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ALKYLATION OF CYSTEINYL RESIDUES OF PIG HEART NAD-SPECIFIC ISOCITRATE DEHYDROGENASE BY IODOACETATE

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Summary

Pig heart NAD-specific isocitrate dehydrogenase is inactivated by reaction with iodoacetate at pH 6.0. Loss of activity can be attributed to the formation of 1—2 mol of carboxymethyl-cysteine per peptide chain. The rate of inactivation is markedly decreased by the combined addition of Mn^{2+} and isocitrate, but not by α-ketoglutarate, the coenzyme NAD or the allosteric activator ADP. The substrate concentration dependence of the decreased rate of inactivation yields a dissociation constant of 1.6 mM for the enzyme-manganous-dibasic isocitrate complex, a value that is 50 times higher than the K_m for this substrate. This result suggests that in protecting the enzyme against iodoacetate, isocitrate may bind to a region distinct from the catalytic site. Isocitrate and Mn^{2+} also prevent thermal denaturation, with an affinity for the enzyme close to that observed for the iodoacetate-sensitive site. The alkylatable cysteine residues may contribute to a manganous-isocitrate binding site which is responsible for stabilizing an active conformation of the enzyme.

Introduction

Mammalian tissues contain two distinct isocitrate dehydrogenases: the allosteric NAD-dependent enzyme (*threo*-D_S-isocitrate: NAD-oxidoreductase (decarboxylating), EC 1.1.1.41) and the non-regulatory NADP-specific enzyme (*threo*-D_S-isocitrate: NADP oxidoreductase (decarboxylating), EC 1.1.1.42) [1]. The kinetics of the two enzymes isolated from pig heart muscle have been compared extensively [2] and while both exhibit profound ^2H_2O solvent isotope effects with a corresponding lack of primary isotope effect when [2-^{2}H] isocitrate is the substrate [3], the enzymes differ in the ionic form of isocitrate which

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serves as substrate [3—5]. The amino acid residues in the active site of the pig heart NADP-specific isocitrate dehydrogenase have been investigated by means of chemical modification: the enzyme is inactivated by reaction of a methionyl residue with iodoacetate [6], of 1—2 cysteinyl residues with *N*-ethylmaleimide [7,8] or 5,5′dithio-bis-(2-nitrobenzoic acid) [9], and of a glutamyl residue with a water-soluble carbodiimide [10]. Less is known about the amino acid residues in the active site of NAD-dependent enzyme, although a lysyl residue has been designated as essential for catalysis [11]. It is anticipated that a comparison of the amino acid residues critical for the function of these two proteins from the same species and tissue will contribute to an understanding of the similarities and differences between an allosteric and a non-regulatory enzyme. The present reports that at pH 6.0 iodoacetate inactivates the pig heart NAD-specific isocitrate dehydrogenase by reaction with 1—2 cysteine residues, in contrast to the modification of a methionyl residue in the NADP enzyme under similar conditions. These cysteinyl residues appear to be involved in the binding of isocitrate and Mn^{2+}; however, this substrate binding site may not be the active site, but rather a second type of binding site for isocitrate and Mn^{2+} which is important for stabilizing the conformation of the enzyme.

Experimental Procedure

Materials. The NAD-specific isocitrate dehydrogenase was isolated from pig heart and purified, as described previously [12], by chromatography on DEAE-cellulose and cellulose phosphate, followed by gel filtration on Sepharose 6B. The resultant enzyme preparation has a specific activity of 20—30 enzyme units/mg and exhibits a single band on electrophoresis in polyacrylamide gels containing 2% sodium dodecyl sulfate. The protein concentration was determined from the difference in absorbance at 224 and 233 nm by the method of Groves et al. [13]. A molecular weight of 40 000 for the constituent polypeptide chains was used in calculations of enzyme concentration [12].

The enzymes bovine liver glutamate dehydrogenase and pig heart NADP-specific isocitrate dehydrogenase were purchased from Boehringer Chemical Co. The coenzymes and substrates, as well as piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer were supplied by Sigma Chemical Co. The non-radioactive iodoacetic acid was obtained from Schwarz-Mann and was recrystallized from water and benzene. Iodo[1-^{14}C]acetic acid, purchased from New England Nuclear Corp. was extracted with carbon tetrachloride in order to remove contaminating iodine.

Carboxymethyl-methionine sulfonium salt was prepared by the method of Gundlach et al. [14,15]. Carboxymethylated derivatives of tyrosine, histidine, glutamic, and aspartic acid were synthesized as described previously [6]. *S*-Carboxymethyl-L-cysteine was purchased from Sigma Chemical Co.

L-Isocitrate was prepared from DL-isocitrate by enzymatic conversion of the D-isocitrate to L-glutamic acid as catalyzed by the NADP-specific isocitrate dehydrogenase and glutamate dehydrogenase. The reaction mixture (5.0 ml) contained 125 μmol DL-isocitrate, 25 μmol NADP, 10 μmol $MnSO_4$, 250 μmol NH_4Cl, 313 μmol piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.9, and was 12% in glycerol. To this solution was added one enzyme unit of NADP-specific

isocitrate dehydrogenase and eight enzyme units of glutamic dehydrogenase. After 18 h at 4° C, the reaction was terminated by placing the reaction mixture in a boiling water bath for 5 min. Precipitated protein was removed by centrifugation. An aliquot (0.01 ml) of the reaction mixture was assayed for the presence of *threo*-D_S-isocitrate in the NAD-specific isocitrate dehydrogenase assay. There was no increase in absorbance at 340 nm, indicating that the reaction had proceeded to completion.

