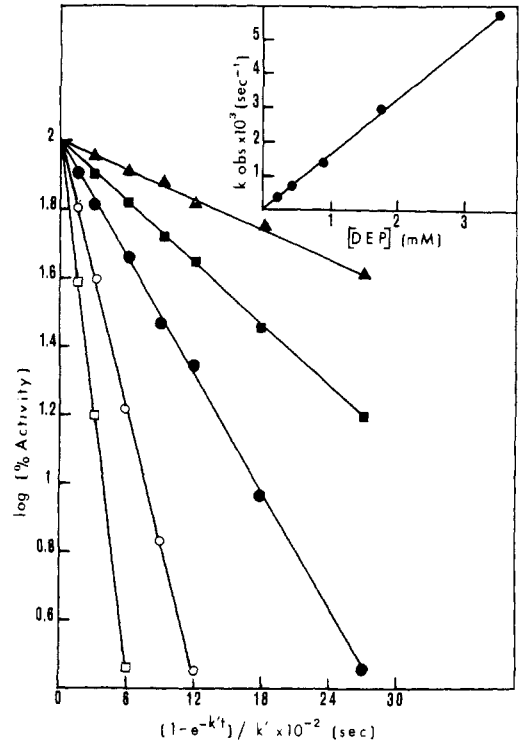

FIGURE 1: Inactivation of 3α,20β-HSDH by DEP. The enzyme (5.5 μM) was incubated with 0.22 (▲), 0.44 (■), 0.88 (●), 1.75 (○), or 3.50 (□) mM DEP at pH 6 and 25 °C in 0.05 M potassium phosphate buffer. (Inset) Plot of apparent first-order rate constants for inactivation (k_{obsd}) at various concentrations of DEP against concentrations of the reagent.

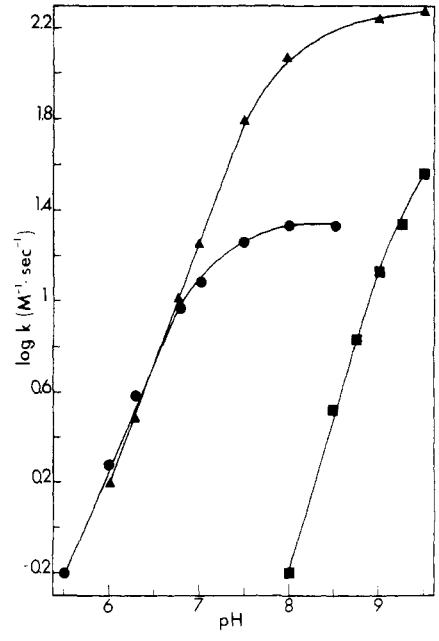

FIGURE 2: Effect of pH on the logarithm of second-order rate constants of inactivation of 3α,20β-HSDH by DEP (▲) and on the logarithm of second-order rate constants for reaction of DEP with N^{α}-acetylhistidine (●) and *N*-acetyltyrosine (■). The enzyme (2 μM) was incubated with DEP (0.1–1 mM) in 0.05 M potassium phosphate buffer, pH 6–9.4, at 25 °C. For the determination of the second-order rate constants of reaction of N^{α}-acetylhistidine or *N*-acetyltyrosine, ϵ_{240} of 3200 M^{-1} cm^{-1} and ϵ_{278} of 1310 M^{-1} cm^{-1} were used (Miles, 1977).

Table I: Reactivation of 3α,20β-HSDH with Hydroxylamine[a]

pH	% reactivation			
	2 h	5 h	21 h	28 h
7.0	5	9	20	21
8.0	11	23	43	46
8.5	16	30	60	67
9.0	20	38	79	81

[a] Enzyme (2 μM) inactivated with 1 mM DEP (2% residual activity) was incubated at 25 °C with 0.1 M hydroxylamine at the pH indicated. The percentage of reactivation is referred to untreated enzyme similarly incubated. Data are means for two determinations.

fast reaction rates at higher pHs. This prevented the exact determination of the pK_a of inactivation of 3α,20β-HSDH.

