Role of Tyrosyl and Arginyl Residues in Rat Liver Microsomal Stearylcoenzyme A Desaturase†

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ABSTRACT: Stearyl-CoA desaturase is rapidly inactivated in borate buffer by the arginine-specific reagents 2,3-butanedione and 1,2-cyclohexanedione. In the presence of butanedione, inactivation is correlated with the loss of arginyl residues. The loss of 75% of the initial enzymatic activity is accompanied by the modification of two arginyl residues. Butanedione-treated enzyme can be reactivated by the removal of the reagent and borate, and the recovery of activity is correlated with the regeneration of arginyl residues. The substrate, stearyl-CoA, protects desaturase from inactivation by butanedione, whereas free CoA, acetyl-CoA, and stearate, which do not bind to the active site, are not effective. In contrast, the interactions of desaturase with cytochrome b_5 and oxygen, as well as the artificial substrate *p*-cresol, are not affected by treatment with butanedione. These data suggest that arginyl residues may play a specific role at the binding site for the negatively charged CoA moiety of the substrate. Desaturase is also inactivated by nitration of tyrosyl residues with tetranitromethane or O-acetylation of tyrosyl residues by *N*-acetylimidazole or acetic anhydride. The enzyme cannot be reactivated by deacetylation of the *O*-acetyltyrosyl residues with hydroxylamine. Both nitration and acetylation of desaturase caused a loss of the iron prosthetic group from the protein. Furthermore, upon nitration, the rate of oxidation of *p*-cresol and the autoxidizability of desaturase both decreased to the same extent as the stearyl-CoA desaturase activity. This suggests that tyrosyl residues may be involved in the chelation of the iron prosthetic group.

The formation of monounsaturated fatty acids in rat liver is catalyzed by a microsomal enzyme, stearyl-CoA[1] desaturase (Oshino et al., 1966; Marcel et al., 1968; Shimakata et al., 1972; Strittmatter et al., 1974; Enoch et al., 1976). This enzyme, which has been obtained in a highly purified form (Strittmatter et al., 1974; Strittmatter and Enoch, 1978), requires NADH, NADH–cytochrome b_5 reductase, cytochrome b_5, stearyl-CoA, oxygen, and phospholipid for maximal activity (Strittmatter et al., 1974). Initial characterization of the desaturase reaction was restricted because of the instability of the desaturase in the detergents required in the purification procedure. Stable, detergent-free preparations of purified desaturase have subsequently been prepared by incorporation of the enzyme into phospholipid vesicles (Enoch et al., 1976; Strittmatter et al., 1978). Such preparations were used to examine the substrate specificity of the desaturase, the interaction of acyl-CoA with the enzyme, and the possible roles of phospholipid in the mechanism of desaturation (Enoch et al., 1976).

More detailed studies have now been carried out using group-specific reagents to modify enzyme activity. In this paper, we describe the treatment of desaturase with reagents which are highly specific for arginyl and tyrosyl residues. The results of these studies suggest that positively charged arginyl residues of the desaturase may be involved in the binding of the CoA moiety of the negatively charged substrate, stearyl-CoA, and that tyrosyl residues on the desaturase may function in the binding of the iron prosthetic group at the catalytic center of the enzyme.

Materials and Methods

Cytochrome b_5, NADH–cytochrome b_5 reductase, and the catalytic fragment of the reductase, formed by lysosomal cathepsins, were prepared from steer liver (Strittmatter, 1973; Spatz and Strittmatter, 1973; Strittmatter et al., 1978). Acetic anhydride (Aldrich Chemical Co.), 2,3-butanedione, tetranitromethane, 5,5′-dithiobis(2-nitrobenzoic acid), *p*-chloromercuriphenylsulfonic acid (Sigma), *N*-acetylimidazole, and ethyl acetimidate (Pierce) were used without further purification. Unless otherwise stated, all studies were carried out in 20 mM Tris–acetate buffer (pH 8.1) containing 100 mM sodium chloride.