Enzyme assay. The NAD-specific isocitrate dehydrogenase activity was measured spectrophotometrically at 340 nm using a Gilford Model 240 spectrophotometer equipped with a recorder with an expanded scale (0.1 absorbance full scale). The standard assay was performed in cuvettes of 1 cm light path with a volume of 1.0 ml which contained 20 mM DL-isocitrate, 1.33 mM $MnSO_4$, and 1 mM NAD in Tris/33 mM acetate buffer, pH 7.2. One enzyme unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per min at 22° C.

Calculation of the concentration of ionic species. In order to elucidate the active species of isocitrate and manganese, calculations as described previously [4] were conducted to obtain the concentrations of all ionic species present. Under the conditions of the experiments described in this paper, the total isocitrate is equal to the sum of the free tribasic and dibasic forms of isocitrate and their respective manganese chelates. When present, the total ADP is also distributed between two ionic forms and their corresponding metal complexes. The total manganese concentration is distributed among the free divalent metal cation and all the chelated complexes found in solution. The ionization constants used for isocitrate and ADP are 1.79 and 0.398 μM, respectively. The dissociation constants for the manganese complexes of dibasic and tribasic isocitrate are 17.4 and 0.869 mM, respectively. The dissociation constants of the ADP chelates of manganese are 1.88 mM and 49.0 μM. A computer program [4] was used to facilitate the calculation of ionic species in each reaction mixture using mass conservation equations and the appropriate dissociation constants.

Preparation and acid hydrolysis of radioactive carboxymethyl-isocitrate dehydrogenase. The NAD-dependent isocitrate dehydrogenase (34 enzyme units) was incubated in a total volume of 2.0 ml with 40 mM iodo[1-^{14}C]acetic acid in 0.05 M sodium piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) buffer, pH 6.0, containing 2.5 mM $MnSO_4$ and 20% glycerol. In addition, reaction mixture A was 25 mM in propanetricarboxylate, which does not appreciably decrease the rate of inactivation by iodoacetate; whereas reaction mixture B was 25 mM in DL-isocitrate. After incubation at 22° C for 160 min, the residual activity of reaction mixture A was 16%, while that of reaction mixture B was 67%. Each sample was dialyzed at 4° C against four changes, of 1 l each, of sodium/0.05 M citrate buffer, pH 6.0, containing 2.5 mM $MnSO_4$ and 20% glycerol. An aliquot of each dialyzed sample was removed and counted in a Packard liquid scintillation counter to determine the total radioactivity incorporated. The remainder of each sample was then passed over a column of Sephadex G-25 (0.9 × 20 cm) equilibrated with 0.1 M NH_4HCO_3 to remove the non-volatile buffer salts. The samples were dried under vacuum, dissolved in 2.0 ml 6 M HCl and hydrolyzed in sealed, evacuated tubes at 105° C for 22 h. After removal of the acid, the

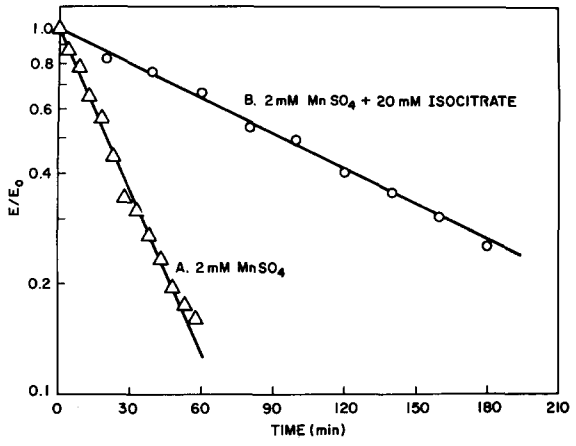

Fig. 1. Inactivation of NAD-specific isocitrate dehydrogenase by 0.08 M iodoacetate at pH 6.0 in the presence and absence of isocitrate. Enzyme was incubated at 22°C with 0.08 M iodoacetate in 0.05 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer, pH 6.0, containing 2 mM $MnSO_4$ and 20% glycerol. DL-isocitrate (20 mM) was added as indicated and the ionic strength was maintained at 0.2 in both solutions by appropriate additions of KCl. At given times, aliquots were withdrawm, diluted 20-fold and assayed for enzymatic activity as described in Experimental Procedure. The pseudo first-order rate constants calculated are 0.0345 and 0.00815 min^{-1} for Lines A and B, respectively.

samples were subjected to descending paper chromatography on Whatman No. 1 paper along with standard carboxymethylated amino acid derivatives.