Protection of 3α,20β-HSDH against Inactivation. NADH, tested between 4 and 705 μM, protected the enzyme (0.6 μM) against inactivation by DEP (1 mM). Determination of the K_d for NADH as protective agent by the procedure of Scrutton and Utter (1965) gave a value of 10 μM, similar to the K_m for NADH (7 μM). The straight line obtained in the plot passed through the origin, which implies that the enzyme–NADH complex is not inactivated by DEP. Instead, NAD^+ (up to 2.80 mM, 39 K_m) or progesterone (up to 0.32 mM, 15 K_m) did not protect the enzyme, while NAD^+ (1.40 mM) plus progesterone (0.32 mM) partially protected the enzyme (half-time of inactivation about 50% longer). Identical results were obtained when the 3α activity of the enzyme was followed, and this is further confirmation that the same active site is responsible for both 3α and 20β activity (Sweet & Samant, 1980, 1981).

Reversibility of Inactivation. Incubation of the inactivated enzyme with hydroxylamine partially regenerated the activity of 3α,20β-HSDH (Table I). The reactivation process, which was pH dependent, was very slow. The highest reactivation yield (81%) was obtained after 28 h of incubation with 0.1 M hydroxylamine, pH 9. Higher concentrations of hydroxylamine (0.3 or 0.5 M) did not substantially increase the reactivation yield.

Amino Acid Residues Modified. DEP modified only one of the four (Edwards & Orr, 1978) histidyl residues present in each subunit. The second-order rate constant for reaction of the residue was 1.8 M^{-1} s^{-1} at pH 6 and 10.6 M^{-1} s^{-1} at pH 7. These values are similar to the second-order rate constant values for enzyme inactivation (Figure 2), and this might suggest that the modification of the histidyl residues was responsible for enzyme inactivation. However, the results shown in Figure 3 disprove this hypothesis. NADH, which protected the enzyme against inactivation, did not protect the histidyl residues against modification. Furthermore, the treatment with hydroxylamine rapidly reversed the modification of histidyl residues but did not restore substantial enzymatic activity (Figure 3). Therefore, the histidyl residues modified by DEP are not essential for activity.

Progesterone plus NAD^+, which poorly protected the enzyme against inactivation, markedly protected the histidyl residues against modification by DEP, whereas NAD^+ or progesterone alone had no effect (Table II). The fact that 3α,20β-HSDH operates by an ordered mechanism, in which the cofactor binds first (Betz & Warren, 1968), strongly suggests that the histidyl residues modifiable by DEP are located in or near the steroid binding site of the enzyme. The presence of residual histidyl residues in enzyme that was inactivated in buffer alone may be ascribed to partial hydrolysis of *N*-carbethoxyhistidyl residues—which have a half-life of about 55 h at pH 6 and 25 °C (Melchior & Fahrney, 1970)—during processing of the samples.

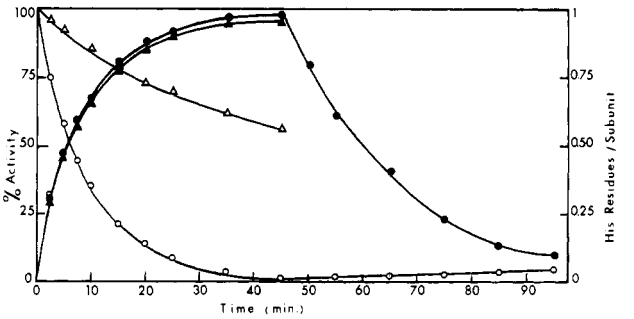

FIGURE 3: Inactivation (open symbols) and modification of histidyl residues (solid symbols) of 3α,20β-HSDH with DEP. The enzyme (5.5 μM) was incubated with 1 mM DEP in 0.05 M potassium phosphate buffer, pH 6, at 25 °C in the presence (△, ▲) or absence (○, ●) of 70 μM NADH. After 45 min of incubation, hydroxylamine was added to a final concentration of 0.1 M and pH 7. Corrections have been made for dilutions.

