A Glutamyl Residue in the Active Site of Triphosphopyridine Nucleotide-dependent Isocitrate Dehydrogenase of Pig Heart*

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SUMMARY

The maximum velocity of the reaction catalyzed by the pig heart TPN-specific isocitrate dehydrogenase depends on the basic form of an enzymatic group of pK 5.7. This pK is independent of temperature from 10-30° and increases in 20% ethanol, suggesting the ionization of a carboxyl group. The enzyme is inactivated by incubation at pH 7.0 with 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide either alone or in the presence of glycine ethyl ester, glycynamide, or glycine. Both the dehydrogenase and decarboxylase functions of the enzyme are affected. The addition of manganese ion and isocitrate or α-ketoglutarate to the incubation mixture produces a striking decrease in the inactivation rate, implying that reaction takes place in the active site. The inactive glycynamide enzyme exhibits a decreased ability to bind manganese ion in the presence of isocitrate, which suggests that the integrity of those amino acid residues susceptible to the carbodiimide and glycynamide are important for the binding of the manganese-isocitrate complex by the enzyme.

The most probable sites of reaction of the carbodiimide are tyrosine, cysteine, glutamic and aspartic acids. Inactivation is not reversed by hydroxylamine indicating that tyrosine modification is not responsible, and no significant change in the measurable sulfhydryl content is noted upon inactivation. Treatment of the enzyme with the carbodiimide in the presence of tritium label leads to incorporation of radioactivity into the carbodiimide and glycynamide, respectively. Reaction of the carbodiimide with the enzyme results in a decrease in the inactivation rate, providing the basis for formulation of the mechanism of the enzymatic reaction. Alkylation of the enzyme with iodoacetate has revealed that a single methionyl residue is essential for catalysis (1, 2); and reactions with 5,5'-dithiobis(2-nitrobenzoic acid) (3) and N-ethylmaleimide (4, 5) have implicated sulfhydryl groups in enzymatic function. A study of the pH dependence of Vmax indicated the requirement for activity of the basic form of an ionizable group in the enzyme-substrate complex, with a pK of 5.7 (6). This observed pK lies between the values generally thought to be characteristic of carboxyl and imidazolium groups in a protein (7) ; however, the increase in the pK of the enzymatic group when measured in 20% ethanol suggests that it represented the ionization of an essential carboxyl group (2). The present investigation represents an attempt to evaluate more conclusively the possible role of an acidic amino acid in the function of isocitrate dehydrogenase.

Water-soluble carbodiimides have been used to modify extensively the carboxyl groups of proteins under mild conditions (8, 9). It has been proposed that the reaction is initiated by the protonation of a nitrogen of carbodiimide followed by the attack of the carboxylate anion on the central carbon atom of the carbodiimide (10). Thus, the reaction generally proceeds more rapidly at lower pH values (11); indeed the standard procedure of Carraway and Koshland designates 4.75 as the pH of choice (9). In the case of isocitrate dehydrogenase, it was reasoned that since the postulated critical carboxyl group seemed to exhibit a relatively high pK, more specific modification of that residue might be achieved by conducting the reaction with carbodiimide around neutrality. This paper reports the inactivation of isocitrate dehydrogenase at pH 7.0 by 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate, either alone or in the presence of suitable nucleophiles and presents evidence that modification of a glutamyl residue is responsible for the loss in catalytic function.

EXPERIMENTAL PROCEDURE

Materials—Pig heart TPN-dependent isocitrate dehydrogenase (albumin-free), with a specific activity of approximately 5.5 pmoles of TPNH per min per mg of enzyme, was supplied as a
solution in 50% glycerol by Boehringer Mannheim Corp. The enzyme was purified to homogeneity by column chromatography on carboxymethylcellulose and gel filtration on Sephadex G-150, as previously described (2), and exhibits a specific activity of 29 pmoles of TPNH reduced per min per mg of protein. A molecular weight of 85,000 was used in the calculations (12, 13).