Results

Kinetics of reaction of isocitrate dehydrogenase with iodoacetic

Pig heart NAD-dependent isocitrate dehydrogenase is irreversibly inactivated

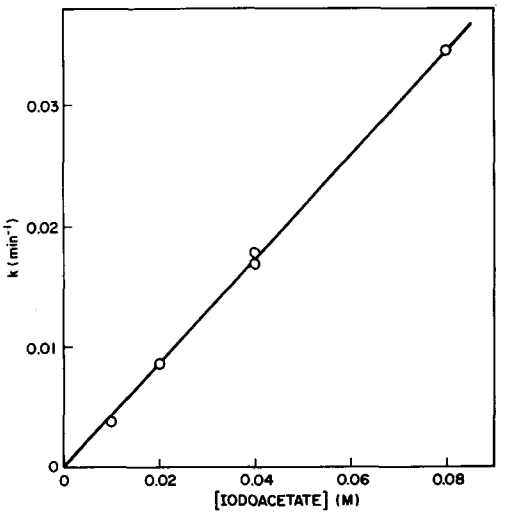

Fig. 2. Dependence of rate constant for inactivation on iodoacetate concentration. The pseudo first-order rate constants for inactivation of isocitrate dehydrogenase were obtained from plots such as that of Fig. 1.

upon incubation with 0.08 M iodoacetate at pH 6.0 and 22° C in the presence of $MnSO_4$ (Fig. 1A). Under these conditions, but in the absence of iodoacetate, the enzymatic activity remains stable. As shown in Fig. 1, the reaction obeys pseudo first-order kinetics with $k = 0.0345\ min^{-1}$ and the semi-logarithmic plot of E/E_o versus time is linear for at least three half-lives. There is specificity for the alkylating agent which reacts with isocitrate dehydrogenase since 0.08 M iodoacetamide ($k = 0.0117\ min^{-1}$) inactivates the enzyme at only one-third the rate exhibited by iodoacetate.

The pseudo first-order rate constant for inactivation by iodoacetate is directly proportional to the concentration of the reagent over the range of 0.01—0.08 M (Fig. 2), indicating that there is no enzyme-iodoacetate complex formed prior to the irreversible reaction. This result contrasts with the reaction of cyanate with a lysyl residue of NAD-specific isocitrate dehydrogenase which proceeds via an enzyme-cyanate complex [11].

The rate of inactivation by iodoacetate is decreased markedly by the combined addition of Mn^{2+} and the substrate isocitrate (Fig. 1B). (This result contrasts with the lack of effect of Mn^{2+} and isocitrate on the rate of inactivation of the enzyme by 0.08 M iodoacetamide ($k = 0.0108\ min^{-1}$ when 10 mM isocitrate and 2 mM $MnSO_4$ were included in the reaction mixture as compared with $0.0117\ min^{-1}$ when substrates were absent)). The metal ion by itself is neither required for the reaction of the enzyme with iodoacetate, nor does it protect appreciably against inactivation, as indicated by the observation of a similar rate constant when 2 mM $MnSO_4$ is present alone (Fig. 1A) and when 2.5 mM EDTA is added to complex the metal ion (Fig. 3). However, Mn^{2+} is essential for the protective effect of isocitrate; when EDTA is added to a reaction mixture containing $MnSO_4$ and isocitrate, the rate of inactivation is the same as in the absence of substrates (Fig. 3).

In the presence of a ligand which complexes with the enzyme at the site of reaction with iodoacetate, the rate of inactivation will be affected in accor-

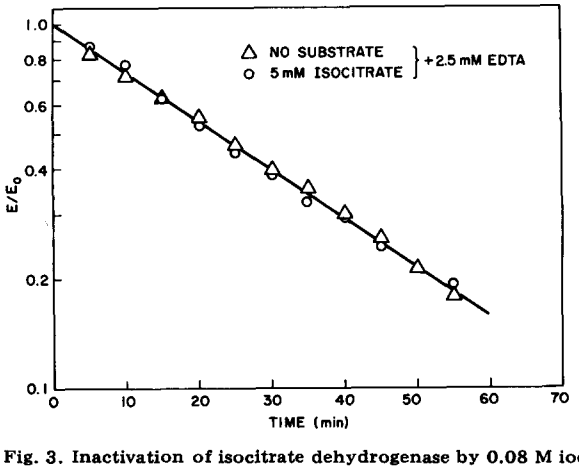

Fig. 3. Inactivation of isocitrate dehydrogenase by 0.08 M iodoacetate in the absence of Mn^{2+}. Incubation conditions were the same as those in Fig. 1 except that 2.5 mM EDTA was added in the absence and presence of 5 mM DL-isocitrate. The points are colinear and are described by a pseudo first-order rate constant of $0.0306\ min^{-1}$.

dance with the following equation:

$$\frac{-d(E)}{dt} = \frac{kE_0}{1 + [L]/K_L} \quad (1)$$

where k is the pseudo first-order rate constant in the absence of ligand, [L] is the concentration of free ligand and K_L is the dissociation constant for the enzyme-ligand complex. The dissociation constant can be calculated from the relationship:

$$K_L = \frac{k_{obs}[L]}{k - k_{obs}} \quad (2)$$

where k_{obs} is the pseudo first-order rate constant obtained in the presence of a given concentration of ligand [L]. Table I reports the observed pseudo first-order rate constants for inactivation of isocitrate dehydrogenase by 0.08 M iodoacetate when various concentrations of Mn^{2+} and DL-isocitrate are included in the reaction mixture. When the manganese concentration is held constant (Table I, upper section), the inactivation rate constant decreases with increasing levels of isocitrate. An average value of 6.3 mM may be estimated for the dissociation constant in terms of the total isocitrate concentration present.

