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Evidence for a Critical Glutamyl and an Aspartyl Residue in the Function of Pig Heart Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The pH dependence of the maximum velocity of the reaction catalyzed by diphosphopyridine nucleotide (DPN) dependent isocitrate dehydrogenase indicates the requirement for the basic form of an ionizable group in the enzyme–substrate complex with a pK of 6.6. This pK is unaltered from 10 to 33 °C, suggesting the ionization of a carboxyl rather than an imidazolium ion. The enzyme is inactivated upon incubation with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide in the presence of glycinamide or glycine ethyl ester. This inactivation is dependent on pH and the rate constant (k) increases as the pH is decreased in the range 7.3 to 6.25. A plot of $1/(H^+)$ vs. $1/k$ suggests that the enzyme is inactivated as a result of the modification of a single ionizable group in this pH range. The coenzyme DPN and substrate α-ketoglutarate do not affect the rate of inactivation. In contrast, manganous ion (2 mM) and isocitrate (60 mM) produce a sevenfold decrease in the rate constant. The allosteric activator ADP (1 mM) does not itself influence the rate of inactivation; however, it reduces the concentration of Mn^{2+} (1 mM) and isocitrate (20 mM) required to produce the same decrease in the inactivation constant. These observations imply that the modification occurs at the substrate-binding site. Experiments employing [1-^{14}C]glycine ethyl ester show a net incorporation of 2 mol of glycine ethyl ester per subunit (40 000), concomitant with the complete inactivation of the enzyme. The radioactive modified enzyme, after removal of excess reagent by dialysis, was exhaustively digested with proteolytic enzymes. High voltage electrophoretic analyses of the hydrolysate at pH 6.4 and 3.5 yield two major radioactive spots with approximately equal intensity, which correspond to γ-glutamylglycine and β-aspartylglycine, the ultimate products of reaction with glutamic and aspartic acids, respectively. Modification in the presence of manganous ion and isocitrate results in significant reduction in the incorporation of radioactivity into the two dipeptides. These results suggest that carbodiimide attacks one glutamyl and one aspartyl residue per subunit of the enzyme and that the integrity of these residues is crucial for the enzymatic activity.

As with most mammalian tissues, the pig heart muscle contains two distinct isocitrate dehydrogenases: a TPN[1]-dependent enzyme (*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42), found in both mitochondria and cytoplasm, and a DPN-dependent enzyme (*threo*-D$_S$-isocitrate:NAD$^+$ oxidoreductase (decarboxylating), EC 1.1.1.41) located in the mitochondria (Plaut, 1963). Though both enzymes catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate in the presence of a divalent cation and the respective coenzyme, considerable differences exist in their physicochemical properties. Thus the TPN-dependent enzyme is relatively small and consists of a single polypeptide chain with a molecular weight of 58 000 (Colman, 1972), while the DPN-dependent enzyme is considerably larger, having a molecular weight of 340 000 and is composed of multiple subunits (Cohen and Colman, 1971). Only the

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[1] Abbreviations used are: CMC, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); GlyOEt, glycine ethyl ester; DPN and TPN, diphosphopyridine and triphosphopyridine nucleotides, respectively; Tris, tris(hydroxymethyl)aminomethane.

DPN-dependent enzyme is activated by ADP and is considered as an important site of regulation of the citric acid cycle (Plaut, 1970).

For the past few years this laboratory has been involved in the comparative study of these two enzymes isolated from pig heart muscle so as to elucidate the differences between an allosteric and a nonregulatory enzyme. The amino acid residues in the active site of the TPN-specific isocitrate dehydrogenase have been studied by means of chemical modification. A methionyl residue (Colman, 1968), a glutamyl residue (Colman, 1973), and 1–2 cysteinyl residues (Colman, 1969; Colman and Chu, 1970; Johanson and Colman, 1974) have been shown to be essential for the catalytic activity of the enzyme. Similarly in the case of the DPN-dependent enzyme isolated from the same source, the integrity of a lysyl residue (Shen and Colman, 1975; Hayman and Colman, 1977) and a cysteinyl residue (Mauck and Colman, 1976) has been shown to be important for function.