All enzyomes and substrates were purchased from Sigma Chemical Co. Aldrich Chemical Co. supplied 1-cyclohexyl-3-(2-morpholinoethyl)-carboxydimide metho-p-toluenesulfonate, glycineamide hydrochloride, aminomethanesulfonic acid, and 5,5'-dithiobis(2-nitrobenzoic acid). The unlabeled glycine ethyl ester hydrochloride was obtained from Nutritional Biochemical Co., and the radioactive [1-14C]glycine ethyl ester hydrochloride from New England Nuclear Corp. Aquasol was also purchased from New England Nuclear Corp. Cyclo Chemical Co. provided γ-glutamylglycine and β-l-aspartylglycine. Pronase (grade B) came from Calbiochem and carboxypeptidase A and B from Worthington Biochemical Corp.

Kinetic Studies—Unless otherwise indicated, isocitrate dehydrogenase activity was measured at 23° in 0.03 M triethanolamine buffer, pH 7.4, with 0.1 mM TPN, 4 mM di-isocitrate, and 2 mM manganese sulfate, in a total volume of 1.0 ml. Initial velocities were determined spectrophotometrically at 340 nm with an expanded scale recorder (0.1 absorbance full scale) using either a Gilford model 242 or Cary model 15 spectrophotometer. In determining the Michaelis constants, each substrate was varied in turn, with the remaining substrates being maintained at the concentrations given in this section. In the case of manganese sulfate only, the Michaelis constant was measured in 0.2 mM sodium acetate buffer, pH 5.5, containing 0.1 mM TPN and 4 mM di-isocitrate.

Reducive carboxylase activity was measured spectrophotometrically at 340 nm and 23° in 0.04 M triethanolamine chloride buffer, pH 7.4, with 0.9 mM TPNH, 90 mM α-ketoglutarate, 40 mM potassium bichromate, and 2 mM MnSO4 in a total volume of 1.0 ml. The potassium bichromate stock solution was saturated with carbon dioxide prior to use. In determining the Michaelis constants, the nonvaried components were maintained at the concentrations here given.

The pH dependence of Vmax for the isocitrate dehydrogenase reaction was measured from pH 4.8 to 7.5 in sodium acetate, imidazole chloride, and triethanolamine chloride buffers (0.03 M in anion). In these experiments the concentrations of isocitrate, TPN, and MnSO4 were 4, 0.1, and 2 mM, respectively, and the rates were independent of substrate concentration. The effects of pH on enzymatic activity were shown to be reversible throughout this pH range.

Reaction with CMC and Nucleophile—Unless specified otherwise, isocitrate dehydrogenase (0.13 mg per ml) was incubated with 0.064 M CMC in the presence of 0.36 M glycaminide (or other nucleophile) in imidazole chloride buffer (0.029 M in chloride). The reaction was conducted at 22° and pH 7.0 and substrates were added as indicated. At specified times, aliquots were withdrawn, diluted 10-fold with 0.1 M triethanolamine chloride buffer, pH 7.7, containing 0.3 mM sodium sulfate and 10% glycerol, at 0°. Aliquots were assayed for isocitrate dehydrogenase activity.

Manganous Ion-binding Experiments—Manganous ion-binding experiments were conducted at 23° in 0.03 M triethanolamine chloride buffer, pH 7.4. Reaction mixtures contained manganese sulfate at concentrations from 3.0 to 80 μM, threo-δ-isocitrate as indicated and enzyme at concentrations from 3.0 to 26.5 μM. Aliquots of the original solutions were withdrawn to assess the total manganese concentration by atomic absorption spectrophotometry as previously described (14). Free metal was separated from protein-bound manganese by the method of ultrafiltration, using an Amicon model 10-PA ultrafiltration cell equipped with a PM-10 membrane, as detailed earlier (15, 16). The concentration of enzyme-bound manganese was calculated from the difference between the concentrations of total and free metal.