Table II: Residual Histidyl Residues in DEP-Inactivated 3α,20β-HSDH[a]

	residual histidyl residues[b]
enzyme in buffer	0.20
enzyme + 1.40 mM NAD^+	0.19
enzyme + 0.32 mM progesterone	0.23
enzyme + 1.40 mM NAD^+ + 0.32 mM progesterone	0.91

[a] Enzyme (5.5 μM) was inactivated (3–4% residual activity) with 1 mM DEP, pH 6, at 25 °C, and then treated with 2 mM histidine for 30 min to block unreacted DEP. The samples were dialyzed extensively at pH 6 for 20 h to remove the small molecular weight compounds (NAD^+, progesterone, free histidine), and then the residual histidyl residues were determined spectrophotometrically at 240 nm with DEP. A control experiment with 3α,20β-HSDH not inactivated with DEP but otherwise processed in the same way showed that the histidyl residues that reacted with DEP were 0.98 per enzyme subunit. [b] Expressed as moles per mole of enzyme subunit. The values do not indicate the total residual histidyl residues but only the histidyl residues accessible to DEP. Since in 3α,20β-HSDH only one histidyl residue per subunit reacts with DEP, the values refer to that. Data are means for two determinations.

The modification of tyrosyl residues can be excluded, since no decrease in absorbance at 278 nm—ascribable to O-carbethoxylation of tyrosyl residues (Miles, 1977)—was observed when the enzyme was treated with DEP under conditions producing complete inactivation of 3α,20β-HSDH (30-min incubation with 3 mM DEP, pH 6–7, 25 °C). The DEP treatment did not appreciably change the content of cysteinyl residues, either. When the sulfhydryl content of the inactivated enzyme (2% of residual activity) was examined, the value was the same as that of the untreated enzyme (one residue per enzyme subunit). The involvement of cysteinyl residues in the process of enzyme inactivation by DEP is also excluded by the fact that 3α,20β-HSDH is fully active after modification with DTNB of cysteinyl residues, which, therefore, are not essential for activity. The modification of tryptophanyl residues was also ruled out, because the intrinsic protien fluorescence of 3α,20β-HSDH was not modified by inactivation with DEP.

To find out if lysyl residues react with DEP, dinitrophenylation of the enzyme was carried out. The experiments were based on the ability of the carbethoxyl group to offer protection against reaction with FDNB (Melchior & Fahrney, 1970; Wells, 1973). The data in Table III show that DEP protected approximately one lysyl residue per enzyme subunit against dinitrophenylation. The correlation between the amount of lysyl residues found and the extent of loss of enzymatic activity was good, at both pH 6 and pH 8. It should be emphasized that at pH 8 the reaction with DEP was blocked

Table III: Residual Lysyl Residues in FDNB-Treated Enzyme[a]

sample	% inactivation by DEP	residual lysyl residues after FDNB treatment[b]
control	0	0.36
enzyme inactivated with DEP at pH 6[c]	96	1.32
enzyme inactivated with DEP at pH 8[d]	99	1.43
enzyme treated with DEP in the presence of NADH[e]	16	0.50

[a] For details see Experimental Procedures. [b] Expressed as moles per mole of enzyme subunit. Arginine was used as a reference, at 10 mol/mol of enzyme subunit (Edwards & Orr, 1979). Data are means for two determinations. [c] 3α,20β-HSDH was incubated at 25 °C with 1 mM DEP in 0.05 M potassium phosphate buffer, pH 6, for 35 min. [d] 3α,20β-HSDH was incubated at 25 °C with 1 mM DEP in 0.05 M potassium phosphate buffer, pH 8, for 2 min. [e] 3α,20β-HSDH was incubated as in footnote *c* except for the presence of 0.3 mM NADH ($30K_d$).

after 2 min of incubation, and this indicates that the rate of reaction of the lysyl residues was very fast. The fact that the lysyl residues were dinitrophenylated after enzyme treatment with DEP in the presence of a saturating concentration of NADH ($30K_d$) demonstrates that these residues were protected by the coenzyme against modification by DEP. Therefore, the data in Table III indicate that a lysyl residue essential for activity is located in or near the NADH binding site of 3α,20β-HSDH.

