Modification and Inactivation of CoA Transferase by 2-Nitro-5-(thiocyanato)benzoate†

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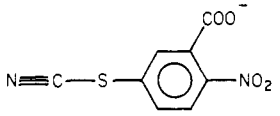
ABSTRACT: Succinyl-CoA:3-ketoacid coenzyme A transferase undergoes a biphasic reaction with 2-nitro-5-(thiocyanato)-benzoate, giving ~70% loss of activity in the initial phase. Active-site titration shows that this inactivation represents the complete loss of activity of 75 ± 5% of the enzyme molecules. The remaining 25 ± 5% of the active sites is protected against inactivation by methyl methanethiosulfonate and 5,5′-dithiobis(2-nitrobenzoate); this protection is removed upon treatment of the modified enzyme with dithiothreitol. Values of k_{cat}/K_m for the two half-reactions catalyzed by the enzyme are the same for the native and modified enzymes on the basis of number of remaining active sites. The modified enzyme shows a smaller decrease in activity with increasing pH in the range pH 7.5–8.7 than the native enzyme. It is concluded that the "essential" thiol group of the enzyme is not involved directly in catalysis and that it reacts with 2-nitro-5-(thiocyanato)-benzoate by two pathways, to form active and inactive enzymes. This can be explained by the attack of the thiol on carbon to form active enzyme–SCN and the attack on sulfur to form enzyme–SSAr, which is blocked at the active site and rapidly undergoes irreversible inactivation.

Succinyl-CoA:3-ketoacid coenzyme A transferase from pig heart is inactivated by the thiol reagents DTNB,[1] *N*-ethylmaleimide, and *p*-(hydroxymercuri)benzoate (Hersh, 1966; White et al., 1976). The initial reaction of one thiol group per subunit with DTNB causes loss of activity to >99%, is blocked by the presence of both substrates, is accelerated 3–100-fold in enzyme–CoA, and presumably occurs at or near the active site; this is followed by a concentration-dependent, irreversible denaturation of the enzyme and reaction of additional thiol groups with DTNB (White et al., 1976). The enzyme from *Escherichia coli* contains six thiol groups that react at an increased rate in enzyme–CoA to give partial inactivation (Sramek et al., 1977).

The experiments described here were carried out to determine whether the active-site thiol group of the pig heart enzyme is required for catalytic activity of the enzyme or whether the loss of activity with bulky thiol reagents is simply due to blocking of the active site. We have therefore examined the reaction of the enzyme with NTCB,[1] which is expected to



NTCB

transfer its cyano group to the active-site thiol to form enzyme–SCN (Degani et al., 1970). The small cyano group and the thiomethyl group, which is attached to thiols by reaction with methyl methanethiosulfonate, can block thiol groups without causing the loss of enzyme activity that is found with larger thiol reagents when the thiol group is not required for activity (Vanaman & Stark, 1970; Smith & Kenyon, 1974). The results show that NTCB reacts with CoA transferase by two pathways to form irreversibly inactivated enzyme and a modified, fully active enzyme that is protected against inhibition by thiol reagents, presumably by transfer of the –SAr and –CN groups, respectively, to enzyme–SH.

Materials and Methods

Materials. CoA transferase, with a specific activity of 280 μmol of acetoacetyl-CoA consumed min^{-1} mg^{-1} under standard conditions (White et al., 1976), was kindly donated by Susan Moore; it was prepared from pig heart by a slight modification of a published procedure (White & Jencks, 1976a).[2] *N*-Ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), acetohydroxamic acid, disodium ethylenediaminetetraacetate, succinic acid, and boric acid were recrystallized before use. Tris and magnesium sulfate were Mann Ultrapure. Diketene was distilled just prior to use for the synthesis of acetoacetyl-CoA (White & Jencks, 1976b). Methyl methanethiosulfonate was kindly donated by Professor Henry Mautner. 2-Nitro-5-(thiocyanato)benzoic acid, half-potassium salt (mp 242–245 °C), was prepared according to Degani & Patchornik (1971). Water was deionized and glass distilled. Stock solutions of NTCB, DTNB, dithiothreitol, and *N*-ethylmaleimide were prepared shortly before use in solutions containing 0.01 M EDTA, pH 7, that had been deaerated by bubbling with argon for 30 min.

