INACTIVATION OF ESCHERICHIA COLI SUCCINIC THIOKINASE BY SELECTIVE OXIDATION OF THIOL GROUPS BY PERMANGANATE.

Jonathan S. Nishimura, Theresa Mitchell, and J. Michael Matula

The Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

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Summary

Succinic thiokinase from Escherichia coli was rapidly inactivated by permanganate ion at 25° and 0°. On the basis of the cysteic acid content of hydrolysates of treated protein, oxidation of 3 sulfhydryl groups appeared to effect total loss of thiokinase activity. However, titration of the same protein samples revealed that 4 important sulfhydryl groups (a fraction of which was possibly in disulfide form) were more likely oxidized during the inactivation process. Significant protection of the enzyme against permanganate inactivation was obtained by the following additions: ATP-Mg^{2+} and succinate (51%); desulfo-CoA alone (53%); and ATP-Mg^{2+}, succinate, and desulfo-CoA (93%). No protection was observed when either inorganic phosphate or arsenate was added.

Introduction

Various studies have been undertaken to achieve an understanding of why sulfhydryl groups are important to both the structure and function of succinic thiokinase from Escherichia coli (1-6). Most recently, it has been demonstrated by selective methanethiolation that 4 of the 16 thiol groups of the enzyme appear to be important for catalytic activity (6). It is not known, however, how these groups are related to catalysis and(or) binding of substrates.

The observation that permanganate ion appears to oxidize important sulfhydryl groups after binding to certain enzymes as an inorganic phosphate analog (7,8) led us to explore this approach as a method of ascertaining the identity of reactive groups at the inorganic phosphate binding site(s) of succinic thiokinase.

Materials and Methods

Succinic thiokinase was isolated from E. coli and assayed as described previously (9). Microcomplement fixation analyses were carried out by the method of Levine (10), as reported previously (5). Sulfhydryl groups

were titrated with 5,5'-dithiobis (2-nitrobenzoic acid) in the presence of sodium dodecyl sulfate (5). The molecular weight of the enzyme was taken as 141,000 (1).

Amino acid analyses were performed in a Beckman Model 118 Amino Acid Analyzer on samples that had been dialyzed exhaustively against water and hydrolyzed in 6N HCl in evacuated tubes at 110° for 21 hrs. The authors are indebted to Dr. B. P. Yu and Mr. Jim Downs for their help and to Dr. James Manning of the Rockefeller University for the initial analysis.

Desulfo-CoA was prepared as described by Chase et al. (11), except that about 3 grams of catalyst were employed. Under these conditions virtually no CoA -SH was detectable. However, the recovery of adenine nucleotide was only about 50%.

All other chemicals used were of reagent grade and were obtained from the usual commercial sources.

Results

The results depicted in Fig. 1 show that thiokinase activity was effectively inhibited at low ratios of permanganate to enzyme. At a ratio of 2 moles of permanganate per mole of thiol, one cysteic acid residue should be formed (7) (see Equation 1). As can be seen in the figure, this was essentially the result. It would appear from these data that there were approximately 3 moles of important -SH groups per mole of thiokinase and that no other kinds of amino acid side chains of the enzyme were affected by permanganate. However, titration of sulfhydryl groups remaining after inactivation showed that more -SH groups had disappeared than predicted

(1) $RSH + 2MnO_4^- \rightarrow RSO_3^- + 2MnO_2 + OH^-$

(2) $6RSH + 2MnO_4^- \rightarrow 3RS\text{-}SR + 2MnO_2 + 2OH^- + 2H_2O$

from the simple stoichiometry of 2 moles MnO_4^- per mole -SH. It is possible that the discrepancy observed here is due to the formation of disulfide bonds between -SH groups that are located closely to each other on the enzyme's surface. The oxidizing environment furnished by the presence of