Stearyl-CoA desaturase was purified and suspended in egg lecithin as described previously for *fraction II* (Strittmatter et al., 1974). Butylated hydroxytoluene (Sigma) was included as an antioxidant (1 mg/500 mg of lipid). *Fraction II* was then filtered through 20 volumes of Sephadex G-25 at 0 °C to remove residual deoxycholate (Enoch et al., 1976). This preparation, which was used in all the studies described below, contained approximately 200 mol of phospholipid and less than 1 mol of detergent per mol of desaturase. The enzyme in this form could be stored under nitrogen without loss of activity for several weeks at 0 °C or for at least 1 year at −75 °C. Desaturase concentrations were measured spectrophotometrically from the absorbance at 280 nm in Tris buffer containing 0.5% sodium dodecyl sulfate using an extinction coefficient of 125 mM^{-1} cm^{-1} (Enoch et al., 1976).

Before desaturase activity can be measured, cytochrome b_5 and reductase must be incorporated into the desaturase–phospholipid complex. This was accomplished by incubating the desaturase (10–20 μM) with cytochrome b_5 (100 μM) and reductase (4 μM) at 25 °C for 30 to 60 min. The enzyme was routinely assayed, however, following a more rapid method of incorporation. In this method, desaturase (10–20 μM) was incubated with cytochrome b_5 (100 μM) and sodium deoxycholate (0.25%) at 0 °C for 10 min. One milliliter of Tris buffer at 25 °C was then added to 10 μL of this mixture, followed by 5 μL of the catalytic fragment of reductase (20 μM). The rate of stearyl-CoA desaturation was then estimated by the spectrophotometric assay described elsewhere (Strittmatter et al., 1974; Strittmatter and Enoch, 1978). One unit of enzyme activity is defined as that which forms 1 mol of oleyl-CoA/min.

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[1] Abbreviations used are: NADH, nicotinamide adenine dinucleotide; CoA, coenzyme A; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

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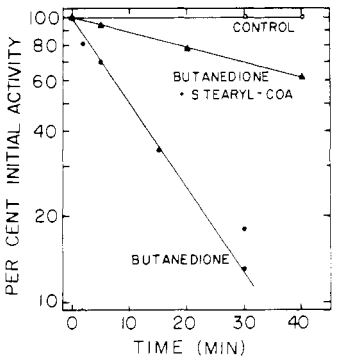

FIGURE 1: The kinetics of inactivation of desaturase by butanedione. Desaturase (17 μM) was incubated in 50 mM sodium borate buffer (pH 8.1) at 25 °C with butanedione (18 mM) and stearyl-CoA (120 μM) as shown above. Aliquots were withdrawn at the indicated times, chilled to 0 °C, and used immediately for the assay of desaturase activity: (○) control, no butanedione; (●) butanedione; (▲) butanedione + stearyl-CoA.

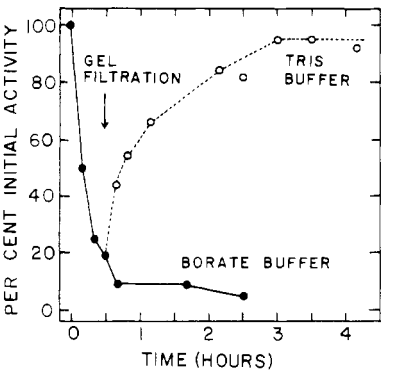

FIGURE 2: Reversibility of desaturase inactivation by removal of borate. Desaturase (16 μM, specific activity = 190 units/mg) was treated with butanedione (40 mM) in 50 mM sodium borate buffer (pH 8.1); after 30 min at 25 °C, 1 mL of this mixture was filtered through Sephadex G-25 (20 × 1 cm) equilibrated either with the same borate buffer or 20 mM Tris-acetate/100 mM NaCl buffer (pH 8.1). The enzyme, which eluted in the void volume within 3 to 4 min, was incubated at 25 °C, and aliquots were withdrawn at the times indicated for measurement of desaturase activity: (●) borate buffer; (○) Tris buffer.

