TPN AND Mn-ISOCITRATE PROTECT ISOCITRATE DEHYDROGENASE AGAINST INACTIVATION BUT INCREASE THE NUMBER OF MODIFIED SULFHYDRYL GROUPS*

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SUMMARY: A complete loss of enzymatic activity results from incubation of methylmethanethiolsulfonate with isocitrate dehydrogenase in the absence of metal ions or substrates. This loss is accompanied by the statistical modification of one of the six sulfhydryl groups on the enzyme as monitored by incorporation of [^{14}C] methanethio groups. Therefore, the modifiable cysteinyl group is implicated in the catalytic activity of the enzyme. When Mn-isocitrate or TPN are present, the enzyme is completely protected against inactivation by methylmethanethiolsulfonate. In fact, the specific activity is increased 20-40%. Enzyme protected from inactivation in this manner incorporates about two [^{14}C] methanethio groups. Thus, the presence of the substrates Mn-isocitrate and/or TPN exposes more sulfhydryl groups for modification but these residues are not implicated in catalysis. The substrates TPNH or α-ketoglutarate do not protect isocitrate dehydrogenase from inactivation by the aforementioned reagent.

Previous work on TPN-dependent, Mn(II)-activated isocitrate dehydrogenase (EC 1.1.1.42) with sulfhydryl modification reagents (1,2) implicated the reaction of two of the six available cysteinyl residues (3) in the subsequent loss of enzymatic activity. The inactivation could result from modification of sulfhydryl groups intimately involved with catalytic steps or from a distortion of the active conformation of the enzyme due to its interaction with the bulky reagents. Also, incubation of enzyme with Mn-isocitrate or Mn-TPNH solutions gave significant, but not complete, protection. In order to supplement these studies, investigations to determine the effect of a newly developed sulfhydryl modifying reagent on isocitrate dehydrogenase were begun.

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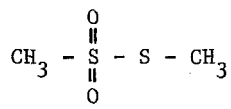
Methylmethanethiolsulfonate (MMTS)[1] is one of a class of sulfhydryl



blocking reagents recently developed by Kenyon and coworkers (4,5). It has many obvious advantages over conventionally used sulfhydryl modifying reagents such as N-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid), in that it has a smaller molecular size and has no tendency to form hydrogen bonds. In addition, it has no charge, reacts under mild experimental conditions, and in many cases is rapidly removable. The methanesulfinate anion is a good leaving group, which facilitates the reaction of the methanethio moiety of MMTS with enzyme-bound cysteinyl groups. Due to the structural features of MMTS noted above, undesirable interactions with the enzyme should be distinctly minimized permitting an evaluation of the participation of -SH groups in catalysis.

The nature of the investigation was twofold: 1) the rate of inactivation of the enzyme by MMTS was measured in the absence and presence of one or more substrates or cofactors, and 2) the enzyme was incubated with [^{14}C] labeled MMTS to determine the amount of reagent incorporated per mole of enzyme (M.W. ~60,000).

EXPERIMENTAL SECTION

Isolation, Purification and Assay of Isocitrate Dehydrogenase

TPN-linked isocitrate dehydrogenase was purified as outlined previously (6). The specific activity of homogeneous enzyme was ~29 μmoles/min/mg enzyme. The purified enzyme used throughout the work was stored in liquid nitroten in the standard buffer system (0.1 M NaCl, 0.1 M triethanolamine, 10% glycerol, pH = 7.7). The specific activity of isocitrate dehydrogenase was measured at 25°C, using the standard assay mixture and techniques described earlier (6), with the exception that $MnCl_2 \cdot 4H_2O$ was substituted for $MnSO_4$.

Preparation of MMTS

MMTS was prepared according to the published method (5) and the colorless oil was purified by distillation (69-71°C, ~0.4 mm). Two proton NMR singlets were observed at δ2.65 and δ3.22. [^{14}C] labeled MMTS of specific activity 2.54×10^{10} dpm/mole was generously provided by Dr. George L. Kenyon.

Inactivation Rate Studies

A typical inactivation experiment involved an initial incubation of enzyme

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[1] Abbrevations used are: MMTS, methylmethanethiolsulfonate; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

(1.72×10^{-2} μmoles) with one or more of the components required for activity. Solution volumes were 0.25 ml, and the average incubation time was ten minutes. Experiments were conducted at 0°C initially, in order to minimize enzyme denaturation. Solutions were assayed in the standard manner described earlier. Upon addition of MMTS, the solutions were returned to the ice bath, and the time of addition was recorded. At varying time intervals, small aliquots were withdrawn and assayed for enzymatic activity.

Incorporation of Labeled MMTS

The enzyme was incubated with [^{14}C] MMTS for the times indicated in the Results Section. Unreacted radioactive MMTS was separated from the enzyme-bound reagent by passing the incubation mixture through a 1 × 20 cm G-25 Sephadex column (sample volumes applied to the column were ∿0.6 ml). Flow rates were less than 0.4 ml/min. In cases where Mn-isocitrate and/or TPN were present in the incubation mixture, the standard column buffer also contained an identical concentration of these reagents in order to avoid dilution and subsequent alterations in MMTS binding during chromatography.