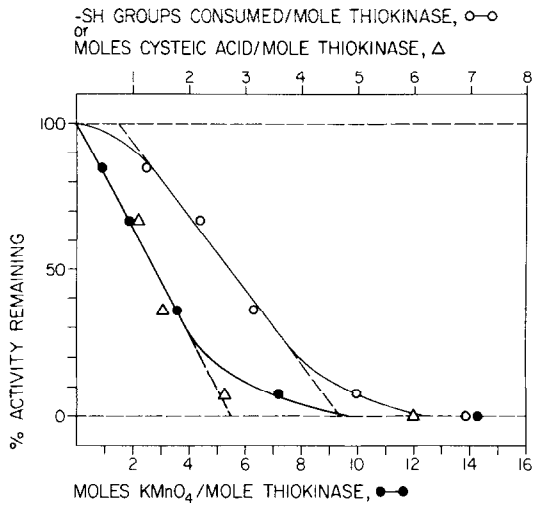

Figure 1. Stoichiometry of permanganate ion oxidation of important sulfhydryl groups of E. coli succinic thiokinase. The reaction mixtures contained succinic thiokinase (98 µg; 0.7 nmole), Tris-HCl pH 7.4 (2 µmoles), KCl (2 µmoles) and potassium permanganate, in a final volume of 45 µl. Incubation was carried out at 25° for 15 min. Thiokinase assays and sulfhydryl groups titration were carried out, as described previously (5). For the purpose of amino acid analyses the reaction mixtures were scaled up 25-fold.

permanganate would facilitate the occurrence of such a reaction. Also, this oxidative process would require an amount of permanganate that is much smaller (one-sixth per mole of -SH) than that needed for cysteic acid production, as suggested in Equation 2. We have observed that with reduced glutathione a similar phenomenon may be observed in the presence of permanganate, i.e., a very high yield of oxidized glutathione (R-S-S-R) and very little of the expected sulfonate derivative were obtained at pH 7.2.

The data shown in Fig. 1 indicate that oxidation of four important sulfhydryl groups is involved in the total inactivation of thiokinase. Although the extrapolated values would indicate 5 such groups, it can be seen in the upper part of the graph that the equivalent of 1 -SH group was oxidized without significant loss of activity. The value of 4 -SH groups of the total of 16 is consistent with the interpretation of experiments in

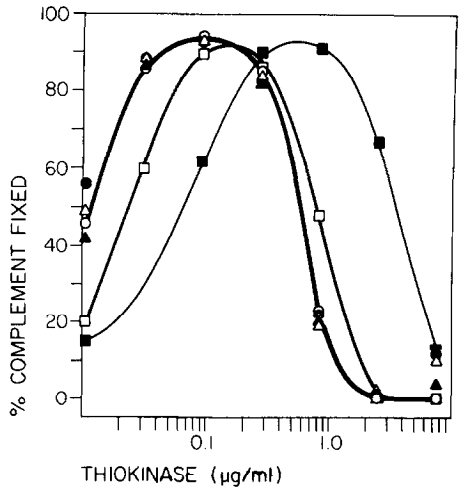

Figure 2. Microcomplement fixation analysis (10) of permanganate ion-oxidized succinic thiokinase. The reaction mixtures were made up as described under Figure 1. Dilutions were made and analyses carried out as described (5). Thiokinase activity remaining: o, 100% (control); ●, 87%; Δ, 62%; ▲, 33%; ◘, 2%; ■, 11%.

which methyl methanethiosulfonate was used to reversibly block important -SH groups (6).

The complement fixation data described in Fig. 2 show that the conformation of the permanganate-oxidized enzyme did not change at least until 67% of its catalytic activity had been lost. Evidence had been presented earlier that shifts in the height and peak position of the complement fixation curve occurred when certain -SH groups were titrated with various reagents (5,6). This adds support to the notion that specific sulfhydryls and not a random selection of such groups are involved in the oxidation by permanganate. This result also suggests that oxidation of the thiol groups to sulfonates does not cause a large perturbation in that area of the enzyme structure.

Finally, the kinetics of permanganate reaction with succinic thiokinase were determined under conditions of dilute enzyme and dilute inhibitor. The results are summarized in Fig. 3 and show that the inactivation process occurs rapidly and apparently in a biphasic manner (see 10^{-3} mM; 0°). At