The rate of *p*-cresol oxidation was estimated by the same procedure using 5 mM *p*-cresol as the substrate instead of stearyl-CoA. The rate of desaturase autoxidation was estimated by measuring the initial rates of cytochrome b_5 oxidation (following reduction by a small amount of NADH) in the presence and absence of 10 mM KCN. The difference in rates was taken to be the portion due to desaturase, since the enzyme is completely inhibited by 10 mM KCN (Oshino et al., 1966; Strittmatter et al., 1974).

Cytochrome b_5 (Rogers and Strittmatter, 1973) and reductase (Rogers and Strittmatter, 1974) concentrations were determined as described previously. Amino groups were determined with 2,4,6-trinitrobenzenesulfonic acid (Sigma) by a modification of the procedure of Habeeb (1966). A 10-μL sample of desaturase containing 0.1 to 1 nmol of protein was added to 100 μL of a solution containing 0.1 M sodium borate buffer (pH 8.3)/2-propanol/25 mM trinitrobenzenesulfonic acid (50:50:5), and the mixture was incubated at 50 °C. After 15 min, 0.4 mL of 0.1 M HCl in 50% 2-propanol was added and the absorbance was measured at 340 nm against a reagent blank in which desaturase was omitted. The concentration of amino groups was calculated using an extinction coefficient of 14.9 mM^{-1} cm^{-1} (Ozols and Strittmatter, 1966). *O*-Acetyltyrosine was determined spectrally from the increase in absorbance at 275 nm following deacetylation with 0.25 M neutralized hydroxylamine using an extinction difference of 1.16 mM^{-1} cm^{-1} (Riordan et al., 1965). Arginine was measured after acid hydrolysis by the modified Sakaguchi method of Izumi (1965).

Sulfhydryl groups were determined using 5,5′-dithiobis(2-nitrobenzoic acid) according to the method of Ellman (1959). Iron determinations on desaturase (Enoch et al., 1976), phospholipid determinations (Chen et al., 1956), and gel electrophoresis in the presence of sodium dodecyl sulfate (Strittmatter et al., 1974) were carried out as described previously. Amino acid analyses were performed on acid hydrolysates by the method of Adams et al. (1977) using a glass capillary column in a Perkin-Elmer Sigma 3 gas chromatograph. Protein samples were prepared by precipitation with 10 volumes of 0.1 N HCl in acetone; the precipitates were washed once with acetone. Acid hydrolysis in 6 N HCl containing 0.1% mercaptoethanol was carried out in evacuated, sealed ampules for 22 h at 110 °C. Tryptophan was determined spectrally in the presence of 1% sodium dodecyl sulfate by the method of Beaven and Holliday (1952).

Results

Inactivation of Desaturase by Butanedione. Incubation of stearyl-CoA desaturase with butanedione in 50 mM borate buffer (pH 8.1) results in a rapid loss of enzyme activity (Figure 1). Prolonged incubation (1–2 h) resulted in complete inactivation of the enzyme. The substrate stearyl-CoA provides considerable protection from this inactivation. In either case, inactivation follows pseudo-first-order kinetics. Borate is essential, as shown previously for butanedione reaction with arginyl residues (Riordan, 1973; Borders and Riordan, 1975; Riordan, 1977); no activity was lost in 30 min when the borate buffer was replaced with Tris-acetate buffer (pH 8.1). The inactivation of desaturase in borate buffer is reversible. After the enzyme activity was reduced to 20% of the initial activity in the presence of butanedione, the preparation was filtered through Sephadex G-25 to remove the reagent and borate. The activity of the modified enzyme increased with time until 95% of the native activity was regained after 2.5 h at 25 °C (Figure 2). In a number of experiments, the recovery of native activity varied from 75 to 100%. This could be due to the fact that some of the butanedione-modified enzyme undergoes irreversible inactivation during the 2- to 3-h incubation (see Riordan, 1973). When the reagent was removed leaving the modified enzyme in the presence of borate buffer, there was no recovery of enzyme activity (Figure 2).