Methods. Enzyme activity was determined by following the disappearance of the absorbance of acetoacetyl-CoA at 310 nm in the presence of 10 mM potassium succinate, 5 mM magnesium sulfate, and 0.067 M Tris–sulfate buffer, pH 8.10, at 25 °C. Incubations with NTCB were generally carried out in 0.2 M boric acid–potassium borate buffer with a final pH of 8.1; Tris buffer was generally avoided because of its slow reaction with NTCB. Phosphate buffer (0.23 M) at pH 8.1 was found to inactivate enzyme–CoA (Hersh & Jencks, 1967).

Active-site titration with acetoacetyl-CoA and acetohydroxamic acid was carried out as described by Pickart & Jencks (1979). The enzyme was incubated with acetohydroxamic acid for 5–10 min after formation of enzyme–CoA by addition of acetoacetyl-CoA. It was shown that NTCB-modified enzyme that was converted to the hydroxamic acid

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[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; NTCB, 2-nitro-5-(thiocyanato)benzoic acid; Tris, tris(hydroxymethyl)aminomethane.

[2] The enzyme preparation examined here, while of higher specific activity than that studied previously (White & Jencks, 1976a), was found to have a break in its primary structure that gives two fragments on gel electrophoresis in sodium dodecyl sulfate (A. Cheung, unpublished experiments).

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Table 1: Effect of NTCB on the Inactivation of CoA Transferase by Thiol Reagents[a]

	acetoacetyl-CoA (μM)	k_{obsd} (min^{-1}) no NTCB	1.8 mM NTCB 0 min	80 min	120 min	170 min
methyl methanethiosulfonate[b]	0		0.69		0.0028	
	10		1.73		0.0039	
	0				0.0025[c]	
DTNB[d]	0	0.69	0.26			0.019
	10	2.0	0.59			0.009
N-ethylmaleimide[e]	13	0.59		0.004		

[a] Rate constants for loss of enzyme activity were determined before and after incubation of 0.2–0.4 μM enzyme with 1.7–2.0 mM NTCB for 80–170 min at 25 °C. [b] Methyl methanethiosulfonate (0.4 mM), 8 mM potassium phosphate, 0.8 mM EDTA, and 0.25 M borate buffer, pH 8.3. [c] Methyl methanethiosulfonate omitted. [d] DTNB (0.33 mM), 6 mM potassium phosphate, 1.0 mM EDTA, and 0.18 M borate buffer, pH 8.1. [e] *N*-Ethylmaleimide (0.86 mM), 7 mM potassium phosphate, 0.9 mM EDTA, and 0.17 M borate buffer, pH 8.1.

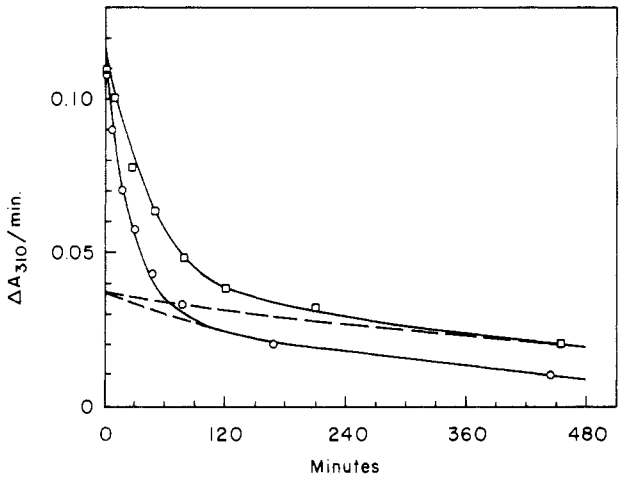


FIGURE 1: Inactivation of 0.3 μM enzyme (□) or enzyme–CoA (○) in the presence of 1.0 mM NTCB, 5.7 mM potassium phosphate, and 1.0 mM EDTA in 0.2 M potassium borate buffer, pH 8.1, at 25 °C. Acetoacetyl-CoA, 6 μM, was added to form enzyme-CoA (○).