A study of the kinetics of the pig heart DPN-specific isocitrate dehydrogenase (Ramachandran et al., 1974) indicated that the maximum velocity requires the basic form of an ionizable residue of the enzyme–substrate complex having a pK of 6.6. In identifying the types of amino acid residues responsible for the pH dependence of this reaction, possible candidates that must be considered are glutamyl or aspartyl residues with abnormally high pK values since values of 6.3 and 6.8 have been reported for lysozyme (Donovan et al., 1960) and trypsin (Blow et al., 1969), respectively. This paper describes the investigation of the possible involvement of the carboxylic amino acids in the activity of isocitrate dehydrogenase. Water-soluble carbodiimides in the presence of a nucleophile have been extensively used to modify the carboxyl groups of proteins under controlled conditions (Carraway and Koshland, 1972). This reaction is thought to proceed through the protonated form of the carboxyl group (Kurzer and Douraghi-Zadeh, 1967) and hence is usually conducted around pH 4.5 (Carraway and Koshland, 1972). Since the group under consideration in isocitrate dehydrogenase exhibits a high pK for a carboxyl group, it was decided to conduct the modification reaction at neutral pH in order to achieve a controlled and more specific modification. By reaction of DPN-dependent isocitrate dehydrogenase with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide in the presence of the nucleophiles glycine ethyl ester or glycinamide, it is here shown that one residue each of aspartic and glutamic acid is modified at the substrate-binding site of the enzyme and that the integrity of these residues is essential for the maintenance of the catalytic function of the enzyme.[2]

Experimental Procedure

Materials. All coenzymes and substrates were purchased from Sigma Chemical Co; 1-cyclohexyl-3-(2-morpholinoethyl)carbodidimide (CMC) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and glycinamide were obtained from Aldrich Chem. Co. The nonradioactive glycine ethyl ester (GlyOEt) was supplied by Nutritional Biochemical Corp. and [1-^{14}C]glycine ethyl ester hydrochloride from New England Nuclear Corp. The compounds γ-L-glutamylglycine and β-L-aspartylglycine were obtained from Cyclo Chemical Co. Pronase (grade B) came from Calbiochem and carboxypeptidase A and B and leucineaminopeptidase from Worthington Biochemical Corp. All other chemicals used were of the reagent grade.

The pig-heart DPN-dependent isocitrate dehydrogenase was purified by modification of the procedure reported earlier (Shen et al., 1974). Pig hearts were obtained locally soon after the sacrifice of the animals, rather than from Pel Freez Biologicals as in the previous procedure. The initial crude extracts had a content of active enzyme three to five times greater than that of the heart tissue obtained earlier. The enzyme was purified by chromatography on DEAE-cellulose (50 × 4.5 cm) and cellulose phosphate (28 × 2 cm) as described previously, except that 2000 enzyme units were applied to the first column. The final step reported previously (viz., gel filtration on Sepharose 6-B) was omitted since the enzyme preparations now obtained after chromatography on cellulose phosphate exhibited specific activities greater than 25 enzyme units/mg, a value comparable to those of the preparations previously obtained after gel filtration. The purity of the enzyme prepared by this method was confirmed by electrophoresis in polyacrylamide gels containing 2% sodium dodecyl sulfate, which gave results identical with those reported using enzyme obtained from the Sepharose 6B column. A subunit molecular weight of 40 000 was used in all calculations (Shen et al., 1974).