Determination of Sulfhydryl Groups—The free sulfhydryl groups in the native and 7% active glycaminide enzyme were measured by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) under denaturing conditions in 0.2 M Tris chloride buffer, pH 8.4, containing 0.2% sodium dodecyl sulfate (17). Under these conditions, native enzyme exhibits an average of 5.93 free —SH groups per mole. It is not clear why this value is lower than that previously reported (1). The starting material for the enzyme purification is not the same as in the earlier study, as noted under “Materials.” However, the final enzyme preparation, which is homogeneous by disc gel electrophoresis, exhibits the same specific activity and kinetic constants as those observed earlier (2, 3). The cysteic acid content of the enzyme used in the present investigation is 6.04 moles per mole of enzyme, as determined by performic acid oxidation followed by hydrolysis in 6 N HCl at 110° for 20 hours, which is in reasonable agreement with the value obtained by reaction with DTNB.

Incorporation of [1-14C]Glycine Ethyl Ester in Presence and Absence of CMC—Isocitrate dehydrogenase (0.47 mg) was incubated with 0.36 M [1-14C]glycine ethyl ester in the presence or absence of 0.064 M CMC in a total volume of 1.0 ml under the conditions given above. At the end of 2.5 hours, the residual activity was 1.7%. The protein was precipitated by the addition of 9 ml of 15% trichloracetic acid, the suspension centrifuged, and the supernatant drawn off. The precipitate was washed four times with a solution of ethanol-diethyl ether (1:1), followed by four washes with diethyl ether. Aquasol was added to dissolve the precipitate and it was quantitatively transferred to a vial for counting in a Packard Tri-Carb liquid scintillation counter, model 3320.

Protoplastic Digestion of [1-14C]Glycine Ethyl Ester Enzyme—Isocitrate dehydrogenase (2.9 mg) was incubated for 2.5 hours at 22° with 0.36 M [1-14C]glycine ethyl ester in the presence of 0.064 M CMC in a total volume of 3.0 ml as described above. The resultant preparation, was dialyzed at 4° against two changes of 1 liter of 0.1 M triethanolamine chloride buffer, pH 7.7, containing 0.3 mM sodium sulfate and 10% glycerol in order to remove the excess reagent. Subsequently, the enzyme was dialyzed against distilled water to remove salts, and finally against 0.2 M ammonium bicarbonate buffer, pH 7.0.

Modified isocitrate dehydrogenase was incubated with 0.32 mg of pronase for 48 hours at pH 7.6 and 40°. At the end of this period, 0.16 mg each of carboxypeptidase A and carboxypeptidase B were added and the incubation was continued for another 48 hours. Hydrolysis of isocitrate dehydrogenase has been shown
to be approximately 93% complete under these conditions (5). The preparation was desalted by repeated evaporation under vacuum.

Isolation of Radioactive Peptide—Digests of the desalted radioactive glycine ethyl ester enzyme were subjected to descending paper chromatography on Whatman No. 3MM (46 × 57 cm) for 18 hours, using as solvent 1-butanol-pyridine-acetic acid-water (15:10:3:12). The chromatograms were dried, turned 90°, and subjected to high voltage electrophoresis in pyridine acetate buffer (0.070 M in anion), pH 6.4, for 40 min at 2.5 kV. Active spots were located either by scanning strips with the Vanguard Autoscaner, or by cutting the chromatogram into pieces 0.75 × 0.50 inch, and counting in the liquid scintillation counter. In the case of the strips which were scanned, the radioactive spots were eluted and were further purified by high voltage electrophoresis on Whatman No. 3MM in pyridine acetate buffer (0.041 M in anion), pH 3.5, for 110 min at 3.5 kV.