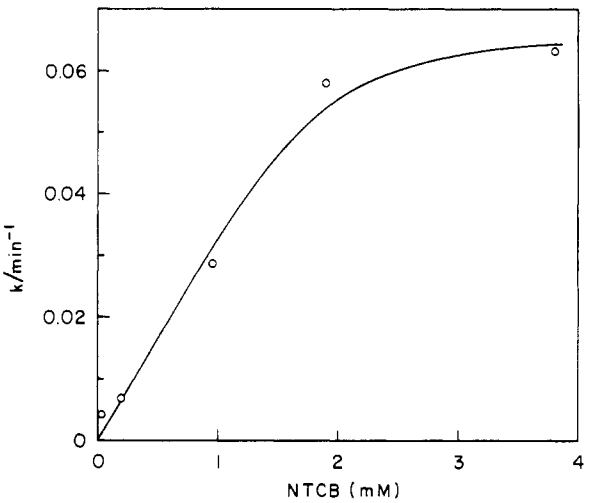


FIGURE 2: Dependence on NTCB concentration of the rate constant for the initial phase of enzyme–CoA inactivation with 0.29 μM enzyme, 5.7 μM acetoacetyl-CoA, 5.7 mM potassium phosphate, and 1.0 mM EDTA in 0.19 M potassium borate buffer, pH 8.1, at 25 °C.

derivative gave 83–95% reactivation in the presence of 1.9 mM coenzyme A and that the modified enzyme gave <10% inactivation in the presence of acetohydroxamic acid for 40 min under the same conditions, but in the absence of acetoacetyl-CoA.

Pseudo-first-order rate constants for the initial phase of the biphasic enzyme inactivation with NTCB were obtained by subtracting the extrapolated activity for the slow phase of inactivation from the observed activity and plotting the difference semilogarithmically against time; the rate constant was obtained from $0.693/t_{1/2}$.

Results

In the presence of 1 mM NTCB at pH 8.1, CoA transferase is inactivated with a biphasic time course (Figure 1, squares). The initial, faster phase gives approximately 70% inactivation and is followed by a slower phase; in the presence of 1.8 mM NTCB, <2% activity remained after 26 h. The time course can be fit by the sum of two pseudo-first-order reactions with rate constants of 0.021 and 0.0014 min^{-1}, as shown by the calculated solid line through the squares in Figure 1. The inactivation is accelerated in the presence of 6 μM acetoacetyl-CoA, which converts the enzyme to enzyme–CoA (White et al., 1976), and the solid line through the circles in Figure 1 for this reaction is calculated from first-order rate constants of 0.046 and 0.0032 min^{-1}. The dashed lines show the calculated rate constants for the slow phase, corresponding to 30% of the total inactivation. The biphasic kinetics were found to be independent of enzyme concentration in the range 0.3–3.1 μM. The addition of more NTCB after 140 min, to bring the concentration from 2.2 to 3.8 mM, did not give another rapid phase of inactivation, and identical results were obtained with two different preparations of NTCB and with NTCB that had been subjected to additional recrystallization. The inactivation was found to be faster at higher pH, with rate constants for the fast phase with enzyme–CoA of 0.019, 0.082, and 0.20 min^{-1} at pH 7.3, 8.1, and 9.0, respectively, in 0.2 M Tris–sulfate buffer, 2 mM NTCB, and 4.4 μM acetoacetyl-CoA.

The rate constants for the rapid phase of inactivation show saturation behavior with increasing NTCB concentration and a half-maximal rate at approximately 1 mM NTCB (Figure 2). The initial slope of Figure 2 gives a second-order rate constant of 40 M^{-1} min^{-1} for the reaction of NTCB with enzyme–CoA. This is approximately 100 times smaller than the rate constant for the reaction of NTCB with glutathione under similar conditions (Degani & Patchornik, 1974), suggesting that access of NTCB to the thiol group of the enzyme is hindered even in enzyme–CoA. Further evidence that NTCB forms an initial noncovalent complex at the active site is provided by the finding that the rate constant for inactivation of the enzyme in the presence of 3.3 mM DTNB under the same conditions is decreased from 0.69 to 0.26 min^{-1} when 1.8 mM NTCB is present; the rate constant for enzyme–CoA is reduced from 2.0 to 0.59 min^{-1} in the presence of 1.8 mM NTCB (Table I). The enzyme is protected against inactivation by the presence of both substrates and shows 80% protection, compared with enzyme–CoA, in the presence of 0.045 M acetoacetate and 2.3×10^{-4} M acetoacetyl-CoA; a