In the physiological range of pH, the total isocitrate is distributed between free dibasic isocitrate (with two of the three carboxyl groups ionized), free tribasic isocitrate and the corresponding metal chelates. Previous investigations of the kinetics of the NAD-specific isocitrate dehydrogenase as a function of pH indicated that the dibasic forms of isocitrate are the active forms of substrate for this enzyme [4,5]. The kinetics are consistent with a model which allows for the formation of the catalytically active enzyme-Mn^{2+}-dibasic isocitrate complex by either of two pathways, the predominant one being dictated by the concentration of the free divalent Mn^{2+}. In the presence of low Mn^{2+} concentrations the free enzyme combines with the preformed metal chelate of dibasic isocitrate. Alternatively, in the presence of high manganese concentrations (1—10 mM $MnSO_4$), the enzyme preferentially binds the metal ion initially and the enzyme-manganese complex subsequently reacts with free dibasic isocitrate [5]. The high manganese concentrations were those used for the determination of the Michaelis constant for isocitrate and under this range of conditions the K_m for free dibasic isocitrate was independent of both pH and manganese concentration [4]. Similar magnanese concentrations have been used in the present experiments in which isocitrate and metal ion are observed to decrease the rate of inactivation of the enzyme by iodoacetate. Thus, the equilibrium constants in Table I are also given in terms of the dissociation of free dibasic isocitrate from the enzyme-Mn^{2+}-dibasic isocitrate complex, yielding an average value of 1.6 mM.

When the isocitrate concentration is held constant and the metal ion concentration varied, the rate constant for inactivation increases with increasing manganous levels, indicating that protection by isocitrate is less effective at higher metal concentrations (Table I, lower section). An increase in the total metal concentration might be expected to modify the distribution among the various

TABLE I

ISOCITRATE PROTECTION OF NAD-DEPENDENT ISOCITRATE DEHYDROGENASE AGAINST INACTIVATION BY 0.08 M IODOACETATE

Additions to reaction mixture		Inactivation rate constant k_{obs} (min^{-1})	Calculated K_S for total DL-isocitrate * (mM)	Calculated K_S for free dibasic DL-isocitrate ** (mM)
$MnSO_4$ (mM)	DL-isocitrate (mM)			
2	—	0.0345	—	—
2	2	0.0284	9.3	2.4
2	5	0.0167	4.7	1.2
2	10	0.0110	4.7	1.9
2	20	0.00835	6.4	1.9
2	10	0.0110	4.7	1.2
5	10	0.0146	7.3	1.5
10	10	0.0219	17.4	2.0

* These dissociation constants were calculated from Eqn. 2 using the total concentration of DL-isocitrate.

** These dissociation constants were calculated from Eqn. 2 using the concentration of free dibasic isocitrate determined by the procedure of Cohen and Colman [4].

forms of isocitrate by decreasing the relative amounts of free isocitrate and increasing the relative amounts of the metal-isocitrate complexes in solution. Thus, if free dibasic isocitrate is the active form of the substrate, higher manganese concentrations should raise the K_m in terms of total isocitrate but have no effect on the K_m calculated in terms of free dibasic isocitrate. Indeed, the results of Table I show that the dissociation constant varies almost 4-fold when calculated in terms of total isocitrate, but is relatively constant when calculated in terms of free dibasic isocitrate, yielding the same average value of 1.6 mM. These data suggest that protection against inactivation of isocitrate dehydrogenase by iodoacetate is produced by free isocitrate binding to the enzyme-manganese complex. However, the dissociation constants observed are considerably higher than the Michaelis constants for total isocitrate (0.15 mM) or for free dibasic isocitrate (0.030 mM) which were previously measured for the NAD-dependent isocitrate dehydrogenase at pH 6.0, under somewhat different buffer conditions [4]. It does not appear as if the discrepancy between the dissociation constant as measured from protection against iodoacetate and the Michaelis constant measured kinetically can be attributed to the difference in ionic conditions, since the same value of 0.15 mM total isocitrate was obtained for the K_m when measured in 0.05 M piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) buffer, pH 6.0, containing 20% glycerol and 2 mM $MnSO_4$, the conditions used in the inactivation experiments. Similarly, the discrepancy cannot be explained by postulating that the reversible binding of iodoacetate alters the Michaelis constant for isocitrate since the K_m was elevated only 2-fold (0.33 mM total isocitrate) when determined in the presence of 0.04 M iodoacetate. (In this experiment the assays were completed in less than 2 min after the enzyme was exposed to iodoacetate, whereas the half-life for inactivation is approx. 40 min.) One possible explanation of the data is that in protecting against inactivation by iodoacetate, isocitrate may bind at a different site from that involved in the catalytic reaction.

TABLE II

EFFECT OF OTHER LIGANDS ON INACTIVATION BY 0.08 M IODOACETATE

The reaction conditions are the same as these described in Fig. 1A. $MnSO_4$ (2 mM) was present in all samples.