RESULTS

Effect of Temperature on pH Dependence of $V_{\text{max}}$—The maximum velocity of the reaction catalyzed by the TPN-specific isocitrate dehydrogenase has been shown to depend on the basic form of an ionizable group in the enzyme-substrate complex with a pK of 5.7 at room temperature (6). The heat of ionization of a group with a pK in this region can be of great value in identifying the type of amino acid responsible for the pH dependence of the reaction. The pK of the imidazolium group of histidine decreases as temperature is increased, with a characteristic ΔH of 6.0 to 7.5 Cal per mole; whereas the pK of the carboxyl groups of aspartic or glutamic acids is relatively insensitive to changes in temperature, with values of ΔH varying from -1.5 to +1.5 Cal per mole (7). Fig. 1 illustrates the pH dependence of $V_{\text{max}}$ at 10.0, 23.0, and 29.7°C. The data have been normalized by plotting for each temperature the ratio of the observed maximum velocity at a given hydrogen ion concentration ($V_{\text{max}}$) to the intrinsic maximum velocity ($V_{\text{max}i}$). It is apparent that the points at all three temperatures describe approximately the same curve. The data at each temperature may be treated separately and a dissociation constant ($K_{\text{D}}$) may be calculated for an essential ionizable group in the enzyme-substrate complex by means of the following equation:

$$V_{\text{max}} = \frac{V_{\text{max}i}}{1 + ([H^+] / K_{\text{D}})}$$

These calculations yield estimates of 5.72, 5.71, and 5.69 for the pK of this residue at 10.0, 23.0, and 29.7°C, respectively. Since these small differences in pK lie within the experimental error, the heat of ionization is clearly close to zero. These results strongly support the postulate (2) that the pK of 5.7 represents the ionization of a carboxylic amino acid.

Inactivation by CMC and Glycinamide—Isocitrate dehydrogenase is inactivated progressively upon incubation with the water-soluble carbodiimide, CMC, in the presence of the nucleophile, glycine amide, at pH 7.0 and 22°C. During incubation under similar conditions in the absence of these reagents, the activity of the enzyme is not significantly altered. The concentrations of CMC and Gly-NH$_2$ are in great excess over that of the enzyme and, therefore, pseudo-first order kinetics is obeyed, as indicated in Fig. 2; the half-life for inactivation as shown in this graph is approximately 15 min. When isocitrate and MnSO$_4$ are present in the incubation mixture, no appreciable inactivation is produced by CMC at times as long as 8 hours. This result implies that carbodiimide reacts with an amino acid residue at or near the active site.

Table 1 records the effect on the inactivation rate constant of adding various substrates and cofactors to the incubation mixture. Isocitrate dehydrogenase requires a divalent metal ion for activity, and relatively small protective effects are observed when metal ion is not added. Isocitrate, which is known to bind to the enzyme in the absence of metal and coenzyme (15), produces a 3-fold decrease in the rate of inactivation, as does TPNH. It might be argued that isocitrate reacts directly with CMC, thereby decreasing the concentration of reagent available to react with the enzyme and yielding an apparent protection against inactivation. However, citrate, which is not a substrate for the enzyme, produces a lesser degree of protection at the same concentration of carboxylic acid. The other carboxylic acid substrate, α-ketoglutarate, as well as the oxidized coenzyme do not markedly influence the rate of inactivation.

Manganous sulfate alone produces a 5-fold decrease in the inactivation rate. The active substrate for this enzyme has been shown to be the metal-isocitrate complex (14) and the most
TABLE I
Rate of inactivation by CMC and glycinamide in presence of substrates and coenzymes

<table>
<thead>
<tr>
<th>Additions to reaction mixture</th>
<th>k (10^-3 min^-1)</th>
<th>No metal ion</th>
<th>+2 mm MnSO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>45.8</td>
<td>9.44</td>
<td></td>
</tr>
<tr>
<td>4.0 mm isocitrate</td>
<td>13.1</td>
<td>&lt;0.41</td>
<td></td>
</tr>
<tr>
<td>4.0 mm citrate</td>
<td>29.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 mm α-ketoglutarate</td>
<td>38.3</td>
<td>1.95</td>
<td></td>
</tr>
<tr>
<td>0.1 mm TPN</td>
<td>20.8</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>0.2 mm TPNH</td>
<td>12.3</td>
<td>3.40</td>
<td></td>
</tr>
</tbody>
</table>

striking protection against inactivation by CMC is provided by MnSO4 plus isocitrate. A ternary enzyme-metal-substrate complex has also been proposed in the case of α-ketoglutarate (16) and the addition of α-ketoglutarate together with MnSO4 produces a further 4- to 5-fold decrease in the rate of inactivation as compared with the effect of metal ion alone. Reaction of CMC probably does not take place directly at the coenzyme binding site since TPN causes no significant alteration in the protective effect of MnSO4 when added together with the metal ion, and TPNH effects the same small decrease (approximately 3-fold) in the rate constant when added alone or in combination with manganous ion.