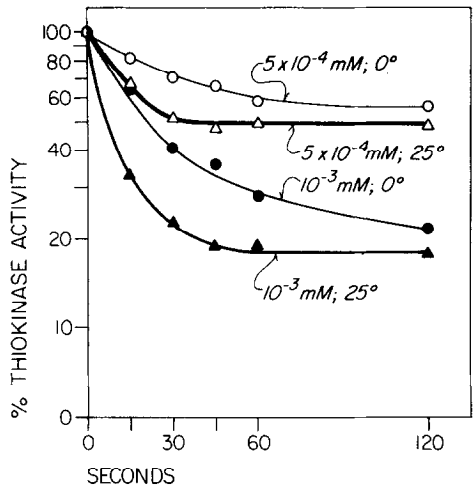

Figure 3. Kinetics of permanganate ion inactivation of succinic thiokinase at 0° and 25°. Incubation mixtures contained succinic thiokinase (0.016 nmole), Tris-HCl pH 7.4 (5 µmoles), KCl (5 µmoles) and permanganate at the indicated final concentrations, in a final volume of 0.1 ml. The reaction was begun by the addition of permanganate and quenched with 2 ml of thiokinase assay medium.

high thiokinase and permanganate concentrations the inactivation process occurred so rapidly that rates could not be measured.

Experiments in which various substrates and substrate analogs were added individually and in combination in order to protect succinic thiokinase against inactivation by permanganate were carried out. Some of these experiments are summarized in Table I. It can be seen that, while desulfo-CoA or ATP-Mg^{2+}-succinate gave significant protection, almost complete protection was obtained with desulfo-CoA, ATP-Mg^{2+} and succinate. Since it was possible that a contaminant of CoA of as little as 1 in 7200 molecules of desulfo-CoA would be adequate to quench enough permanganate to obtain the observed protection, the desulfo-CoA itself was pre-incubated with permanganate (2 moles/7200 moles desulfo-CoA) prior to the experiment. No difference was observed in the outcome of the experiment. Both arsenate and inorganic phosphate, added at 100 mM, did not prevent inhibition of the enzyme by permanganate.

Experiments with succinyl-CoA as a protective agent were not carried out because of the uncertainty in assessing the consumption of permanganate by free CoA under the conditions of incubation.

Discussion

Permanganate appears to react specifically with about 4 -SH groups of E. coli succinic thiokinase. This is consistent with the results of experiments described earlier (6), in which methanethiolation of about 4 important sulfhydryl groups was observed. As in previous work, permanganate oxidation did not result in significant perturbation of the enzyme's structure until most of its catalytic activity had been lost.

According to the stoichiometry of 2 moles of permanganate required to effect oxidation of an -SH group to the sulfonate state (Equation 1), the formation of cysteic acid could account within experimental error for almost all of the permanganate added. However, it does appear that some disulfide groups may be formed. If this is true, it would seem that some of the disulfide is composed of important sulfhydryl groups. Other oxidized states for cysteine sulfur are possible. One of the objectives of our future work will be to examine this subject more closely.

The simplest interpretation of the protection experiment (Table 1) is that there are two kinds of important sulfhydryl groups. One kind is involved with ATP-Mg^{2+}-succinate interaction with the protein and the other with the CoA-thiokinase interaction. In the $\alpha_2\beta_2$ structure of E. coli succinic thiokinase (12), the 4 important sulfhydryl groups might be completely accounted for in this manner. We are presently engaged in experiments involving peptide mapping which should show how many different types of -SH groups are critical to thiokinase activity and if these groups specifically interact with different substrates.

In both the catalytic subunit of E. coli aspartate transcarbamylase (7) and Salmonella phosphoribosylpyrophosphate synthetase (8) permanganate ion appears to react at inorganic phosphate binding sites. This does not appear to be the case with E. coli succinic thiokinase.

TABLE I

Protection of Succinic Thiokinase
Against Inactivation by Permanganate

Compound(s) Added	% Loss of Activity	% Protection
None	51	0
ATP-Mg^{2+}	51	0
Succinate	53	0
Desulfo-CoA	27	47
ATP-Mg^{2+}, succinate	25	51
ATP-Mg^{2+}, desulfo-CoA	24	53
ATP-Mg^{2+}, succinate, desulfo-CoA	7	86

The incubations were basically the same as those described for Fig. 3. The concentrations of critical components were: enzyme (0.16 μM), $KMnO_4$ (1.0 μM), ATP-Mg^{2+} (10mM), disodium succinate (100 mM) and desulfo-CoA (1.2 mM). Incubation was carried out at 0° for 30 seconds.

Acknowledgement

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