Modification of Arginyl Residues by Butanedione and Cyclohexanedione. The known specificity of butanedione, the borate effect, and the reversibility of inactivation (Riordan, 1973; Riordan et al., 1977; Marcus et al., 1976; Lange et al., 1974) strongly suggest that the inactivation of desaturase is due to the modification of essential arginyl residues. Treatment with cyclohexanedione, another arginine-specific reagent (Patthy and Smith, 1975a, b) also causes inactivation of desaturase. Desaturase (16 μM) lost 80% of its original activity when incubated with 10 mM cyclohexanedione in 0.1 M sodium borate buffer (pH 8.1) at 28 °C for 2 h.

Arginine analysis showed that inactivation of desaturase by butanedione was accompanied by a modification of arginyl residues in the enzyme (Figure 3). The loss of two arginyl residues was accompanied by the loss of 75% of the original activity. When the butanedione-modified enzyme was filtered to remove the reagent and borate, an increase in enzyme activity occurred concomitantly with the regeneration of the two arginyl residues.

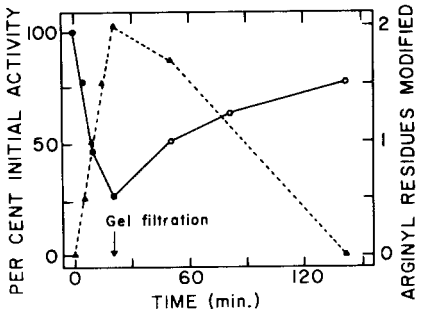

FIGURE 3: The correlation of the reversible modification of desaturase activity with the modification of arginyl residues. The treatment of desaturase with butanedione and the removal of reagent and borate by gel filtration were carried out as described under Figure 2. At various times, samples of the reaction mixture were chilled rapidly to 0 °C and used immediately for measurement of desaturase activity, (●) before and (○) after gel filtration, and arginine, (▲) before and (△) after gel filtration.

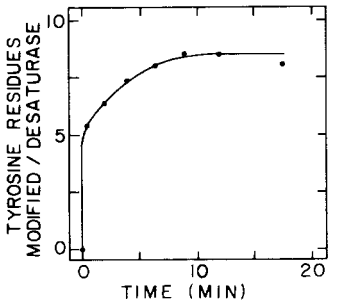

FIGURE 4: Modification of the tyrosines in desaturase by tetranitromethane. The enzyme (1.4 μM) was incubated with 1.2 mM tetranitromethane in Tris buffer at 28 °C. The reaction was carried out in a cuvette so that spectra could be taken at intervals. The amount of 3-nitrotyrosine formed was determined from the absorbance at 428 nm using a molar extinction coefficient of 4100 cm^{-1} (Sokolovsky et al., 1966).

The protective effect of stearyl-CoA is concentration dependent; the half-time for inactivation with 1 mM stearyl-CoA was 2.3 times longer than with 0.2 mM stearyl-CoA and 13 times longer than no stearyl-CoA (Table I). In contrast, smaller effects were observed with free CoA, acetyl-CoA, and ammonium stearate, each of which was shown previously not to bind to the enzyme active site (Enoch et al., 1976). Butanedione-treated desaturase preparations with as little as 9% of the original stearyl-CoA desaturase activity showed the same rate of *p*-cresol and autoxidation as the native enzyme (Table II), indicating that the reduction of the iron prosthetic group by cytochrome b_5 and its reoxidation by oxygen or oxygen plus *p*-cresol were unaffected.

Tyrosine Modification by Tetranitromethane. Tetranitromethane has been found to be a selective, mild reagent for the nitration of tyrosine at pH 8 (Sokolovsky et al., 1966) and is frequently used in the assignment of functional significance to tyrosyl groups (Banerjee et al., 1975; Markland et al., 1975; Wolff et al., 1975; Yazawa and Noda, 1976). Incubation of desaturase with 1 mM tetranitromethane results in the formation of 8.5 mol of 3-nitrotyrosine per mol of enzyme (Figure 4). The reaction is rapid (half-time <0.5 min), and no further modification is observed after 10 min of reaction. Since desaturase contains 24 tyrosyl residues (Strittmatter et al., 1974), most of the tyrosyl residues appear to be unreactive with tetranitromethane. Enzyme activity is completely abolished by incubation of desaturase with a 70- to 200-fold molar excess of tetranitromethane, conditions which lead to the modification of two to five tyrosyl residues. Reaction with as little as a 20-fold molar excess leads to the modification of one to two tyrosyl residues and the loss of 83% of the original activity (Table III), indicating that the enzyme may contain a limited number of essential tyrosyl residues. Tetranitromethane is also known to react with sulfhydryl groups in proteins and, under certain conditions, with the amino acids tryptophan, histidine, and methionine (Sokolovsky et al., 1969). Amino acid analysis of desaturase treated with a 20-fold molar excess of tetranitromethane indicated that of the potentially reactive residues

