EVIDENCE FOR A METHIONYL RESIDUE IN THE ACTIVE SITE OF ISOCITRATE DEHYDROGENASE*

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The importance of methionine in the function of enzymes has come to be appreciated only relatively recently and few studies implicate this amino acid directly in catalysis. Modification of a methionine residue in chymotrypsin leads to loss of activity under some conditions, but this effect has been attributed to a change in the Michaelis constants for the substrates rather than to a change in the basic catalytic power of the enzyme (Lawson and Schramm, 1965; Weiner et al., 1966). The effects of alkylation of methionyl residues of ribomuclease or of its S-peptide have been ascribed to an inability of the modified polypeptide to refold into native conformation instead of to an alteration within the active site (Stark and Stein, 1964; Vithayathil and Richards, 1960). Ray and Koshland (1962) offered some kinetic evidence for the participation of methionine in the catalytic function of phosphoglucomutase and more recently, Fanger et al. (1967) proposed that in Pseudomonas cytochrome C, the heme iron is coordinated to the thioether group of methionine. Inactivation of myokinase is produced by the alkylation of 2.5 methionyl residues (Kress and Noda, 1967); however, it is unclear whether this effect reflects a structural change in the protein or modification of an essential amino acid residue.

In this paper, it will be demonstrated that treatment of TPN-dependent isocitrate dehydrogenase with iodoacetate below pH 6 results in complete inactivation concomitant with specific alkylation of one methionyl residue. Significant protection against inactivation is obtained when the substrate

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isocitrate is included in the reaction mixture, suggesting that alkylation occurs at the active site. Loss of activity cannot be explained in terms of weaker binding of substrates by an intrinsically active modified enzyme, since the Michaelis constants are unaltered in partially active alkylated enzymes.

Experimental - Porcine heart TPN-dependent isocitrate dehydrogenase obtained from Boehringer and Soehne was purified twelve-fold by ion exchange chromatography and gel filtration (Colman, to be published). The resultant preparation exhibited a specific activity of 29 μmoles TPN reduced per min per mg protein at pH 7.4 and 25° and was homogeneous in the ultracentrifuge and on cellulose acetate electrophoresis from pH 6.2 to 9.5. The enzymatic activity was assayed spectrophotometrically at 340 mμ in .03 M triethanolamine chloride buffer, pH 7.4 using concentrations of 1×10^{-4} M, 4×10^{-3} M and 2×10^{-3} M for TPN, DL-isocitrate and manganese sulfate, respectively, in a total volume of 1.0 ml. Iodoacetic acid purchased from Mann Research Laboratories was recrystallized from water and benzene and iodoacetic acid-1-^{14}C was obtained from New England Nuclear Corporation. Carboxymethyl methionine sulfonium salt was prepared according to Gundlach et al. (1959) using ^{14}C-labeled or cold iodoacetate. Homocysteine, tyrosine, histidine and glutamic acid derivatives were synthesized by incubation of the corresponding amino acid with a 25-fold excess of iodoacetate at pH 8 and 40° for 20 to 40 hours. The reaction mixtures were acidified and the excess iodoacetic acid extracted with ether. DL-homoserine, DL-homocysteine and S-carboxymethyl-L-cysteine were obtained commercially.

Results and Discussion - Isocitrate dehydrogenase is totally inactivated by incubation with iodoacetate at pH 5.5 and 30°, whereas enzyme in the absence of reagent remains fully functional. In the alkylation reaction mixture, the concentration of iodoacetate is high compared to that of the enzyme; therefore, pseudo first order kinetics are obeyed as is evidenced in Fig. 1. The presence of isocitrate in the reaction mixture causes an 11-fold decrease in the rate constant for inactivation, indicating that iodoacetate produces a specific modification in the active site of the enzyme. A lesser degree of protection