Additions to reaction mixture	Inactivation rate constant, k_{obs} (min^{-1})
None	0.0345
10 mM α-ketoglutarate	0.0338
25 mM propanetricarboxylic acid	0.0359
1 mM NAD	0.0377
1 mM ADP	0.0336
1 mM NAD + 1 mM ADP	0.0359
2 mM DL-isocitrate	0.0284
2 mM DL-isocitrate + 2.5 mM ADP	0.0129

It has been reported that the enzyme catalyzes the oxidative decarboxylation of *threo*-D_S-isocitrate exclusively [16]. Stereospecificity is also exhibited in the mode of binding of isocitrate which leads to protection against iodoacetate. For total *threo*-D_S-isocitrate, the dissociation constant calculated from protection experiments is 3.7 mM. Slight protection is afforded by the corresponding L-isomer, allowing the estimation of 18.9 mM as a dissociation constant for the enzyme complex with *threo*-L_S-isocitrate.

Table II shows the effect of other ligands of the enzyme on the rate of inactivation by iodoacetate. NAD-dependent isocitrate dehydrogenase has been shown to catalyze the reductive carboxylation of α-ketoglutarate [11], but the substrate α-ketoglutarate fails to protect against inactivation by iodoacetate. Similarly, the isocitrate analogue propanetricarboxylic acid does not appreciably decrease the rate of inactivation. The coenzyme NAD and allosteric activator ADP do not themselves affect the course of reaction of iodoacetate with the enzyme when present in concentrations in considerable excess of their Michaelis constants. However, in correspondence with the known effect of ADP in decreasing the K_m of isocitrate [4], the presence of 2.5 mM ADP improves the effectiveness of a given concentration of isocitrate in providing protection against iodoacetate. From these protection experiments, the dissociation constant measured in the presence of ADP for free dibasic isocitrate from the enzyme-manganese-dibasic isocitrate complex is 0.44 mM as compared with an average value of 1.7 mM in the absence of the nucleotide.

Incorporation of radioactive iodoacetate

The NAD-dependent isocitrate dehydrogenase was incubated with ^{14}C-labeled iodoacetate at pH 6.0 in the presence of manganese and either DL-isocitrate (25 mM) or propanetricarboxylate (25 mM). The latter has only a slight effect on the rate of inactivation by iodoacetate (Table II) and was added to maintain comparable conditions of ionic strength in the presence of isocitrate. After 160 min incubation, the residual activity had fallen to 16% in the absence of substrate and 2.8 mol of radioactive iodoacetate per peptide chain were incorporated. In contrast, when the incubation was conducted in the presence of

isocitrate, the residual activity was still 67% and 1.9 mol of radioactive iodoacetate per peptide chain were incorporated. The amino acid residues protected by isocitrate (2.8—1.9 = 0.9 residues per peptide chain) are likely to be responsible for the difference in residual activity observed in the presence and absence of the substrate (i.e. 67%—16% = 51%). Thus, it appears that the complete loss in activity is correlated with the modification of 1—2 groups on the enzyme.

Identification of the class of amino acid residues modified by iodoacetate

Iodoacetate, under various conditions, has been reported to react with methionyl, glutamyl, aspartyl, histidyl, lysyl and cysteinyl residues of proteins. Reaction at a methionyl residue in the NAD-specific isocitrate dehydrogenase does not seem to be responsible for inactivation since treatment of a 75% inactive enzyme preparation with 0.1 M dithiothreitol at pH 6.9 failed to restore activity. It has previously been shown that sulfur nucleophiles, including dithiothreitol, are capable of regenerating methionine residues from sulfonium salts contained in peptide and proteins [17—19]. Similarly, the carboxylic amino acids do not appear to be the major locus of reaction of iodoacetate, since dialysis at pH 9.5 of a ^{14}C-labeled carboxymethyl enzyme did not lead to an appreciable decrease in the protein-bound radioactivity. Carboxymethylated derivatives of carboxylic amino acids are known to be unstable under these conditions [20].

Direct identification of the class of amino acid residues of NAD-specific isocitrate dehydrogenase which reacts with iodoacetate was accomplished by analysis of acid hydrolysates of the radioactive carboxymethyl enzymes prepared in the presence or absence of isocitrate and manganese, as described in Experimental Procedure. After hydrolysis of the protein samples in 6 M HCl for 22 h at 105°C, they were subjected to paper chromatography along with standard carboxymethylated amino acids, as well as glycolic acid, the acid decomposition product of carboxymethylated glutamate and aspartate [21]. The results shown in Fig. 4 indicate that in the case of both types of enzyme sample approx. 83% of the radioactivity chromatographs at the position of authentic carboxymethyl-cysteine. Only minor amounts of carboxymethyl-methionine, carboxymethyl-histidine and glycolic acid are produced. The decrease in incorporation of radioactivity observed with added isocitrate and manganese thus reflects reduced formation of carboxymethyl-cysteine. In the absence of substrate (Fig. 4A), the enzyme has lost 84% of its original activity and contains approx. 2.3 mol of carboxymethyl-cysteine per subunit (i.e. 83% of 2.8 mol of radioactive iodoacetate incorporated). For comparison, when the radioactive enzyme was prepared in the presence of the substrate (Fig. 4B), it lost only 33% of its activity and contained about 1.6 mol of carboxymethyl-cysteine per subunit (i.e. 83% of 1.9 mol of radioactive iodoacetate incorporated). The inactivation of the NAD-specific isocitrate dehydrogenase by iodoacetate thus seems to be produced by modification of 1—2 cysteine residues per peptide chain.