The rate-determining step in the over-all oxidative decarboxylation of isocitrate has been shown to be the initial dehydrogenation reaction (16, 18) whereas the slow step in the reductive decarboxylation is probably the carboxylation step (19, 20). Any difference in the effect of chemical modification of an amino acid on the two functions of the enzyme would be reflected in a different rate of inactivation as assessed by the over-all isocitrate dehydrogenase reaction as compared with the reductive carboxylation reaction, and in a difference in the residual activities in these two assays after the same period of incubation with a reagent. Fig. 3 shows that the same rate of inactivation by CMC in the presence of glycinamide is obtained whether the enzyme activity is measured using the isocitrate dehydrogenase or reductive carboxylase assay. Similarly an enzyme which was incubated with CMC and glycinamide, and then dialyzed to remove excess reagent, exhibited 4.7% of its original isocitrate dehydrogenase activity and 3.9% of its initial reductive carboxylation activity.

Properties of Modified Enzyme—A loss of activity under standard assay conditions may be observed when the modified enzyme is intrinsically active but has a weaker affinity for substrates. Isocitrate dehydrogenase was incubated with CMC and glycinamide as described under "Experimental Procedure." At appropriate intervals, aliquots were taken, diluted 10-fold, and assayed for isocitrate dehydrogenase or reductive carboxylation activity as described under "Experimental Procedure." The pseudo-first order rate constant calculated for Line A is 0.0477 min⁻¹ while that for Line B is 0.0469 min⁻¹.

Fig. 3. Rate of inactivation by CMC in the presence of glycinamide as assessed by the over-all isocitrate dehydrogenase (A) or reductive carboxylation (B) reactions. Isocitrate dehydrogenase was incubated with CMC and glycinamide as described under "Experimental Procedure." At appropriate intervals, aliquots were taken, diluted 10-fold, and assayed for isocitrate dehydrogenase or reductive carboxylation activity as described under "Experimental Procedure." The pseudo-first order rate constant calculated for Line A is 0.0477 min⁻¹ while that for Line B is 0.0469 min⁻¹.

Since it is not possible to assess by kinetic means whether an inactive enzyme is capable of combining with substrates, the ability of glycinamide enzyme (with only 8% remaining activity) to bind manganous ion was tested. Table III shows that for the native enzyme the dissociation constant of the enzyme-manganous complex is decreased 20-fold when measured in the presence of isocitrate, which is consistent with the proposal that the manganous-isocitrate complex is the actual substrate of this enzyme (14). The inactive glycinamide enzyme still binds approximately 1 mole of metal ion in the presence and absence of substrate and the dissociation constant for this enzyme-manganous complex in the absence of isocitrate is similar to that
The Michaelis constants were determined at pH 7.4 under "Experimental Procedure," with the exception of the $K_m$ for MnSO$_4$, which was measured at pH 5.5.

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>Native enzyme</th>
<th>Glycinamide enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ threo-2,3-isocitrate (μM)</td>
<td>3.9</td>
<td>2.9</td>
</tr>
<tr>
<td>$K_m$ TPN (μM)</td>
<td>4.6</td>
<td>4.8</td>
</tr>
<tr>
<td>$K_m$ MnSO$_4$ (μM)</td>
<td>11.1</td>
<td>9.3</td>
</tr>
<tr>
<td>$K_m$ α-ketoglutarate (μM)</td>
<td>0.51</td>
<td>0.74</td>
</tr>
<tr>
<td>$K_m$ TPN + (μM)</td>
<td>12.0</td>
<td>8.7</td>
</tr>
<tr>
<td>pK$_{diss}$ (at 23°)</td>
<td>5.71</td>
<td>5.75</td>
</tr>
</tbody>
</table>

The binding measurements were conducted at room temperature by the method of ultrafiltration, as described under "Experimental Procedure." The data were analyzed graphically in accordance with the equation:

$$\frac{r}{(Mn)_{free}} = \frac{n}{K} \cdot \frac{r}{K}$$

where $r = $ moles of metal ion bound per 58,000 g of protein; $n = $ number of metal binding sites; and $K = $ dissociation constant for the enzyme-substrate complex.