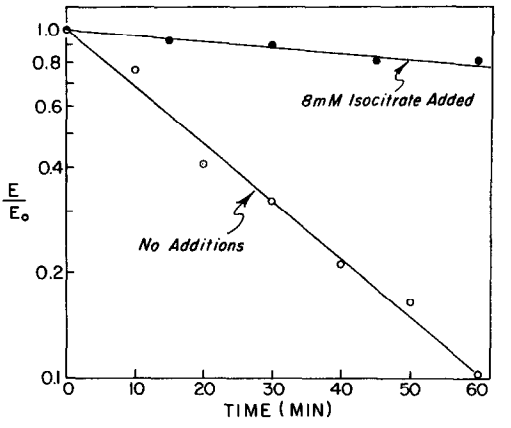

Fig. 1 - Rate of Inactivation by Iodoacetate at pH 5.58 in the Presence and Absence of 8 mM Isocitrate. In these experiments, 2 mg/ml isocitrate dehydrogenase was incubated at 30° with 2.83 x 10^{-2} M iodoacetate in 0.2 M sodium acetate buffer, pH 5.58. Isocitrate (8 x 10^{-3} M) was added as indicated. At the given times, aliquots were taken, diluted immediately 20-fold with 0.12 M triethanolamine chloride buffer at 0° and assayed as described in Experimental Procedure. The pseudo first order rate constant calculated in the absence of substrate, is .0402 min^{-1}; while that with added substrate is .00359 min^{-1}.

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is afforded by α-keto glutarate; while TPN, TPNH and Mn^{++} exert little or no effect on the course of alkylation. Lotspeich and Peters (1951) failed to observe inhibition of isocitrate dehydrogenase by 1 mM iodoacetate in 20 min incubations at pH 7.2. However, studies of the pH dependence of inactivation by iodoacetate (Colman, in preparation) demonstrate that under the conditions used in the earlier study inhibition could not have been expected.

The binding of radioactive iodoacetate by isocitrate dehydrogenase was measured after appropriate incubation of the enzyme and reagent at pH 5.5 and 30°, as will be described in greater detail elsewhere. Approximately one mole of ^{14}C-labeled iodoacetate is incorporated per mole of enzyme of molecular weight 58,000.

Methionine was identified as the site of carboxymethylation on the basis of analysis of proteolytic digests of ^{14}C-labeled alkylated enzyme. Modified isocitrate dehydrogenase, freed of excess reagent, was exposed to Pronase (10:1) for 48 hours at pH 7.7 and 40°, followed by treatment with carboxypeptidase A

and B (20:1) for 18 hours. Hydrolysis was approximately 83% complete as monitored by quantitative reaction of the digest with ninhydrin using leucine as a standard (Spies, 1957). Digests were desalted on Dowex-50, concentrated, and fractionated on a 1.0 x 38 cm column of Sephadex G-10. Calibration of the column indicated a void volume of 9.9 ml and elution of carboxymethyl (CM) tyrosine, which is adsorbed by Sephadex, at 18.8 ml. One radioactive peak was found for the hydrolysate at 11.7 ml with no radioactivity being observed between 15 and 25 ml, thereby indicating the absence of a radioactive derivative of tyrosine.

Paper chromatography of the peak radioactive fraction was conducted using a solvent system consisting of phenol-water (4:1) with .3% NH_4OH in a separate beaker. The R_fs of standard compounds along with the probable assignment of structures to the spots of the methionine derivative and the hydrolysate are given in Table I.

TABLE I. PAPER CHROMATOGRAPHY OF PROTEOLYTIC DIGESTS OF ^{14}C-ALKYLATED ENZYME

Probable Compound	^{14}C-Alkylated Enzyme Hydrolysate	^{14}C-Alkylated Methionine		Nonradioactive Standards
	Radioactivity R_f	Radioactivity R_f	Ninhydrin R_f	Ninhydrin R_f
Methyl CM-Thioether	.20	.21	--	
				.26 Glu
				.29 CM-Cys
				.34 CM-Homocysteine
CM-Homocysteine	.37*	.38*	.38*	.37 CM-Tyr
				.39 CM-His, CM-Glu, Homocysteine
Homoserine	--	--	.54	.53 Homoserine
				.55 Tyr, Cys
				.63 His
CM-Methionine Sulfonium Salt	.68	.69	.69	.69 CM-Met
				.76 Met

* Minor component, < 5% total.