Collection vials each contained 10.0 ml of scintillation cocktail, consisting of a mixture of PPO/POPOP as the fluor (7). Fractions of ∿0.1 ml per vial were collected and counted on a Beckman liquid scintillation counter.

In each run, the protein and the unbound ^{14}C-MMTS were well separated with at least two vials recording zero counts between the bands. The total recovery of radioactivity for each experiment was between 91-95%, with the small discrepancy in the total recovery probably being due to dilution quenching or systematic pipeting inaccuracies.

A typical experiment consisted of 1.72×10^{-2} μmoles enzyme in 0.7 ml total volume. Mn-isocitrate or TPN ratios with respect to enzyme were 50:1; MMTS to enzyme ratios were 30:1. Increasing this latter ratio did not alter the results reported herein.

RESULTS

Inactivation of Isocitrate Dehydrogenase by MMTS

Addition of MMTS to a solution containing isocitrate dehydrogenase results in a progressive loss of enzymatic activity. The inactivation followed saturation kinetics with a rate of inactivation of $\sim 4 \times 10^{-4}$ sec^{-1} at MMTS to enzyme ratios of 9:1 and 30:1. The enzyme exhibited no measurable activity (<0.01%) after incubation periods exceeding 180 minutes. Enzymatic activity could only be partially restored (∿10%) by incubation of the MMTS treated enzyme with excess mercaptoethanol over a 45 minute period, although it had been reported

TABLE I

MMTS INACTIVATION OF ISOCITRATE DEHYDROGENASE

Additions to Reaction Mixture[1]	% Residual Activity[2]
I. Enzyme Alone	< 0.1
II. Substrate and Cofactors Alone	
8.6×10^{-2} µmoles Mn^{2+}	40
8.6×10^{-2} µmoles threo-D_s-isocitrate	45
1.72 µmoles α-ketoglutarate	40
3.96×10^{-2} µmoles TPNH	70
3.96×10^{-2} µmoles TPN	140
III. 8.6×10^{-2} µmoles Mn^{2+} plus Substrates and Cofactors	
8.6×10^{-2} µmoles threo-D_s-isocitrate	120
1.72 µmoles α-ketoglutarate	35
3.96×10^{-2} µmoles TPNH	60

[1] 1.72×10^{-2} µmoles of enzyme and 5.16×10^{-1} µmoles of MMTS were used according to the procedure described in METHODS. T ≈ 0°C.

[2] The percent activity was measured after ∿100 minutes of incubation with MMTS. At this point the level of inactivation or activation had plateaued.

that such regenerations proceeded rapidly and completely in most cases (5). Recently Nishimura et al. (8) were able to remove a CH_3S- group from succinic thiokinase with tributylphosphine. Reaction of MMTS-treated isocitrate dehydrogenase with tributylphosphine did not regenerate enzymic activity.

Since the MMTS inactivation implies that the group(s) modified are sulfhydryls located in the vicinity of the active site, the possibility that substrates or cofactors would protect against modification was explored. Several solutions containing different combinations of substrates or cofactors (at saturating levels) were incubated with enzyme prior to MMTS addition. Previously published K_M or K_D values (2,6,9,10) were used to establish saturating conditions.

TABLE II

[^{14}C] CH_3S- INCORPORATION INTO ISOCITRATE DEHYDROGENASE

Additions to Reaction Mixture[1]	Moles [^{14}C] CH_3S-/Mole Enzyme
I. Enzyme Alone	1.07[2]
II. Enzyme plus 8.6×10^{-2} µmoles Mn^{2+} 8.6×10^{-2} µmoles threo-D_s-isocitrate	1.85[3]
III. Enzyme plus 8.6×10^{-2} µmoles of TPN	1.93
IV. Enzyme plus 8.6×10^{-2} µmoles Mn^{2+} 8.6×10^{-2} µmoles threo-D_s-isocitrate (1.72×10^{-3} µmoles TPN)[4]	1.39

[1] 1.72×10^{-2} µmoles of enzyme and 5.16×10^{-1} µmoles of MMTS were incubated from 1-3 hrs according to the procedures described in Methods. Columns were run at 25°C.

[2] When the enzyme was incubated at 4°C for 1-2 hrs and the column run at 4°C, this value was ∿0.6-0.7.

[3] This represents the total incorporation of [^{14}C] CH_3S- groups after 3.2 hr. After 1.7 hr of incubation, this value was 1.56.

[4] After incubation of enzyme Mn^{2+} and isocitrate for 2 hr, TPN (1.72×10^{-3} µmoles) glutamate dehydrogenase (1.7×10^{-4} µmoles) and NH_4^+ (70 µmoles) were added to convert isocitrate to α-ketoglutarate and then α-ketoglutarate to glutamate. Glutamate does not protect isocitrate dehydrogenase from inactivation (Levy & Villafranca, unpublished observations).

The results of this study appear in Table I. In all cases, where inactivation was observed, the rate of inactivation was not affected greatly but the final extent of inactivation plateaued at the levels reported in Table I. The major exceptions to this behavior were solutions that contained Mn-isocitrate or TPN. In these two instances, the specific activities actually increased upon addition of MMTS.