TABLE I: Effects of Substrate and Substrate Analogues on the Inactivation of Stearyl-CoA Desaturase by Butanedione.[a]

compd added	desaturase act. remaining[b] (%)	half-time for inact.[c] (min)	binding to desaturase
none	24	10	
stearyl-CoA, 0.2 mM	78	57	+
stearyl-CoA, 1.0 mM	90	130	+
CoA, 1 mM	47	18	−
acetyl-CoA, 1 mM	32	12	−
ammonium stearate, 0.1 mM	36	14	−

[a] The enzyme was incubated with butanedione as described under Figure 1 with the additions shown above. After 20 min, reaction mixtures were chilled and samples were used immediately for measurement of desaturase activity. [b] Desaturase activity of untreated enzyme was taken as 100%. [c] Based on the assumption of pseudo-first-order kinetics of inactivation which was found in Figure 1.

TABLE II: Effect of Butanedione on *p*-Cresol Oxidation and the Autoxidation of Stearyl-CoA Desaturase.[a]

time (min)	desaturase activity[b] stearyl-CoA oxidation	*p*-cresol oxidation	autoxidation
0	59	111	3.9
30	24	110	4.0
60	9	104	4.0

[a] The enzyme was treated with 18 mM butanedione as described under Figure 1. After 30 min, the samples were chilled to 0 °C and used immediately for the measurement of desaturase activities as described under Materials and Methods. [b] nmol of NADH oxidized min^{-1} (mg of desaturase)$^{-1}$.

TABLE III: Effect of Tyrosine Modification on Stearyl-CoA Desaturase Activity.[a]

treatment	molar excess of reagent	desaturase act. remaining (%)	Tyr modified per desaturase	amino groups modified per desaturase
none		100[b]	0	0
tetranitromethane (5 min, 25 °C)	20	17	1-2	0
N-acetylimidazole (30 min, 25 °C)	1600	14	3	15
acetic anhydride (10 min, 0 °C)	100 or 400	0	4	23

[a] The enzyme (31 μM) was incubated as shown below in Tris buffer; the reaction mixtures were then chilled to 0 °C. Samples were taken immediately for the measurement of desaturase activity and modified tyrosine and amino group concentration as described under Materials and Methods and Figure 4. [b] The initial specific activity was 85 units/mg of desaturase. Desaturase activity was not affected by any of the reaction conditions in the absence of reagent.

TABLE IV: Amino Acid Composition of Native and Tetranitromethane-Modified Desaturase.[a]

amino acid	native desaturase	tetranitromethane-modified desaturase[b]	Δ[c]
Ala	36	37	+1
Val	26	28	+2
Gly	33	33	0
Ile	23	24	+1
Leu	64	63	−1
Pro	19	19	0
Thr	24	23	−1
Ser	30	29	−1
Asx	31	32	+1
Met	10	11	+1
½-Cys	7	7	0
Phe	25	25	0
Glx	26	25	−1
Tyr	25	21	−4
His	11	13	+2
Lys	21	20	−1
Arg	25	26	+1
Trp	17	17	0

[a] The values are amino acid residues per mol of desaturase (453 residues, Strittmatter et al., 1974) and are nearest integer values based on the average of four determinations. They are expressed relative to the proline content. [b] Prepared as described in Table III. [c] The difference between native and tetranitromethane-modified desaturase.