Properties of Modified 3α,20β-HSDH. Inactivated 3α,20β-HSDH had the same intrinsic protein fluorescence, molar ellipticity, and elution volume (Bio-Gel A-1.5m column) as the native enzyme, and this indicates that DEP treatment did not induce conformational modifications, dissociation, or aggregation of the protein. The partially inactivated enzyme (35% residual activity) had K_m values for NADH and progesterone identical with those of the native enzyme, and this rules out the possibility that the loss of activity after modification might also be due to an increase in K_m values.

Discussion

The treatment of 3α,20β-HSDH with DEP did not alter the conformation or molecular size of the enzyme. Thus, the inactivation can be ascribed to the modification of amino acid residue(s) essential for catalytic action of the enzyme.

Of the several amino acid side chains (imidazole, phenolates, sulfhydryl, α- and ε-amines) that can be modified by DEP, histidyl residues are believed to react in a more specific way, although carbethoxylation of amino and tyrosyl groups has also been reported (Melchior & Fahrney, 1970; Wells, 1973; Burstein et al., 1974). In the case of 3α,20β-HSDH, the treatment with DEP modified a single histidyl residue per enzyme subunit, with a second-order rate constant of 1.8 M^{-1} s^{-1} at pH 6 and 25 °C. However, the histidyl residue is not essential for activity because in the presence of NADH the modification takes place without enzyme inactivation and, furthermore, there is no correlation between reversal of modification by hydroxylamine and reactivation (Figure 3). The protection by progesterone against modification indicates that the histidyl residue is located in the steroid binding site of the enzyme. This is in agreement with the data of Sweet and Samant (1978), who identified a histidyl residue in the steroid binding site of 3α,20β-HSDH by affinity radioalkylation experiments with ([2-^{3}H]bromoacetoxy)progesterone derivatives. The affinity labeling also inactivated the enzyme. However, the present results strongly suggest that the irreversible occupancy of the binding site by the bulky steroid derivatives, rather than the modification of the histidyl residue, was responsible for inactivation.

DEP also modified one lysyl residue per enzyme subunit, as demonstrated by dinitrophenylation experiments (Table III). The correlation between inactivation and modification of lysyl residues at different pHs and the protection by NADH against inactivation and modification of lysyl residues indicate that the lysyl residue is essential for activity and is located in or near the NADH binding site. The modification of lysyl residues by DEP has been described previously for pepsin (Melchior & Fahrney, 1970), which was not inactivated by the treatment, and for phospholipase A_2 (Wells, 1973), which was inactivated at pH 6 and 25 °C with a second-order rate constant of 0.25 M^{-1} s^{-1}. The second-order rate constants of inactivation of 3α,20β-HSDH are 1.63 M^{-1} s^{-1} at pH 6 or 190 M^{-1} s^{-1} at pH 9.4, at 25 °C, values much higher than those of primary amines in model systems (Miles, 1977).

However, there are many examples of residues in proteins that are much more reactive than expected. For instance, the essential histidyl residues of lactate dehydrogenase (Holbrook & Ingram, 1973), yeast alcohol dehydrogenase (Dickenson & Dickinson, 1975), and horse liver alcohol dehydrogenase (Hennecke & Plapp, 1983) are 10–20 times more reactive with DEP than free histidine, and in the case of horse liver alcohol dehydrogenase the pK_a value is also markedly higher than that of histidine (9.6 instead of 7). Other examples are the ε-amino group of Lys-41 of ribonuclease and the amino group of the terminal valyl residue of the α chain of human hemoglobin, which are dinitrophenylated at an unusually high rate (Glazer, 1970). The high reactivity of horse liver alcohol dehydrogenase was attributed to the ionization of the zinc–water system of the enzyme (Hennecke & Plapp, 1983) and that of ribonuclease to the proximity of an arginyl residue, which decreases the pK_a of the lysyl group (Glazer, 1970). The data presently available do not enable us to propose an explanation for the high reactivity with DEP of the essential lysyl residues of 3α,20β-HSDH.