<table>
<thead>
<tr>
<th>Enzyme preparation and activity</th>
<th>No substrates</th>
<th>0.5 mM threo-2,3-isocitrate present</th>
</tr>
</thead>
<tbody>
<tr>
<td>K$_{diss}$ (μM)</td>
<td>n</td>
<td>K$_{diss}$ (μM)</td>
</tr>
<tr>
<td>Native enzyme, 100%</td>
<td>45.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Glycinamide enzyme, 8%</td>
<td>28.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Direct binding of manganous ion by native and inactive glycinamide enzyme

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**Effect of Other Nucleophiles**—The experiments described up to this point were conducted using the nucleophile glycine ethyl ester (0.36 μM) in combination with CMC (0.064 μM) to chemically modify isocitrate dehydrogenase. Under these conditions, a typical rate constant for inactivation is 0.0423 min$^{-1}$. Inactivation is also produced by incubation of enzyme with the carbodiimide alone ($k = 0.0369$ min$^{-1}$), or with CMC and the nucleophiles glycine ethyl ester ($k = 0.0324$ min$^{-1}$) or glycine ($k = 0.0213$ min$^{-1}$). The lower inactivation rate in the presence of glycine may be attributable to a decreased effective concentration of CMC resulting from its reaction with the α-carboxyl group of glycine. Similarly, the slightly lower inactivation rate observed with glycine ethyl ester may be caused by partial hydrolysis to glycine. However, it is possible to completely inactivate the enzyme with CMC alone, CMC plus glycine, CMC plus glycine ethyl ester, or CMC plus glycine. In all of these cases, inactivation is prevented by incubation with reagent in the presence of isocitrate and manganous ion, suggesting that reaction occurs at the same site.

**Incorporation of [1-14C]Glycine Ethyl Ester—Isocitrate dehydrogenase** which is incubated with [1-14C]glycine-ethyl ester for 2.5 hours in the absence of CMC retains all of its original catalytic activity. However, after precipitation and extensive washing with ethanol and diethyl ether, it retains approximately 1 mole of radioactive Gly-OEt per mole of enzyme (Table IV, Line 1a). Similarly, when isocitrate and MnSO$_4$ are included with Gly-OEt in the incubation mixture in the absence of CMC, full activity is retained but 1 mole of radioactive glycine ethyl ester remains tightly bound (Table IV, Line 2a). In contrast, when the water-soluble carbodiimide (but no substrates) is present together with [1-14C]glycine ethyl ester, the enzyme is inactivated and 2 moles of the radioactive compound are incorporated (Table IV, Line 1b). The amount of ester bound in the absence of CMC, which is unrelated to the activity of the enzyme, has been subtracted from that recorded in Line 1b to yield a net value of moles of glycine ethyl ester bound per mole of protein as a result of reaction of enzyme with carbodiimide. These data imply that 1 mole of radioactive compound is incorporated concomitantly with inactivation and therefore that modification of a single amino acid residue may be responsible for the loss of activity.

The postulate is supported by the observation that when the enzyme is treated with CMC and [1-14C]glycine ethyl ester in the presence of isocitrate and metal ion, both the extent of inactivation and the net incorporation of radioactive compound are reduced proportionately (Table IV, Line 2b).