L-Isocitrate was prepared from DL-isocitrate by the overall enzymatic conversion of D-isocitrate to L-glutamate as catalyzed successively by TPN-isocitrate dehydrogenase and glutamate dehydrogenase. The reaction mixtures contained 140 μmol of DL-isocitrate, 2.7 μmol of TPN, 25.0 μmol of ammonium chloride (pH 8.0), and 10 μmol of $MnSO_4$, in a total volume of 5 mL of 50 mM Pipes buffer (pH 7.6). The enzymes TPN-isocitrate dehydrogenase (1 unit) and glutamate dehydrogenase (9 units) were added to the reaction mixture and the solution was left overnight in the cold at 5 °C. The reaction was stopped by heating the solution in a boiling water bath for 2–3 min, after which the precipitated protein was removed by centrifugation. The solution was then assayed for D-isocitrate using TPN-isocitrate dehydrogenase, indicating that the conversion was virtually complete. L-Isocitrate was isolated from the reaction mixture using a Dowex-1-formate column as described previously (Ramachandran et al., 1974). Prior to the application of the reaction mixture onto the column, 300 μL of radioactive DL-isocitrate (less than 1 μmol and approximately 5000 cpm) was added to a 3-mL aliquot containing about 70 μmol of L-isocitrate. Since both D and L forms of isocitrate elute from the column at the same position, the addition of radioactive DL-isocitrate as a marker allowed the detection and isolation of L-isocitrate upon elution from the column. This method of preparation yielded 80–85% of the theoretical yield of L-isocitrate.

Kinetic Studies. Unless otherwise specified, 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 at 25 °C in 1.0-mL cuvettes of 1-cm light path containing 20 mM DL-isocitrate, 1.33 mM $MnSO_4$, 1 mM DPN 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 DPNH per min.

For the determination of the pH dependence of reaction catalyzed by the DPN-specific isocitrate dehydrogenase in the pH region 5–7, three buffer systems were used: sodium–0.072 M acetate, imidazole–0.072 M chloride, and triethanolam-

[2] A preliminary report of these studies has appeared (Ramachandran and Colman, 1975).

ine–0.072 M chloride buffers. The substrate concentrations used were those given for the standard assay, which are all high relative to the Michaelis constants over this pH range (Cohen and Colman, 1974). Doubling the concentrations of substrates did not have any effect on the reaction rate, implying that even at the lower concentration levels, the enzyme was saturated with substrates at all pH values and temperatures. In order to determine the heat of ionization of the enzyme-catalyzed reaction, the pH dependence of V_{max} was measured at different temperatures in the region 10–33 °C. In these experiments, the temperature of the cuvette compartment was regulated by a circulating water bath and was measured in the cuvettes. At each temperature, the pH values of the reaction mixtures were measured immediately after the velocity determination using a pH meter which had been standardized with buffers maintained at the same temperature.

Reaction with CMC and Nucleophile. All chemical modification experiments were performed using enzyme stock solutions dialyzed against 50 mM Pipes buffer containing 20% glycerol and 0.5 mM $MnSO_4$ at the desired pH. The dialyzed enzyme was incubated in the same buffer with 0.032 M CMC in the presence of 0.18 M glycinamide or glycine ethyl ester at 25 °C. As a control, the activity of the enzyme was measured simultaneously as a function of time after the addition of only glycinamide or glycine ethyl ester. In experiments where the reactions were followed in the presence of added ligands, their additions were made prior to those of the reagents. In those experiments in which manganous ion had to be excluded, aliquots of a 50 mM stock solution of EDTA were added to the enzyme solution so as to yield a final concentration of 1 mM. The inactivation rate was followed by measuring the activity of the enzyme at regular time intervals; when necessary, enzyme was diluted 20–40-fold with 50 mM Pipes buffer, prior to activity determinations.