Effect of isocitrate on the rate of denaturation of isocitrate dehydrogenase

As indicated in Table I, the dependence on isocitrate concentration of protection of isocitrate dehydrogenase against inactivation by iodoacetate yielded

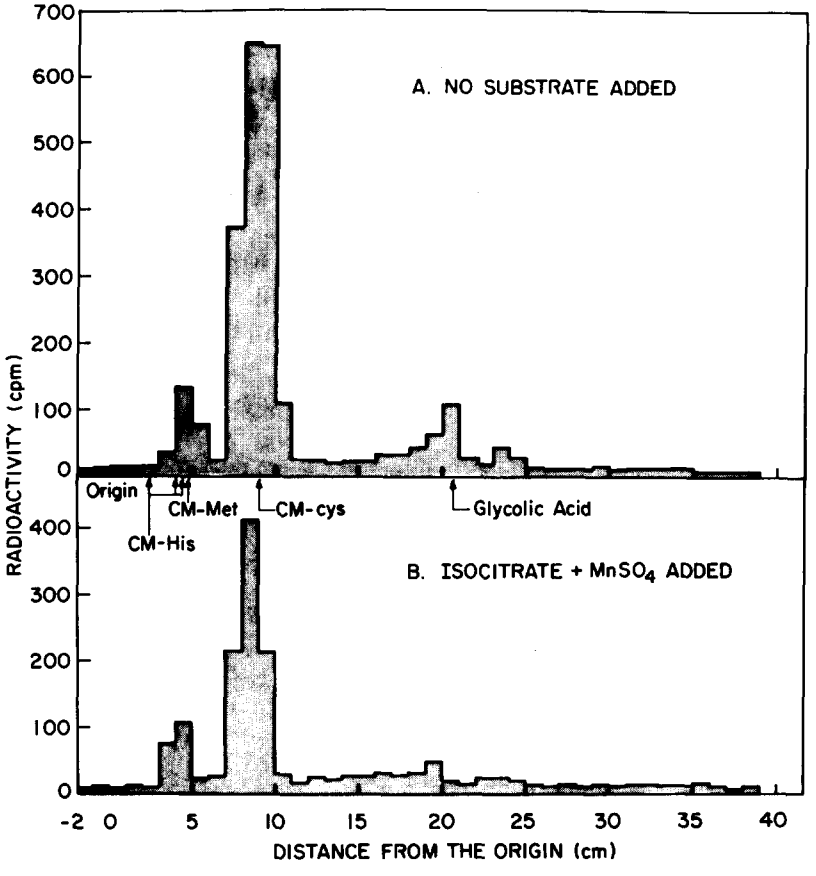

Fig. 4. Paper chromatography of acid hydrolysates of ^{14}C-labeled carboxymethyl-isocitrate dehydrogenase, prepared in the presence and absence of isocitrate and $MnSO_4$. The acid hydrolysates, prepared as described in Experimental Procedure, were subjected to descending paper chromatography on Whatman No. 1 paper for 18 h, using as solvent *n*-butanol/pyridine/acetic acid/water (150 : 100 : 30 : 20, v/v). The radioactivity was detected by cutting the strips containing the enzyme hydrolysates into 1-cm segments and counting each segment in a Packard liquid scintillation counter. The remainder of the chromatogram was sprayed with ninhydrin to visualize the carboxymethyl-amino acid standards. A, carboxymethyl enzyme prepared in the absence of isocitrate; B, carboxymethyl enzyme prepared in the presence of 25 mM isocitrate.

an average value of 6.3 mM for the dissociation constant of the enzyme-isocitrate complex. Since this estimate is at least an order of magnitude higher than the Michaelis constant for isocitrate, attempts were made to ascertain whether the more weakly bound isocitrate served a function different from that of substrate. When isocitrate dehydrogenase is incubated at 37° C in the presence of a low glycerol concentration (1%), the enzyme loses activity progressively over a period of about 10 h. Fig. 5 illustrates the biphasic kinetics that obtain. The first phase may be interpreted as representing a decline of activity to a level characteristic of a partially active enzyme preparation exhibiting 27% of the activity of the original native enzyme. The second phase then represents the slower transformation of the partially active species to a completely inactive enzyme. If the first phase of the inactivation is corrected by subtracting the