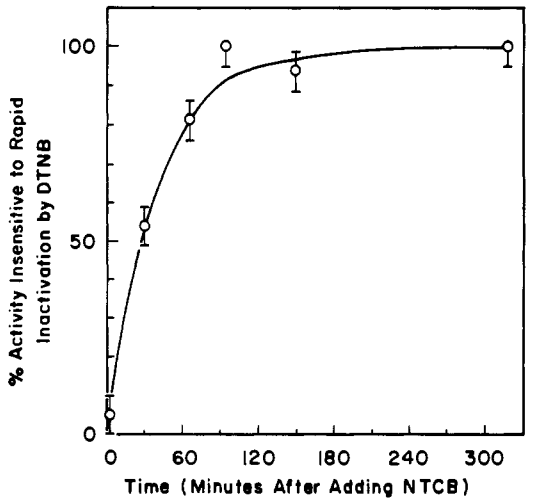


FIGURE 3: Rate of formation of NTCB-modified enzyme that is protected against inactivation by DTNB. Modification was carried out with 0.53 μM enzyme, 6.6 μM acetoacetyl-CoA, 2.1 mM NTCB, 6.3 mM potassium phosphate, and 1.0 mM EDTA in 0.21 M potassium borate buffer, pH 8.1, at 25 °C. Aliquots were removed, and the time course of inactivation was determined with 0.29 μM enzyme in the presence of 0.33 mM DTNB, 10.8 μM acetoacetyl-CoA, 1.8 mM NTCB, 5.4 mM potassium phosphate, and 1.0 mM EDTA in 0.18 M potassium borate buffer, pH 8.1. The fractions of DTNB-sensitive and DTNB-insensitive enzyme were determined from the intercepts at zero time of first-order plots of the remaining activity against time.

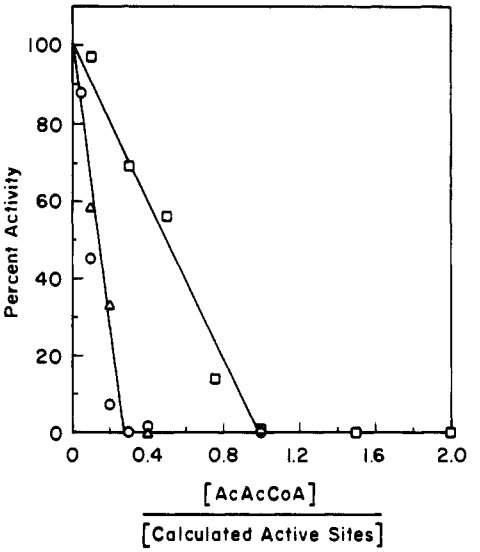


FIGURE 4: Active-site titration with acetoacetyl-CoA of native (□) and NTCB-modified (○, △) CoA transferase. Enzyme, 0.77 μM, was modified with 1.9 mM NTCB in the presence of 0.9 mM EDTA, 14 mM potassium phosphate, and 0.21 M potassium borate buffer, pH 8.1, for 100 min. Measured amounts of acetoacetyl-CoA were added to aliquots of the enzyme solution to form enzyme–CoA, which was then inactivated by incubation with 4 mM acetohydroxamic acid in the presence of 1.4 mM EDTA in 0.1 M Tris buffer at pH 8.1. Inactivation was followed for 25 min and was complete in 5–10 min.

different preparation of enzyme showed 95% protection against inactivation by DTNB under the same conditions (White et al., 1976).

The modified enzyme that remains after the initial phase of inactivation is protected against inactivation by thiol reagents. Methyl methanethiosulfonate (0.4 mM) was found to give rapid inactivation of enzyme and enzyme–CoA that followed first-order kinetics to 97% inactivation. After incubation of the enzyme with NTCB for 120 min, these rate constants are decreased by factors of 250 and 440 for enzyme and enzyme–CoA, respectively (Table I). Since the rate of inactivation of the protected enzyme incubated with NTCB alone accounts for most of the observed rate in the presence of methyl methanethiosulfonate, the actual protection is larger than indicated by these factors. There is a similar protection against inactivation by *N*-ethylmaleimide and a smaller protection by a factor of 14–60 against inactivation by DTNB after incubation with NTCB; there is a much smaller protection by a factor of 2–4 against DTNB when NTCB is added at the same time as the second thiol reagent, as noted above. Figure 3 shows that the time course for the development of protection against inactivation by DTNB corresponds to the fast phase of the reaction with NTCB.