Incorporation of [1-^{14}C]Glycine Ethyl Ester in the Presence of CMC. Isocitrate dehydrogenase was concentrated using an Amicon ultrafiltration cell to yield a protein concentration of approximately 1 mg/mL and was dialyzed, as before, prior to the addition of modifying agents. The enzyme (0.5–1.0 mg) was incubated with 0.18 M [1-^{14}C]glycine ethyl ester and 0.032 M CMC in a total volume of 1.0 mL under conditions described before. As a control experiment, enzyme was incubated under the same conditions with the sole omission of CMC from the incubation mixture. At suitable time intervals from 0 to 90% inactivation, 100-μL aliquots of the enzyme solution from both the control and experimental samples were withdrawn and diluted to 0.6 mL with cold 50 mM Pipes buffer (pH 7.0), containing 20% glycerol and 2 mM Mn^{2+} and dialyzed overnight at 5 °C to remove excess reagents. The dialysis was continued at room temperature against several changes of 20 mM sodium citrate buffer (pH 7.0), containing 1% sodium dodecyl sulfate, in order to solubilize the protein and to facilitate the removal of adsorbed radioactive glycine ethyl ester. When the radioactivity in the dialysate reached background level (about 2–3 days), the samples were dialyzed against multiple changes of 10 mM Pipes buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate.

The dialyzed solutions were removed and brought to approximately 1 mL with the dialysate. The protein concentration was determined spectrophotometrically by the method of Groves et al. (1968) which is based on the difference in the absorbance between 224 and 233.3 nm. Serum albumin dissolved in the dialysate was used as the primary standard. Aliquots of the enzyme solutions were mixed with Aquasol and measured in a Packard TriCarb liquid scintillation counter, Model 3330. The same procedure was followed in experiments where the enzyme was modified in the presence of added ligands.

Determination of Sulfhydryl Groups. In order to ascertain whether CMC was reacting with cysteine residues in isocitrate dehydrogenase, the free sulfhydryl content was measured for enzyme incubated either with 0.032 M CMC and 0.18 M glycine ethyl ester (experimental sample) or with 0.18 M glycine ethyl ester alone (control sample), as described above. Reaction with 5,5′-dithiobis(2-dinitrobenzoic acid) was used to measure the free sulfhydryl content of the enzyme samples (Ellman, 1959). Aliquots of the enzyme (corresponding to 75–100 μg of the protein) were withdrawn at different time intervals from both control and experimental reaction mixtures and dialyzed first against two changes of 1 L of 50 mM Pipes buffer (pH 7) containing 20% glycerol and 0.5 mM $MnSO_4$, and finally against 100 mM Tris-Cl buffer (pH 7.1). An aliquot of 0.32 mL of the dialyzed enzyme solution was mixed in a 1-mL cuvette with 0.48 mL of Tris-Cl buffer containing 10 M urea in order to solubilize the protein and to yield a final urea concentration of 6 M. After determining the protein concentration by the method of Groves et al. (1968), 0.05 mL of 50 mM EDTA in 6 M urea followed by 0.1 mL of 10 mM 5,5′-dithiobis(2-dinitrobenzoic acid) in 6 M urea was added to the same cuvette. The increase in absorbance at 412 nm, together with a molar extinction coefficient of 1.36×10^4 cm^{-1} L^{-1} for the dinitrophenolate ion, was used to calculate the number of sulfhydryl groups per mole of enzyme subunit.

Proteolytic Digestion of [1-^{14}C]Glycine Ethyl Ester Enzyme. The enzyme samples (0.5–1.0 mg) were inactivated in Pipes buffer in the presence of 0.032 M CMC and 0.18 M [1-^{14}C]glycine ethyl ester at 25 °C. In those cases in which no additions of ligands were made, the reaction was allowed to proceed until the enzyme reached 20–25% residual activity. The same time required to reach this residual activity was employed for the modification of the enzyme under protected conditions, when 20 mM DL-isocitrate, 1 mM Mn^{2+}, and 1 mM ADP were added to the reaction mixture. After the initial dialysis against Pipes buffer to remove excess reagents, the modified enzyme samples were dialyzed exhaustively against 50 mM ammonium bicarbonate buffer, pH 7.8. They were subsequently digested at 38–40 °C with sequential additions of trypsin (0.05 mg added in two aliquots 12 h apart), Pronase (0.05 mg), carboxypeptidase A and B (0.01 mg each), and leucine aminopeptidase (0.01 mg) over a period of 4 days. The hydrolyzed samples were desalted by repeated evaporation, after the addition of water, using a Rotovac.