**Identification of Amino Acid Residue Attacked by CMC and Glycine Ethyl Ester**—In considering the amino acid residues attacked by CMC and glycine ethyl ester or glycine, the most likely candidates are the weak acids tyrosine (21), cysteine (22), glutamic and aspartic acid (8). Carraway and Koshland have shown that free tyrosine in proteins can be regenerated from the corresponding O-aryl isourea derivative of carbodiimide by treatment with 0.5 M hydroxyamine at pH 7. In the case of isocitrate dehydrogenase, such hydroxyamine treatment did not restore activity to a 23% active glycinamide enzyme. This result suggests that modification of tyrosine is not primarily responsible for the loss of activity.

To test for reaction of the carbodiimide with sulfhydryl groups in isocitrate dehydrogenase, the enzyme was incubated with CMC and Gly-NH$_2$ until its activity has declined to 7.6% of its original value. Enzyme incubated with Gly-NH$_2$ alone under
the same conditions served as a control. At the end of the reaction period, both preparations were dialyzed extensively to remove excess reagents, and the total sulphydryl groups were titrated with 5,5′-dithiobis(2-nitrobenzoic acid), as described under "Experimental Procedure." The native enzyme exhibited 6.32 and the inactive modified enzyme 6.52 —SH groups per mole of protein, indicating that the carbodiimide had not reacted with cysteine residues in isocitrate dehydrogenase.

If the site of attack of CMC and nucleophile on isocitrate dehydrogenase were one of the two carboxylic amino acids, the expected products would be β-aspartylglycine ethyl ester or γ-glutamylglycine ethyl ester. Since these compounds are unstable upon acid hydrolysis, isocitrate dehydrogenase, which had been inactivated with CMC in the presence of [1-14C]glycine ethyl ester, was extensively digested by Pronase, carboxypeptidase A, and carboxypeptidase B. This proteolytic digest was subjected to descending paper chromatography followed by high voltage electrophoresis at pH 6.4. As shown in Fig. 4, two regions of radioactivity were detected, one in the vicinity of the acidic amino acids (labeled 1) and the other in the region of the neutral amino acids (designated 2). During the long periods used for digestion by proteolytic enzymes, followed by desalting, glycine ethyl ester or its derivatives would be expected to hydrolyze to glycine. Spot 1 is located in the region where authentic γ-L-glutamylglycine and β-L-aspartylglycine migrate; these two peptides do not separate well at pH 6.4. Spot 2 is in the area where glycine might be expected. In a control experiment, enzyme was incubated with [1-14C]glycine ethyl ester in the absence of CMC, and the resulting active enzyme was digested by the same procedures used for the inactive enzyme. The "finger print" corresponding to Fig. 4 revealed only a single radioactive spot for the active enzyme: Spot 2. It is probable that Spot 2 is derived from the radioactive glycine ethyl ester which is tightly bound to the enzyme with or without added CMC, and which is unrelated to catalytic activity. In contrast, Spot 1 may represent the product of the reaction between isocitrate dehydrogenase and CMC in the presence of glycine ethyl ester which is responsible for the inactivation.

**DISCUSSION**

The data here presented provide evidence for the involvement of a carboxylic amino acid in the catalytic function of the pig heart TPN-specific isocitrate dehydrogenase. The pH dependence of the maximum velocity of the dehydrogenase reaction yields a pK of 5.7 which is essentially independent of temperature and which increases when the reaction is conducted in 20% ethanol (2). Extreme caution must be used in attempting to identify any measured ionization constant with a particular amino acid residue. The range of pI within which those groups dissociate may be significantly altered by other functional groups within the protein environment (23); furthermore, in a complex reaction involving more than one enzyme-substrate intermediate, the apparent ionization constant may include terms for any equilibrium reaction prior to the rate-determining step (24). The simplest interpretation of the data, however, is that a carboxylate group in the enzyme-substrate complex participates in the catalytic reaction.