Measurements of Incorporation of [^{14}C] MMTS into Isocitrate Dehydrogenase

Table II lists the various conditions under which experiments were

performed to measure the stoichiometry of incorporation of methanethio groups into the enzyme. The most interesting observation regarding these results is that an enzyme sample with no substrates or cofactors present incorporates significantly smaller amounts of the labeled reagent than do samples protected by Mn-isocitrate and/or TPN. Thus, when enzyme alone is incubated with MMTS, approximately one of the six sulfhydryl groups is modified, whereas when the enzyme has Mn-isocitrate or TPN bound, $\sim$2 groups react.

In experiment IV in Table II, an attempt was made to see whether one of the two sulfhydryls modified in the presence of Mn-isocitrate was the one modified in the absence of substrate (experiment I, Table II). After a 2 hr incubation, isocitrate was converted to α-ketoglutarate by addition of a catalytic amount of TPN. The TPNH and α-ketoglutarate were then converted to glutamate by the addition of NH_4^+ and glutamate dehydrogenase. The TPN produced by the glutamate dehydrogenase reaction was recycled to convert all of the isocitrate to glutamate. The total number of sulfhydryls modified was $\sim$1.4 suggesting that no additional sulfhydryls were modified when isocitrate was removed.

DISCUSSION

The results in this paper clearly demonstrate that statistically one sulfhydryl group of the six present per mole of enzyme can be treated with MMTS to completely inactivate the enzyme. When Mn-isocitrate or TPN are incubated with isocitrate dehydrogenase prior to MMTS addition, a sulfhydryl critical for activity becomes inaccessible (or unreactive) to the modification reagent. However, additional cysteine residues now become available for reaction with the reagent. In this and other studies involving MMTS, the reagent appeared to be specific for -SH groups. In studies from Colman's laboratory, modification of about five sulfhydryl groups by 5,5'-dithiobis-(2-nitrobenzoic acid) inactivates the enzyme (1). Mn-isocitrate protects the enzyme from inactivation and protects two of the sulfhydryls from modification. However, reaction of only one sulfhydryl with N-ethylmaleimide inactivates the enzyme (2). All six sulfhydryls should be susceptible to reaction with MMTS because of the significantly smaller size of this reagent relative to those employed earlier. This, however, does not appear to be the case.

It is difficult to say whether the same sulfhydryl group(s) are being modified when no substrate is present or when TPN or Mn-isocitrate is present to protect the enzyme. The fact that Mn-isocitrate and TPN bind to different sites on the enzyme (9-14) tentatively suggests that the modified sites differ.

An interesting observation is that while the presence of TPN produces a stimulation of enzyme activity in the presence of MMTS, the presence of TPNH results in a loss of activity (Table I). This strongly suggests that these two forms of the nucleotide produce different conformational states of the enzyme when they are bound and significantly alter the reactivity of sulfhydryl groups on the enzyme. Indeed, EPR and NMR results (9,11,12) provide independent evidence that TPN and TPNH stabilize different conformational states of the enzyme. Also, EPR spectra of enzyme-bound Mn(II) (Levy and Villafranca, unpublished observations) show that MMTS itself produces a distinct conformational change at the metal ion site when the reagent has reacted with the isocitrate dehydrogenase-Mn-isocitrate complex.

In summary, preliminary experiments have been conducted with isocitrate dehydrogenase and a thiol modifying reagent, MMTS. These studies have yielded some rather unexpected results concerning the stoichiometry of modified enzymic sulfhydryl groups. More experiments are necessary in order to understand the three dimensional relationships among the -SH groups and substrate and cofactor binding sites. Isolation of the radioactive peptides is underway to establish whether the variously modified sulfhydryl groups are in different regions of the polypeptide chain.

REFERENCES

1. Colman, R. F. (1969), Biochemistry 8, 888.
2. Colman, R. F., and Chu, R. (1970), J. Biol. Chem. 245, 601.
3. Colman, R. F. (1973), J. Biol. Chem. 248, 8137.
4. Smith, D. J., and Kenyon, G. L. (1974), J. Biol. Chem. 249, 3317.
5. Smith, D. J., Maggio, E. T., and Kenyon, G. L. (1975), Biochemistry 14, 766.
6. Colman, R. F. (1968), J. Biol. Chem. 243, 2454.
7. Lindblad, B. (1971), Clinical Chem. Acta 34, 113.
8. Nishimura, J. S., Kenyon, G. L., and Smith, D. J. (1975), Arch. Biochem. Biophys. 170, 461.
9. Villafranca, J. J., and Colman, R. F. (1972), J. Biol. Chem. 247, 209.
10. Villafranca, J. J., and Colman, R. F. (1974), Biochemistry 13, 1152.
11. Levy, R. S., and Villafranca, J. J. (1977a), Biochemistry 16, in press.
12. Levy, R. S., and Villafranca, J. J. (1977b), Biochemistry 16, in press
13. Ehrlich, R. S., and Colman, R. F. (1975), Biochemistry 14, 5008.
14. Ehrlich, R. S., and Colman, R. F. (1976), Biochemistry 15, 4034.