Isolation of Radioactive Peptide. The protein hydrolysate was divided into two aliquots (representing 1/5 and 4/5 of the total material, respectively) for application to Whatman No. 3 MM filter paper. The hydrolysate, along with standard amino acids and peptides, was then subjected to high voltage electrophoresis in pyridine–acetate buffer (0.07 M in anion), pH 6.4, for 35 min at 2.5 kV using a Gilson high voltage electrophoresis apparatus. The portion of the paper containing the smaller aliquot of the protein hydrolysate was stained with ninhydrin and the radioactivity was detected by cutting the strip into 2 cm × 1.2 cm segments, placing each segment in a vial containing 10 mL of Aquasol and counting it in a Packard liquid scintillation counter. The major radioactive spot detected by this method was eluted with water from the corresponding area of the unstained portion of the electrophorogram. It was subjected to further purification, along with standards, by high voltage electrophoresis in pyridine–acetate buffer (0.041 M in anion), pH 3.5, for 2 h at 2.5 kV. As before, after the completion of the

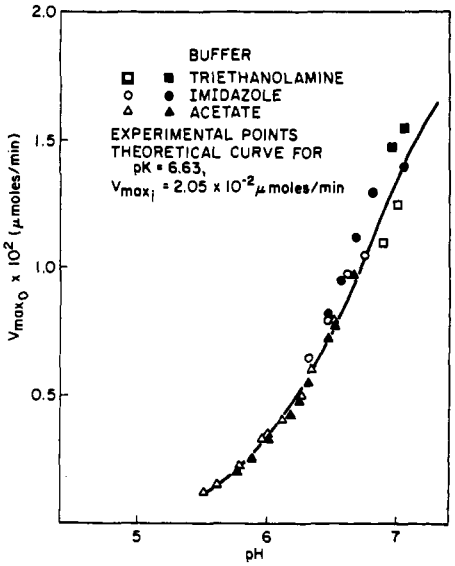

FIGURE 1: pH dependence of V_{max} for the DPN-dependent isocitrate dehydrogenase at 10 °C. The open points (△, ○, □) represent the velocity observed when the reaction mixture contained 0.99 mM DPN, 1.33 mM Mn^{2+}, and 20 mM DL-isocitrate and the solid points (▲, ●, ■) correspond to the velocity observed when the reaction mixture contained 2.0 mM DPN, 2.7 mM Mn^{2+}, and 40 mM DL-isocitrate. The points are experimental and the line is theoretical, calculated from the equation, $V_{max_0} = V_{max_i}/[1 + ((H^+)/K_{es})]$, where V_{max_0} = observed maximum velocity at a given (H^+); V_{max_i} = intrinsic maximum velocity which is independent of pH and K_{es} = dissociation constant of the activity-dependent ionizable group in the enzyme–substrate complex.

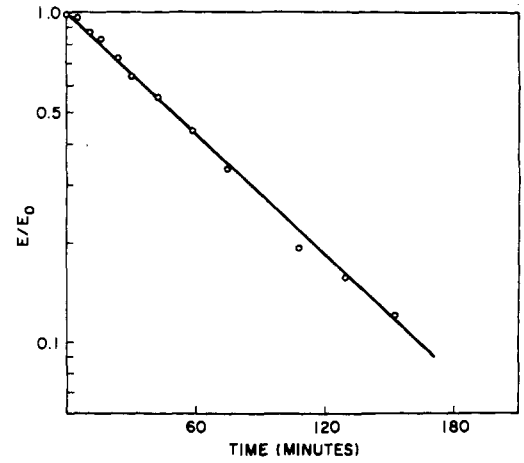

FIGURE 2: Reaction of DPN-dependent isocitrate dehydrogenase with CMC in the presence of glycinamide. Enzyme (about 3 units/mL) was incubated with 0.032 M CMC and 0.18 M glycinamide at pH 7.0 and 25 °C as described in Experimental Procedure. Metal ions were chelated by the addition of EDTA to yield a final concentration of 1 mM. The pseudo-first-order rate constant is 14.2×10^{-3} min^{-1}.

run, the paper was stained with ninhydrin and the electrophorogram was analyzed for radioactivity.