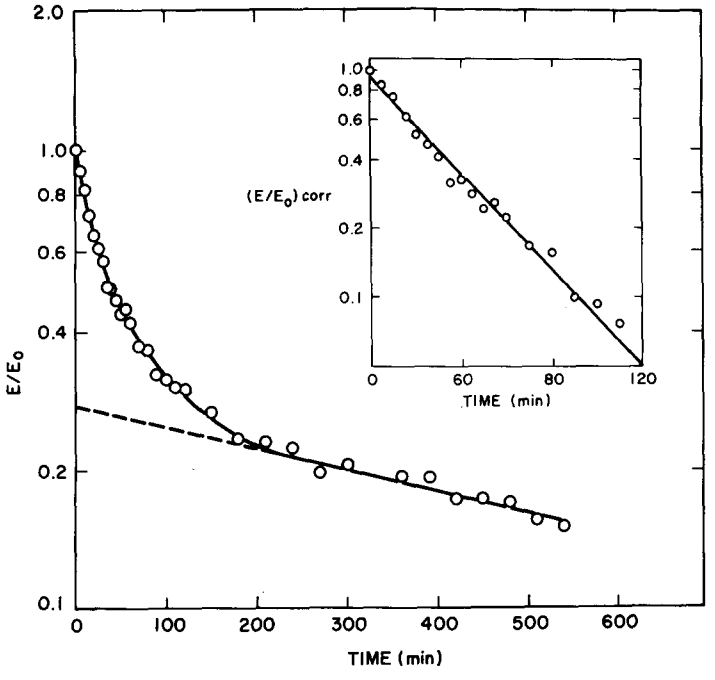

Fig. 5. Thermal denaturation of NAD-dependent isocitrate dehydrogenase. Enzyme was incubated at 37°C in 0.05 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) buffer, pH 6.0, containing 2 mM $MnSO_4$ and 1% glycerol. The ionic strength was adjusted to 0.15 by the addition of KCl. At the times indicated, aliquots were withdrawn and assayed for enzymatic activity as described in Experimental Procedure. The inset is a re-plot of the first phase of the reaction after a correction has been made for the slower rate of inactivation of the second phase (k = 0.00106 min^{-1}) and the data have been re-normalized to E/E_0 = 1.00 at t = 0. The first-order rate constant calculated is 0.0243 min^{-1}.

rate of the second phase of inactivation, it is found to obey first-order kinetics, as illustrated in the inset of Fig. 5. The rate constant for the first phase is more than 20 times greater than that of the second phase.

The addition of isocitrate markedly reduces the rate of denaturation of the

TABLE III

EFFECT OF ISOCITRATE ON THE THERMAL DENATURATION OF NAD-SPECIFIC ISOCITRATE DEHYDROGENASE

Isocitrate dehydrogenase was incubated at 37°C in 0.05 M piperazine-N,N′-bis(2-ethanesulfonic acid) buffer, pH 6.0, containing 2 mM $MnSO_4$ and 1% glycerol, as described in the legend of Fig. 5. Isocitrate was present as indicated. The rate constants for the first phase of the reaction were calculated as indicated in Fig. 5.

Additions to incubation mixture	Denaturation rate constant k^D_{obs} (min^{-1})	Calculated K_S for total DL-isocitrate (mM)
None	0.0243	—
5.0 mM DL-isocitrate	0.00853	2.70
10.0 mM DL-isocitrate	0.00354	1.71
40.0 mM DL-isocitrate	0.00145	2.54
53.0 mM DL-isocitrate	0.00123	2.84

enzyme. Table III records the quantitative effect of increasing isocitrate concentrations on the rate constant for the first phase of denaturation. Over the concentration range from 5 to 53 mM DL-isocitrate, the rate constant decreases about 20-fold. Similar concentrations of citrate did not affect the rate of denaturation. From the relationship given in Eq. 2 and used in Table I, dissociation constants may be calculated for the enzyme-isocitrate complex at each of the isocitrate concentrations tested. The results shown in Table III reveal a reasonably constant K_s with an average value of 2.45 mM in terms of total DL-isocitrate. This value is similar to the average dissociation constant of 6.3 mM total DL-isocitrate calculated from the effect of isocitrate in lowering the rate of inactivation by iodoacetate (Table I).

Discussion

Iodoacetate is considered to be a general reagent capable of reacting with many classes of amino acid residues of proteins. It has, for example, been found to react with a cysteinyl residue in horse liver alcohol dehydrogenase [22], with histidyl and methionyl residues in pancreatic ribonuclease [14,15], with a glutamyl residue in ribonuclease T_1 [20] and with a methionyl residue in NADP-dependent isocitrate dehydrogenase [6]. However, despite its broad reactivity, iodoacetate has frequently been observed to yield quite limited and specific reactions with particular proteins. The specificity may be the result of a non-covalent interaction of iodoacetate with amino acid residues in the vicinity of the residue which reacts covalently. Such an explanation has been postulated to account for the high reactivity of the active site cysteine in alcohol dehydrogenase [23] and for the enhanced rate of inactivation of the NADP-specific isocitrate dehydrogenase as the pH is decreased below 6.5 [6].

A pH of 6.0 was selected for the reactions of NAD-dependent isocitrate dehydrogenase with iodoacetate which are described in this paper in order to approximate the conditions used for the specific reaction with the methionyl residue of the NADP-specific isocitrate dehydrogenase. The reactions of amino, imidazole and sulfhydryl groups are thought to proceed via their unprotonated forms and therefore they might not be expected to be important at pH 6.0; whereas, reaction with methionine is independent of pH. The initial aim was to ascertain whether the allosteric NAD-dependent isocitrate dehydrogenase was similar to the non-regulatory isocitrate dehydrogenase in having a reactive methionyl residue which participates in catalysis. The data here presented indicate that reaction with methionine in the NAD-enzyme is minimal; rather, the inactivation can be accounted for by formation of 1—2 carboxymethyl-cysteine residues. The most direct conclusion is that the methionyl residue, which has been proposed as one candidate for the enzyme group participating in the removal of a proton form the C-2 hydroxyl group of isocitrate [2], is not a universal feature of the active site of isocitrate dehydrogenases. The general base involved may vary with the particular enzyme. Alternatively, the possibility must still be considered that the two enzymes differ not in the existence of an essential methionyl residue, but rather in the amino acids surrounding that residue, so that iodoacetate is specifically attracted to the active site of the NADP enzyme but is not so directed in the case of the NAD enzyme.