The number of active sites that remain after reaction of the enzyme with NTCB for 100 min was titrated by reaction of the enzyme with known concentrations of acetoacetyl-CoA to form corresponding amounts of enzyme–CoA (eq 1), followed by inactivation of enzyme–CoA with acetohydroxamic acid (eq 2; Pickart & Jencks, 1979). The number of active sites

$$E + AcAcCoA \rightarrow E\text{-}SCoA + AcAc \quad (1)$$

$$E\text{-}SCoA + CH_3CONHO^- \rightarrow E\text{-}ONHCOCH_3 \text{ (inactive)} \quad (2)$$

per subunit is decreased by 70–80% after reaction with NTCB (Figure 4), which corresponds to the observed loss of activity of 67% compared with the native enzyme. This shows that the specific activity of the remaining enzyme is the same, within experimental error, as that of the native enzyme. The same result was obtained from an active-site titration using methyl mercaptopropionate (Moore, 1977) instead of acetohydroxamic acid to inactivate enzyme–CoA.

Table II: Catalytic Activity of Native and NTCB-Modified CoA Transferase[a]

enzyme	k_{cat}/K_m for succinate (M^{-1} min^{-1})	k_{cat}/K_m for acetoacetyl-CoA (M^{-1} min^{-1})
native	7.2×10^5	6.1×10^9
NTCB-modified		
observed	2.3×10^5	1.8×10^9
corrected[b]	7.7×10^5	6.0×10^9

[a] Enzyme, 0.31 μM, was modified by incubation for 115–140 min with 2 mM NTCB, 6.1 μM acetoacetyl-CoA, and 1.0 mM EDTA in 0.25 M borate buffer, pH 8.1. Activity measurements were made with 2.2×10^{-9} M enzyme, 10–60 mM sodium succinate, and 0.02–0.17 mM acetoacetyl-CoA in 0.067 M Tris-sulfate buffer, pH 8.1, at 25 °C and are based on the molarity of dimeric enzyme. Rates were corrected for changes in the extinction coefficient of acetoacetyl-CoA with increasing succinate concentration. [b] Corrected for a 70% decrease in the number of active sites in the modified enzyme.

The observed values of k_{cat}/K_m for varying succinate and varying acetoacetyl-CoA concentrations show similar decreases in the NTCB-modified enzyme when the same amount of protein was assayed (Table II). However, after correction for the decreased number of active sites in the modified enzyme, there is no significant difference in these parameters for the native and modified enzymes.

No reactivation of enzyme that had been partially inactivated by NTCB was observed in the presence of 0.095 M dithiothreitol. There was also no reactivation by 2 mM dithiothreitol or 0.09 M mercaptoethanol of 0.6–2.5 μM enzyme that had been inactivated by 0.2 mM DTNB in the presence of 4.4–10.1 μM acetoacetyl-CoA and 0.2 M Tris–sulfate, pH 8.1. A different preparation of enzyme can be partially reactivated immediately after DTNB inactivation but undergoes a rapid, concentration-dependent conversion to irreversibly inactivated enzyme (White et al., 1976).[2]

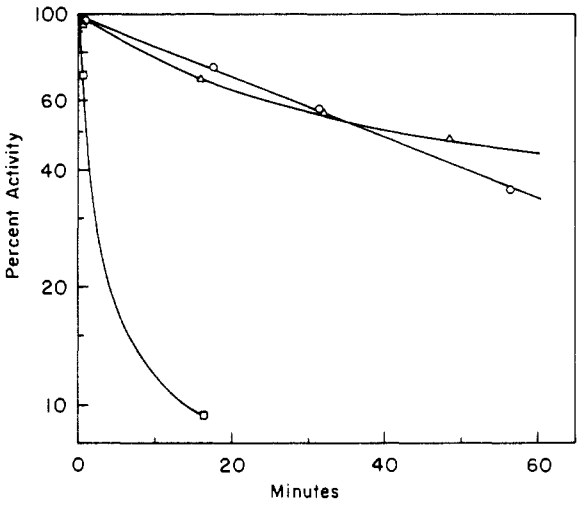


FIGURE 5: Inactivation by 0.19 mM DTNB of NTCB-modified enzyme (△), NTCB-modified enzyme that has been separated from NTCB by passage through a 1-mL column of Sephadex G-25 (○), and NTCB-modified enzyme that has been incubated with 0.095 M dithiothreitol for 1 h in 0.2 M Tris–sulfate buffer, pH 8.1, before passage through the Sephadex column (□). The enzyme, 0.44 μM, was modified by incubation with 2 mM NTCB in the presence of 8.8 μM acetoacetyl-CoA, 8 mM potassium phosphate, and 1 mM EDTA in 0.2 M Tris–sulfate buffer, pH 8.1, for 1 h. Inactivation by DTNB was measured in the presence of 8.8 μM acetoacetyl-CoA.