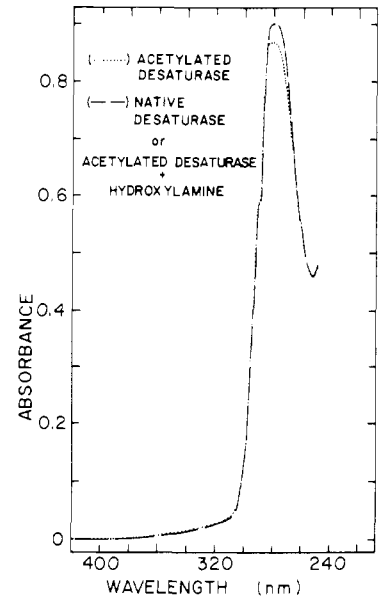

FIGURE 5: Absorption spectra of native and acetylated desaturase. Spectra were recorded in cuvettes with a 1-cm light path on samples at 0.38 mg of protein/mL in Tris buffer containing 0.2% sodium dodecyl sulfate. Acetylated desaturase (· · ·) was prepared by treating enzyme (30 μM) with acetic anhydride (100-fold molar excess) for 10 min at 0 °C in Tris buffer. After recording the spectrum of the acetylated enzyme neutralized hydroxylamine (250 mM, final concentration) was added, and after a 30-min incubation at 25 °C the spectrum was recorded again (- -).

only tyrosine was modified significantly (Table IV). However, since the reproducibility of the analysis on the same sample is approximately ±5%, these data do not absolutely exclude the possibility that the loss of enzyme activity is associated instead with modification of a single residue of one of the other amino acids.

Tetranitromethane may also cause nitration of double bonds (Sokolovsky et al., 1966; Schmidt and Fischer, 1920), although under the reaction conditions used in the present study (Table III) tetranitromethane does not react with the egg lecithin liposomes used in the desaturase preparations. Protein cross-linking has been observed in some studies with tetranitromethane (Doyle et al., 1968). Desaturase treated with a 20-fold molar excess of tetranitromethane as in Table III showed no cross-linking when examined by gel electrophoresis in the presence of sodium dodecyl sulfate.

O-Acetylation of Tyrosyl Residues. Treatment of desaturase with *N*-acetylimidazole or acetic anhydride also results in a loss of enzymatic activity (Table III) and both reagents cause a decrease in the molar absorptivity of the protein at 275 nm (Figure 5), indicating the O-acetylation of tyrosyl residues (Riordan et al., 1965). The *O*-acetyltyrosyl residues of the protein could be deacetylated by hydroxylamine and a quantitative estimate obtained from the resultant increase in absorbance at 275 nm (Figure 5). Complete inactivation of desaturase is observed after the modification of four tyrosyl residues by acetic anhydride. The enzymatic activity was not restored by removal of the *O*-acetyl groups by hydroxylamine. Treatment with hydroxylamine does not effect the iron content or enzymatic activity of native desaturase. The correlation of O-acetylation of tyrosyl residues with the loss of desaturase activity is complicated by the fact that the reagents also cause acetylation of lysyl residues (Table III).

Function of Tyrosyl Residues. Since the desaturase is known to be a nonheme iron protein, we considered the possibility that phenolic oxygens might be involved in iron coordinations and that tyrosine modification might result in the loss of iron from the enzyme. This would explain why the loss of enzymatic activity upon acetylation was not reversed by hydroxylamine. The enzyme was acetylated with *N*-acetylimidazole as described in Table III and then 0.5 M hydroxylamine was added to deacetylate O-acetyltyrosyl residues and destroy excess reagent. After 5 min at 25 °C spectral analysis showed that there were no *O*-acetyltyrosyl groups or *N*-acetylimidazole remaining; however, the enzymatic activity was still only 14% that of the native enzyme. If O-acetylation results in iron loss from the enzyme, this loss may be irreversible under these conditions. We have thus far been unable to reconstitute enzyme activity under any condition using enzyme from which iron had been removed by bathophenanthroline sulfonate.