Although the course of the iodoacetate reaction is different for the NAD enzyme, it is also limited and specific. Modification of 2—3 of the seven half-cystine residues per peptide chain [12] occurs at a pH that is low for reaction with a typical thiol compound; and alkylation of only 1—2 of these residues appears to be responsible for the inactivation of the enzyme. The specificity may be determined in part by the negative charge of the reagent, since the neutral iodoacetamide causes inactivation at a rate that is approximately one-third that obtained with iodoacetate. In contrast to the striking protection afforded by isocitrate and Mn^{2+} against inactivation by idoacetate, the substrates do not influence the course of inactivation by iodoacetamide. It appears that these two closely related reagents must alkylate the enzyme at different sites. There may be a positively charged amino acid proximal to a critical cysteinyl residue which directs the specificity of iodoacetate, in analogy to the arginine which plays that role in alcohol dehydrogenase [23].

The function of the cysteine residue(s) attacked by iodoacetate merits comment. No protection against inactivation is provided by NAD or ADP, suggesting that the susceptible residues lie neither within the coenzyme nor the allosteric site. Similarly, the substrate α-ketoglutarate does not decrease the rate of inactivation, nor do isocitrate or $MnSO_4$ alone. Indeed, marked protection against inactivation is observed only when isocitrate and Mn^{2+} are included together in the reaction mixture. The β-carboxylate group of isocitrate, as well as metal ion, seems to be required for binding to the site attacked by iodoacetate.

Evidence has been presented that 1—2 cysteinyl residues are essential for the activity of the NADP-dependent isocitrate dehydrogenase [7—9] and that these residues participate in the binding of the manganous-tribasic isocitrate complex [24,25]. In that case, the concentrations of isocitrate and metal ion required to reduce the rates of modification of the cysteinyl residues are consistent with the known Michaelis constants for the substrates. These results contrast with the present observations on the NAD-specific isocitrate dehydrogenase. For the NAD enzyme, the Michaelis constant for the free dibasic DL-isocitrate binding to the enzyme-mangenous complex has been measured as 0.030 mM and is independent of pH from 6.0 to 8.0 [4]. Essentially the same value of 0.031 mM was obtained for the dissociation constant of dibasic isocitrate from the enzyme-manganous-dibasic isocitrate complex on the basis of the dependence on isocitrate concentration of the decrease in the rate constant for inactivation by cyanate [11]. From the similarity between the Michaelis constant and the more directly determined dissociation constant, it appears that under the conditions of the cyanate modification reaction (i.e. pH 7.4), K_m is a simple dissociation constant and the lysyl residue modified by cyanate constitutes that part of the active site involved in the binding of substrate. In contrast to these observations, the average dissociation constant for the enzyme-manganous-dibasic isocitrate complex measured from the decrease in rate of inactivation by iodoacetate (Table I) was 1.6 mM, about 50 times higher than the K_m. It is possible that under the conditions of the iodoacetate reaction (i.e. pH 6.0) that the K_m does not represent a dissociation constant. However, in view of the correspondence between K_m and K_d obtained from the cyanate inactivation studies at pH 7.4 [11] and the similarity in values of K_m in terms of dibasic isocitrate between pH 6.0 and 8.0 [4], it seems likely that K_m also represents a dissociation

constant at pH 6.0. If this assumption is correct, the manganous-isocitrate site revealed by the iodoacetate experiments is probably not the catalytically functional site for the substrate. Nevertheless, alkylation of the cysteine residue(s) of this site yields an inactive enzyme. Binding of substrate at this site is quite specific: *threo*-D_S-isocitrate is bound in preference to the L-isomer; and the allosteric activator ADP reduces the dissociation constant for the enzyme-manganous-isocitrate complex as measured by protection against modification by iodoacetate. It is possible that this second type of substrate binding site is responsible for stabilizing an active conformation of the enzyme, and that when the cysteine(s) are carboxymethylated, binding of manganous-isocitrate can no longer take place. The effect of isocitrate in preventing the thermal denaturation of the enzyme yields the dissociation constant for the enzyme-manganous-isocitrate complex of 2.45 mM, a value which is suggestively close to that observed at the site susceptible to iodoacetate. These observations lead to the prediction of two classes of binding sites for manganese and isocitrate in the pig heart NAD-specific isocitrate dehydrogenase.

The NAD-dependent isocitrate dehydrogenase isolated from bovine heart muscle has been reported to be inactivated by reaction of three cysteines with 5,5′dithio-bis-(2-nitrobenzoate) [26]. Partial protection was provided by the coenzymes, NAD and NADH; but the best protection was afforded by manganese and isocitrate. Unfortunately, the substrate concentration dependence of protection was not studied, so it is difficult to relate the cysteine-containing sites in the pig heart and bovine heart enzymes.

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