Results

Effect of Temperature on pH Dependence of V_{max}. A study of the change of maximum velocity of the reaction catalyzed by the pig heart DPN-dependent isocitrate dehydrogenase indicated the requirement for the basic form of an ionizable amino acid in the enzyme–substrate complex with a pK of 6.51 at 25 °C (Ramachandran et al. 1974). A pK in this range might be ascribed to an unusual carboxyl group or alternatively it may represent the ionization of an imidazole group. The pK of the imidazolium group of histidine decreases as the temperature is increased with a characteristic heat of ionization (ΔH) of 6.9–7.5 kcal per mole, whereas the pK of the carboxylic group of aspartic or glutamic acids is relatively insensitive to changes in temperature with a ΔH value of ±1.5 kcal per mol (Cohn and Edsall, 1943). Therefore a determination of ΔH for the ionizable group in the enzyme–substrate complex might aid in identifying the amino acid responsible for the observed pH dependence of the enzyme catalyzed reaction.

Figure 1 illustrates the pH dependence of V_{max} of DPN-dependent isocitrate dehydrogenase at 10 °C, at two different sets of substrate concentrations. These concentrations are high relative to the Michaelis constants for DPN, isocitrate, and Mn^{2+} over this pH range which were previously measured at 23 °C (Cohen and Colman, 1974). It is apparent from the points that doubling the substrate concentration does not have any significant effect on the observed velocity over most of the pH range. From these data a dissociation constant (K_{es}) can be calculated for an essential ionizable group in the enzyme–substrate complex using the equation given in the legend to Figure 1. Calculations of K_{es} have been made at a number of temperature values in the range 10–33 °C. They yield the estimates for the pK of the ionizable residue at different temperatures which are tabulated in Table I. As can be seen from this table, the pK is almost independent of temperature over the range 10–33 °C. Since the small differences in the estimated pK lie within experimental error, the heat of ionization is clearly close to zero. The above results strongly imply that the pK of 6.6 represents the ionization of an abnormal carboxyl group rather than an imidazole group. This observation stimulated the following study on the chemical modification of the enzyme using a water-soluble carbodiimide.

TABLE I: Dependence of pK_{es} on Temperature.[a]

Temp (°C)	pK_{es}
10	6.63
15	6.54
20	6.57
25	6.61
29	6.60
33	6.51
	Av: 6.6

[a] At each temperature, the pH dependence of V_{max_0} was followed. From the slope of a plot of $1/V_{max_0}$ vs. (H^+), the pK_{es} was calculated. The details regarding reaction conditions are described in Experimental Procedure.

Inactivation by 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide and Glycinamide. The enzyme is inactivated at 25 °C upon incubation with the water-soluble 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide (0.032 M) in the presence of the nucleophile, glycinamide (0.18 M), at pH 7.0. The concentrations of both carbodiimide and glycinamide are in great excess over that of the enzyme and, therefore, pseudo-first-order kinetics is obeyed, as can be seen from Figure 2. The same rate was observed when the nucleophile used was either glycinamide or glycine ethyl ester. No activity is recovered upon dialysis of the enzyme against 50 mM Pipes buffer (pH 7.0), containing 0.5 mM Mn^{2+} and 20% glycerol, showing that the modification results in an irreversible loss of enzymatic activity.