The reactivation time of 3α,20β-HSDH by hydroxylamine (Table I) was markedly longer than the time needed to remove the carbethoxyl groups from histidyl (half-time 15 min with 0.1 M hydroxylamine, pH 7, 25 °C) or tyrosyl residues (half-time 42 min with 0.1 M hydroxylamine, pH 8, 25 °C), and this is additional evidence that no essential histidyl or tyrosyl residue was modified by DEP. The reversal of lysine modification, which has been reported to be resistant to hydroxylamine treatment (Melchior & Fahrney, 1970), could be due to the unusual reactivity of this residue in 3α,20β-HSDH. This result also indicates that the reversibility of inactivation by hydroxylamine is not an absolute criterion for exclusive modification of histidine and tyrosine. The finding that NAD^+, unlike NADH, completely failed to protect the enzyme against inactivation suggests that the two nicotinamide cofactors have different positioning in the active site, depending on their oxidation state or charge.

In conclusion, the modification of 3α,20β-HSDH by DEP has demonstrated the presence of an essential lysyl residue in or near the NADH binding site of the enzyme and has shown that the histidyl residue located in the steroid binding site is not essential for activity. Studies with other selective reagents are in progress to obtain further information about the amino acid residues involved in the catalytic process of 3α,20β-HSDH.

Registry No. NADH, 58-68-4; 3α,20β-hydroxysteroid dehydrogenase, 72855-18-6; hydroxylamine, 7803-49-8; L-histidine, 71-00-1; progesterone, 57-83-0; diethyl pyrocarbonate, 1609-47-8; L-lysine, 56-87-1; N^{α}-acetylhistidine, 2497-02-1; *N*-acetyltyrosine, 537-55-3.

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Effect of Hydrogen Peroxide on the Iron-Containing Superoxide Dismutase of *Escherichia coli*†

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ABSTRACT: The iron-containing superoxide dismutase from *Escherichia coli* is inactivated by H_2O_2 to a limit of ~90%. When corrected for the H_2O_2-resistant portion, this inactivation was first order with respect to residual activity and exhibited a pseudo-first-order rate constant of 0.066 min^{-1} at 25 °C in 0.24 mM H_2O_2 at pH 7.8. The superoxide dismutase activity remaining after treatment with H_2O_2 differed from the activity of the native enzyme with respect to heat stability, inhibition by azide, and inactivation by light in the presence of rose bengal and by *N*-bromosuccinimide. The native and the H_2O_2-modified enzymes were indistinguishable by electrophoresis on polyacrylamide gels. Inactivation of the enzyme by H_2O_2 was accompanied by loss of tryptophan and some loss of iron, but there was no detectable loss of histidine or of other amino acids. H_2O_2 treatment caused changes in the optical spectrum of the enzyme. Inactivation of the enzyme by H_2O_2 depends upon the iron at the active site. Thus, the apoenzyme and the manganese-substituted enzyme were unaffected by H_2O_2. We conclude that reaction of H_2O_2 with the iron at the active site generates a potent oxidant capable of attacking tryptophan residues. A mechanism is proposed.

Superoxide dismutases containing copper and zinc (McCord & Fridovich, 1969), manganese (Keele et al., 1970), and iron (Yost & Fridovich, 1973) have been isolated and characterized. The manganese and the iron enzymes show a great deal of amino acid sequence (Steinman, 1978; Harris et al., 1980) and structural (Stallings et al., 1984) homology. H_2O_2 inactivates the Cu,Zn and the Fe, but not the Mn, superoxide dismutases SODs[1] (Steinman, 1982). In the case of the Cu,ZnSOD, bleaching of the Cu(II) preceded inactivation, and the rate of inactivation increased with pH in a manner defining a pK_a of ~10.2 (Hodgson & Fridovich, 1975). This effect of pH was subsequently explained in terms of HO_2^- being the active species, rather than H_2O_2 (Blech & Borders, 1983). Inactivation of Cu,ZnSOD by H_2O_2 is associated with the loss of one histidine residue per subunit (Bray et al., 1974).

Inactivation of the FeSOD from *Pseudomonas ovalis* has been correlated with losses of tryptophan, histidine, and cysteine residues (Yamakura, 1984). We have previously

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[1] Abbreviations: FeSOD, iron-containing superoxide dismutase; Cu,ZnSOD, copper- and zinc-containing superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; SDS, sodium dodecyl sulfate; NBS, *N*-bromosuccinimide; TSY, trypticase–soy yeast extract; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Da, dalton(s).

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