In the case of the methionine derivative, three ninhydrin spots and three peaks of radioactivity (located using the Vanguard Autoscanner) were identified. The major ninhydrin and radioactive peaks exhibit R_fs of 0.69 and must certainly represent carboxymethyl (CM) methionine sulfonium salt. The ninhydrin spot

with an R_f of .54 is identical in R_f with authentic homoserine and, as expected, it is non-radioactive. This compound has been observed as a decomposition product of carboxymethyl methionine (Gundlach, 1959). It is not possible to identify this product in the chromatogram of the hydrolysate since its R_f is close to that of many amino acids which may still be present. The radioactive fragment which must be produced concomitantly with the generation of homoserine is methylcarboxymethyl thioether ($CH_3SCH_2COO^-$). This compound probably represents the radioactive, ninhydrin negative spot with an R_f of .21. In addition, a minor radioactive, ninhydrin positive species is found with an R_f of .38. This value corresponds reasonably well with .34 found for synthetic carboxymethylhomocysteine, and might be expected from decomposition of carboxymethylmethionine to yield non-radioactive methanol as the second product. The pattern of radioactivity obtained from the hydrolysate agrees well with that of the methionine derivative. The major peak, representing approximately 70% of the total radioactivity, is that with R_f 0.68. In view of the similarity of the two other R_fs to those found for the methionine derivative, the minor component with R_f 0.37 probably represents carboxymethylhomocysteine.

Further support for the designation of methionine as the site of carboxymethylation on the enzyme comes from a comparison of the quantitative amino acid analysis of native and alkylated enzymes hydrolyzed in evacuated sealed tubes in 6 M HCl at 110° for 18 hrs.

TABLE II. AMINO ACID COMPOSITION OF NATIVE AND ALKYLATED ENZYMES

	Native	Alkylated		Native	Alkylated
Met	10.2	9.2	Ser	35.3	35.6
CM-Homocys.	--	0.44	Glu	52.0	53.4
Lys	41.0	42.4	Pro	29.4	30.6
His	12.2	12.2	Gly	47.2	49.8
Cysteine*	11.9	12.5	Ala	50.1	52.4
Tyr	12.0	11.8	Val	40.2	43.2
- - - - - - - - - - - -			Ileu	30.9	30.9
Arg	17.1	17.2	Leu	42.5	44.7
Asp	53.4	52.4	Phe**	19.6	20.2
Thr	34.5	35.4	Trp	6.3	6.1

*Ellman Method (1959).

**Spectrophotometric determination (Beaven and Holiday, 1952).

The data of Table II, obtained by the methods of Spackman et al. (1958), show

a decrease of one methionine residue in the alkylated enzyme. Furthermore, there is the appearance of a new peak, occuring between the positions of proline and glycine in the chromatogram, which has been identified as carboxymethyl homocysteine (Gundlach, 1959), the major acid decomposition product of the methionine carboxymethyl sulfonium salt. No other peakswere noted in the chromatogram of the alkylated enzyme that were not evident in that of the native enzyme. In particular, no differences are observed between the lysine, histidine and tyrosine contents of the two enzymes. Carboxymethylation of the dicarboxylic acids could not be determined in an acid hydrolysate since any esters present would be hydrolyzed; however, the decrease in the methionine content can entirely account for the extent of incorporation of radioactive iodoacetate by the enzyme. The sulfhydryl content of the enzyme was measured by reaction with 5,5' dithio bis (2-nitrobenzoic acid) (Ellman, 1959) at pH 8.5 after denaturation in 0.2% sodium dodecyl sulfate. Enzyme alkylated at pH 5.5 and dialyzed to remove excess reagent was found to contain 12.5 sulfhydryl groups as compared to 11.9 for the native enzyme. Iodoacetate does not, therefore, appear to attack the cysteine residues under these conditions.

Experiments to be reported in detail elsewhere indicate that the rate of inactivation of the enzyme by iodoacetate is independent of ionic strength at pH 5.5, strongly implicating an uncharged amino acid as the site of reaction. Furthermore, the mobilities of native and alkylated enzyme were identical in cellulose acetate electrophoresis from pH 6.2 to 9.5, an observation which is also consistent with modification of a thioether group.

The data reported in this paper suggest that iodoacetate alters a single methionyl residue in the active site of isocitrate dehydrogenase. Kinetic studies are now in progress to further elucidate the role of this methionyl residue in the catalytic process.

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