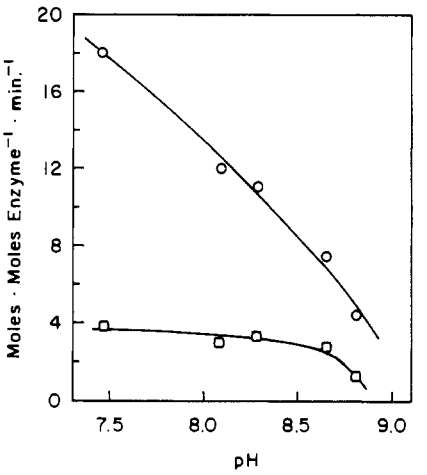


FIGURE 6: Dependence on pH of the activity of 3.6 mM native (○) and 7.2 nM NTCB-modified (□) CoA transferase in the presence of 1.5 mM potassium succinate and 0.16 mM acetoacetyl-CoA in 0.067 M Tris–sulfate buffer. The absorbance changes were corrected for the different extinction coefficients of acetoacetyl-CoA at the different pH values. The enzyme, 0.73 μM, was modified by incubation with 1.8 mM NTCB, 14.6 μM acetoacetyl-CoA, 13 mM potassium phosphate, and 0.8 mM EDTA in 0.16 M potassium borate buffer, pH 8.1. The observed rates were corrected for the change in activity with time during incubation between 75 and 113 min.

However, enzyme that has been made insensitive to inactivation by thiol reagents after reaction with NTCB can be reconverted to native enzyme by reaction with thiols, as shown by the return of sensitivity to inactivation by thiol reagents. Enzyme–CoA that has been modified by reaction with 2 mM NTCB for 50 min at pH 8.1 and separated from small molecules on a Sephadex column is inactivated slowly by 0.19 mM DTNB (Figure 5, circles). Treatment of this enzyme with 0.095 M dithiothreitol (Wiedner et al., 1978) before Sephadex chromatography converts the modified enzyme to a DTNB-sensitive enzyme that undergoes rapid inactivation upon addition of DTNB (Figure 5, squares). In other experiments, it was shown that dithiothreitol converts >98% of the modified enzyme back to DTNB-sensitive enzyme and that this enzyme again undergoes inactivation in the presence of 1.7 mM NTCB with biphasic kinetics.

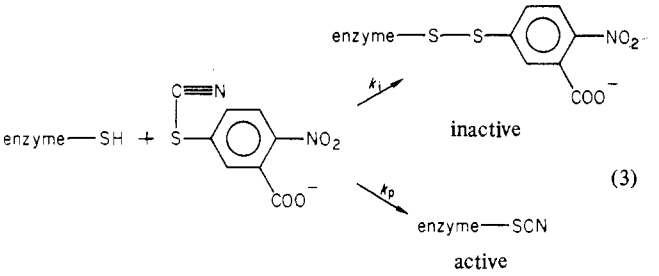
The dependence on pH of the activity of native enzyme and of NTCB-inactivated enzyme in the presence of 1.5 mM succinate and 0.16 mM acetoacetyl-CoA is shown in Figure 6. The native enzyme shows decreasing activity with increasing pH in the range 7.5–8.8, confirming earlier results (Solomon, 1970). In contrast, the modified enzyme shows little sensitivity to pH in the range 7.5–8.6; it does show a decrease in activity at pH 8.8.

Discussion

The rapid inactivation of CoA transferase by NTCB, followed by a leveling off to a much slower rate at about 70% inactivation (Figure 1), could represent either a modification of all enzyme molecules to a form with 30% of the original activity or the complete inactivation of 70% of the enzyme molecules with full retention of activity in the remaining 30% of the enzyme. The latter explanation requires that this 30% be protected against inactivation in some way, because a large excess of NTCB is still present.