We were able to show in a more direct way that nitration of tyrosyl residues causes a loss of iron from the enzyme. Desaturase was treated with tetranitromethane as described in Table III and then subjected to gel filtration on Sephadex G-25 to remove excess reagent and any unbound iron. The modified desaturase, which contained 1.3 residues of 3-nitrotyrosine had only 2% of the native enzymatic activity and 30% as much iron as the native enzyme. In addition, treatment of desaturase with acetic anhydride as described in Table III gave an enzyme with four acetylated tyrosyl residues and no detectable enzymatic activity. Iron analysis on this preparation following gel filtration showed the modified enzyme contained less than 0.02 mol of iron/mol of enzyme.

If tyrosine modification causes a loss of functionally coordinated iron, one would expect that the oxidation of artificial substrates and the autoxidizability of the enzyme might also be lost. This was found to be the case for enzyme modified by tetranitromethane (Table V); the rates of *p*-cresol oxidation and desaturase autoxidation are decreased to the same extent as stearyl-CoA desaturation after treatment with tetranitromethane.

Discussion

Since desaturase has only recently been prepared in a pure form (Strittmatter et al., 1974; Strittmatter and Enoch, 1978), the literature contains little information that implicates specific amino acid residues in the catalytic function. The present studies on the chemical modification of desaturase indicate that several arginyl and tyrosyl residues may be involved directly in its action. In a number of enzymes arginine has been implicated as an essential residue at the substrate recognition site (Riordan et al., 1977; Powers and Riordan, 1975, and references therein). In each of these enzymes, arginyl residues are thought to interact with the negatively charged phosphate moiety of the substrate. Our results suggest that this may also be the case for desaturase, since the substrate, stearyl-CoA, has three phosphate groups and bears four negative charges at pH 8. Since the butanedione-treated enzyme appears to interact with cytochrome b_5, *p*-cresol, and oxygen as well as the native enzyme does, the arginyl residues modified by this reagent are probably not involved in the interaction with these substrates. Oxidation of *p*-cresol and autoxidation are catalytic activities associated with a functional desaturase molecule (Enoch et al., 1976; Oshino and Sato, 1971). The fact that both of these activities remain after stearyl-CoA desaturase activity is lost due to modification of arginyl residues suggests that butanedione treatment does not result in a denaturation or other gross conformational changes in the enzyme. This is also implied by the fact that the loss of stearyl-CoA desaturase activity and the arginyl modifications are reversible. A simple one to one stoichiometry was not obtained for the number of arginyl residues modified by butanedione, and enzymatic activity and, thus, the number of arginyl residues at the recognition site(s) cannot be assessed at this time. However, modification of only a few residues has a profound effect upon the substrate binding as suggested by the fact that most of the activity is lost with the modification of only one or two arginyl residues. Similarly, reactivation of butanedione-treated enzyme is accompanied by regeneration of one or two arginyl residues. Space-filling molecular models of stearyl-CoA show that the four negative charges in the substrate may be present as a cluster. Fung and co-workers (1976a,b) have determined the conformation of propionyl-CoA bound at the active site of transcarboxylase, and this structure, which shows CoA to be in a partially folded conformation, has the phosphate groups spatially oriented such that the molecule contains a single cluster of negative charges. This raises the question as to whether or not there is a complementary positvely charged cluster of arginyl residues on the substrate-binding region of the desaturase.

Several lines of evidence suggest the functional importance of tyrosyl residues at or near the oxidation-reduction site of desaturase. The loss of enzymatic activity was accompanied by the modification of one to four tyrosyl residues using three different reagents. In the tetranitromethane-treated enzyme, the oxidation of an artificial substrate and the autoxidizability of the desaturase were decreased to the same extent as the catalytic activity with stearyl-CoA. Since this enzyme preparation had a significantly lower iron content than native enzyme, we conclude that tyrosyl residue(s) may be involved in the chelation of the iron prosthetic group of desaturase. Tyrosine is involved in iron binding by transferrins and a loss of the iron-binding activity of these proteins is observed upon nitration or O-acetylation of essential tyrosyl residues (Komatsu and Feeney, 1967; Line et al., 1967; Tsao et al., 1974). It has been shown in human transferrin that three tyrosyl residues participate in the binding of each iron atom (Komatsu and Feeney, 1967; Tsao et al., 1974). It appears that 4 of the 24 tyrosyl residues of desaturase are in a different environment based on their reactivity with tetranitromethane (Figure 4) and acetic anhydride (Table III). The reaction of a single tyrosyl residue with tetranitromethane leads to nearly complete loss of the catalytic activity of desaturase. Thus, it is possible that one to four tyrosines of desaturase may participate in iron coordination. Modification of only a single tyrosyl residue may sufficiently destabilize an iron chelate involving several of these residues and, therefore, effect the iron loss that we have observed.