This tentative conclusion is considerably strengthened by the observed inactivation of isocitrate dehydrogenase by a water-soluble carbodiimide and glycinamide. A carboxylic group of glutamic or aspartic acid is the most probable site of attack of CMC. Reaction with tyrosine or cysteine residues does not appear to be responsible for loss of enzymatic function since activity is not regained by treatment with hydroxyamine (21), and the total content of free sulphydryl groups in the enzyme does not change as much as the sulphydryl content of the modified enzyme. The isocitrate dehydrogenase used in this study was heart TPN-specific isocitrate dehydrogenase. The pH dependence of the maximum velocity of the dehydrogenase reaction yields a pK of 5.7 which is essentially independent of temperature and which increases when the reaction is conducted in 20% ethanol (2). Extreme caution must be used in attempting to identify any measured ionization constant with a particular amino acid residue. The range of pI within which those groups dissociate may be significantly altered by other functional groups within the protein environment (23); furthermore, in a complex reaction involving more than one enzyme-substrate intermediate, the apparent ionization constant may include terms for any equilibrium reaction prior to the rate-determining step (24). The simplest interpretation of the data, however, is that a carboxylate group in the enzyme-substrate complex participates in the catalytic reaction.

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not change significantly after incubation with CMC and glycinamide (22). In the absence of esterlyce, alcohols (such as threonine or serine) are relatively inert toward CMC at room temperature (25). Amines can react additively with carbodiimides to form di- and tri-substituted guanidines, and guanidines can react to yield biguanides (25); however, these reactions require the unprotonated form of the amino and guanidino groups and, therefore, would be expected to proceed relatively slowly at pH 7. The correlation between the extent of inactivation by CMC and Gly-OEt, and the incorporation of approximately 1 mole of radioactive glycine ethyl ester, as well as the identification of radioactive \( \gamma \)-glutamylglycine in the modified protein provides strong evidence that a glutamyl residue is the site of reaction of the carbodiimide and nucleophile in isocitrate dehydrogenase.

The explanation for the inactivation of isocitrate dehydrogenase by CMC and nucleophile cannot be merely that there is a loss of the negative charge of the glutamyl side chain. In the case of glycaminamide and glycine ethyl ester the charge of the side chain is indeed altered; however, the enzyme is also inactivated when glycine is used as the nucleophile and, in this case, the negative charge is retained in the glutamyl derivative. It may be that the precise position of the negative charge within the active site of the enzyme is critical to the function of the enzyme.

The role played by the glutamate residue in isocitrate dehydrogenase cannot yet be definitively designated. An effect on the conformation of the enzyme produced by chemical modification has not been excluded. Nevertheless, the protection provided by manganous ion with the same dissociation constant as that of native enzyme in the absence of substrates, but that the affinity of enzyme for metal ion is not strengthened by the addition of isocitrate (16). Concomitantly, the inactive \( N \)-ethylmaleimide enzyme exhibits a dissociation constant for isocitrate in the presence of manganese ion which is similar in value to that of the native enzyme in the absence of metal (4). It appeared that this inactive \( N \)-ethylmaleimide enzyme was capable of separately binding isocitrate and metal ion but did not exhibit preferential affinity for the manganese-isocitrate complex. A similar situation may be operative in the case of the inactive glycaminamide enzyme: modification of a critical glutamate residue may disrupt the interaction between the manganese and isocitrate sites and this interaction may be important in the function of the enzyme.

It is possible, for example, that glutamate may be one of the ligands of the coessential divalent metal ion. Thus, the manganous ion may interact with the carboxylate group of the essential glutamyl residue and with both the \( \alpha \) and \( \beta \) carboxylate groups of isocitrate (or the \( \beta \)-carboxylate group of \( \alpha \)-ketoglutarate) to minimize repulsive forces between the enzyme and substrate and to facilitate productive binding. This would be a chemical version of the cyclic enzyme-metal-substrate complex previously proposed (16). The manganous ion, as bound to the enzyme, may also function as a catalyst for the enzymic decarboxylation of oxaloacetic acid by stabilizing the enolate form of \( \alpha \)-ketoglutarate which is the initial product of that reaction. When the glutamate residue is blocked the manganous ion would still be bound to the enzyme (albeit more weakly) through other ligands; however, it may not be positioned to catalyze the reaction effectively. The precise details of the involvement of the glutamyl residue in this process remain to be elucidated.

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