Active-site titrations with acetoacetyl-CoA and acetohydroxamic acid (Figure 4) or methyl mercaptopropionate show that the number of active enzyme molecules has been reduced to 25 ± 5% after treatment with NTCB, so that the second explanation is correct. The finding that the values of k_{cat}/K_m for varying succinate and varying acetoacetyl-CoA concentrations, calculated from the measured number of active sites, are essentially the same for the native and modified enzymes confirms that the catalytic activity of the remaining enzyme is closely similar to that of the native enzyme for both half-reactions (Table II).

These results require that the initial reaction with NTCB must occur by two concurrent pathways to give inactive and active, protected enzyme with relative rate constants of 7 and 3, respectively. The simplest explanation for this is that a thiol group on the enzyme reacts concurrently with the sulfur atom of NTCB to form a disulfide that gives irreversible inactivation (k_i, eq 3) and with the cyanide carbon atom of NTCB to give



(3)

a fully active enzyme that is protected against further inactivation (k_p, eq 3). Creatine kinase has been shown to react with NTCB through both of these pathways, with 50–70% reaction at carbon, and gives inactive enzyme from the reaction on sulfur; a dicyano enzyme can be prepared that retains 75% of the activity of the native enzyme (Price, 1976; der Terrossian & Kassab, 1976; Degani & Degani, 1979). It is known that CoA transferase is >99% inactivated by reaction with DTNB, which gives the same disulfide as NTCB, and that this inactivation rapidly becomes irreversible in a reaction that is faster at low enzyme concentrations (White et al., 1976).

Modification of the enzyme with NTCB gives protection against inactivation by methyl methanethiosulfonate, *N*-ethylmaleimide, and DTNB by factors ranging from 14 to 440 (Table I). The remaining slow inactivation may represent reaction with other thiol groups that are not at the active site and have not been modified by NTCB. This interpretation

is supported by the observation that the rate constant for inactivation of the modified enzyme with DTNB shows a 60% decrease with enzyme–CoA rather than the increase that is observed for the active-site thiol group of the native enzyme.

Reaction of the modified enzyme with dithiothreitol restores the sensitivity of the enzyme to inactivation by DTNB (Figure 5) and by NTCB. This is the behavior expected from the displacement of cyanide from enzyme–SCN by dithiothreitol to regenerate enzyme–SH (Wiedner et al., 1978).

The retention of full activity in enzyme–SCN shows that the free sulfhydryl group is not required for enzymatic activity. The enzyme is completely inactivated by the larger thiol reagents DTNB and NEM, suggesting that inhibition is caused by steric blockage of the active site. Methyl methanethiosulfonate, which gives the small enzyme–$SSCH_3$ derivative, also causes complete inactivation, but this may be caused by reaction of this reactive disulfide with other thiol groups on the enzyme to give irreversible denaturation.

In the presence of both substrates, the enzyme is protected against inactivation by DTNB (White et al., 1976) and NTCB. However, the rate of inactivation is increased by acetoacetyl-CoA alone, which reacts with enzyme to form enzyme–CoA. This increase is presumably caused by a conformation change that makes the active-site thiol group more accessible (White et al., 1976). The fact that reaction of the active-site sulfhydryl group with DTNB precedes the development of irreversible inactivation, which involves the exposure of additional sulfhydryl groups and probably dissociation of the subunits (White et al., 1976), suggests that occupation of part of the active site by the thionitrobenzoate group also brings about a conformation change that triggers the development of irreversible inactivation.

The only difference that we have observed between the native and the NTCB-modified enzymes, in addition to protection against thiol reagents, is a reduction in the sensitivity to pH of the catalytic activity of the modified enzyme. The smaller rate decrease with increasing pH in the range 7.5–8.7 for the modified enzyme (Figure 6) suggests that ionization of the thiol group decreases the activity of the native enzyme, possibly by inhibiting binding of the β-carboxylate group of succinate. The decrease in activity of the native enzyme with increasing pH is observed only at low succinate concentrations, at which the reaction of enzyme–CoA with succinate is rate determining; it may reflect deprotonation of the thiol or of an ammonium group that binds to the carboxylate group of succinate (Hersh & Jencks, 1967; Solomon, 1970).

The reason why evolution has brought about the inclusion of a nonessential thiol group in the active site of this enzyme (and other enzymes) is unknown.

Acknowledgments

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