TABLE V: Effect of Tetranitromethane on the Rate of *p*-Cresol Oxidation and Autoxidation of Stearyl-CoA Desaturase.[a]

mol of tetranitromethane/mol of desaturase	desaturase activity[b]		
	stearyl-CoA oxidation	*p*-cresol oxidation	autoxidation
0	200 (100)	495 (100)	30 (100)
4	120 (60)	322 (65)	20 (66)
16	60 (30)	160 (32)	10 (33)
40	14 (7)	50 (10)	2 (7)

[a]The enzyme (20 μM) was incubated with tetranitromethane in Tris buffer at 25 °C. After 15 min the samples were chilled to 0 °C and used immediately for the measurement of desaturase activities as described under Materials and Methods. [b]nmol of NADH oxidized min^{-1} (mg of desaturase)$^{-1}$. Values in parentheses are the percent of initial activities.

These data begin to define the essential amino acid residues involved in the desaturation of the acyl chain of stearyl-CoA in a complex four-electron reaction in which oxygen is reduced by the oxidation of two molecules of reduced cytochrome b_5 and dehydrogenation of the substrate. The proposed ionic interactions with arginyl residues in stearyl-CoA binding and the tyrosyl residues involved in iron chelation would represent two of the loci involved in this overall reaction. Affinity labeling with substrate analogues and attempts to cross-link cytochrome b_5 to the desaturase may provide additional information on the precise stoichiometry of these essential residues and their distribution in the primary structure of the single polypeptide chain of the enzyme.

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Kinetic Study on the Reaction Mechanism of Pantothenase: Existence of an Acyl-Enzyme Intermediate and Role of General Acid Catalysis†

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ABSTRACT: A kinetic study was performed on the reaction mechanism of pantothenase (EC 3.5.1.22) catalyzed hydrolysis of the pantothenic acid. A nonlinear progress curve is derived if the reaction occurs at low buffer concentrations. The nonlinearity is due to partial reversibility of the reaction; an acylenzyme (pantoyl-enzyme) is formed during the reaction, and β-alanine, the other end product, is able to react with the acyl-enzyme and return back to pantothenate. The dependence of the β-alanine return reaction on buffer concentration and on pH suggests a general acid catalysis during the reaction. A reaction mechanism is suggested, in which the NH_3^+ form of β-alanine participates in the return reaction, and the deacylation of the acyl-enzyme is acid catalyzed.

Pantothenase (EC 3.5.1.22) decomposes pantothenic acid into pantoic acid and β-alanine:

$$HOCH_2C(CH_3)_2CH(OH)CONHCH_2CH_2COOH \rightarrow HOCH_2C(CH_3)_2CH(OH)COOH + NH_2CH_2CH_2COOH$$

Previous kinetic studies of the enzyme have shown the existence of an ionizable group with pK = 7.0 at the substrate binding site in the enzyme. The ionizable group affects substrate binding (K_m) but not V_{max}, and the substrate is bound to the basic form of the ionizable group. The pK value and its dependence on temperature ($\Delta H = -50$ kJ/mol) suggest that the ionizable group is a histidine imidazole. The decrease in the activity below pH 7 is due to this imidazole. An anomalous, buffer-dependent fall in the pH-activity curve is found above pH 7, and this fall originally caused the further kinetic studies described in the present paper. The fall was found to be due to nonlinearity of the reaction velocity curve, and, further, the nonlinearity is caused by a partial reverse reaction. A scheme of the reaction mechanism is suggested based on kinetic studies on the dependence of the reaction on the pH, buffer concentration, and the buffering substance.

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