Inactivation Rate as a Function of pH. Although the water-soluble carbodiimides that are used for the modification

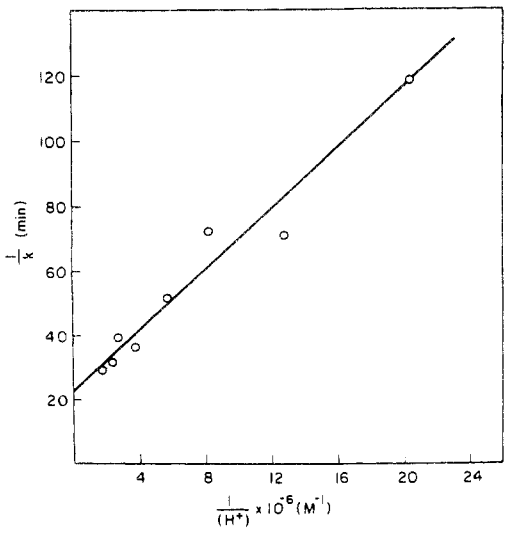

FIGURE 3: pH dependence of inactivation of DPN-specific isocitrate dehydrogenase by CMC and glycinamide. The enzyme (~3 units/mL) was incubated in 50 mM Pipes buffer of the desired pH in the presence of 0.032 M carbodiimide and 0.18 M glycinamide. A concentration of 1 mM EDTA was maintained in all experiments to chelate the metal ions. Additional details are given in Experimental Procedure. The data obtained are plotted here in accordance with the following equation: $1/k_{obsd} = 1/k_{max} + (K_{es}/k_{max})(1/(H^+))$. From the plot the values for k_{max} (44.4×10^{-3} min^{-1}) and pK_{es} (6.68) were calculated.

exhibit a strong preference for carboxyl groups, a number of studies have shown that the modification might occur at other functional groups as well (Carraway and Koshland, 1972). Reactions between carboxyl groups and carbodiimides have been postulated to proceed via the protonated form of the carboxyl group (Kurzer and Douraghi-Zadeh, 1967). This is in agreement with the modification studies with carboxypeptidase A when the rate of inactivation was found to increase as the pH was lowered (Riordan and Hayashida, 1970). The reaction with primary amines as well as histidine requires their unprotonated form to yield substituted guanidines (Riordan and Hayashida, 1970). The effect of pH on the rate of inactivation was thus investigated in order to obtain an indication of the type of group(s) which are modified during inactivation. Such a study showed that the rate of inactivation increased from 6.8×10^{-3} to 36.3×10^{-3} min^{-1} as the pH was decreased from 7.31 to 6.25. The direction of the pH dependence of the rate is consistent with reaction of the carbodiimide with carboxyl group(s) during the inactivation process; in contrast it is opposite to that expected for histidine modification.

The pH dependence of the inactivation of the enzyme by carbodiimide can be expressed by

$$k_{obsd} = \frac{k_{max}}{1 + \frac{K_{es}}{(H^+)}} \quad (1)$$

In reciprocal form this equation becomes

$$\frac{1}{k_{obsd}} = \frac{1}{k_{max}} + \frac{K_{es}}{k_{max}}\left(\frac{1}{H^+}\right) \quad (2)$$

where K_{es} is the ionization constant of a reacting group on the enzyme and k_{max} is the intrinsic pseudo-first-order rate constant when the reacting group is protonated. By plotting $1/k_{obsd}$ vs. $1/(H^+)$, a straight line was observed (Figure 3) which yielded a value of 6.69 for the pK_{es} of the reacting group. This value is close to the pK determined earlier for the pH dependence on V_{max} catalyzed by the enzyme.

TABLE II: Rate of Inactivation of DPN-Dependent Isocitrate Dehydrogenase in the Presence of Substrates and Coenzyme.[a]

Additions to the Reaction Mixture	$k(10^3 \times min^{-1})$ No Metal Ion	$k(10^3 \times min^{-1})$ + 2 mM Mn^{2+}
(1) None	14.2	7.7
(2) DL-Isocitrate (60 mM)	9.2	1.6
(3) 1,2,3-Propanetricarboxylate (60 mM)	9.0	6.5
(4) α-Ketoglutarate (60 mM)	15.1	6.7
(5) DPN (1.5 mM)	13.4	6.4

[a] Isocitrate dehydrogenase was incubated with CMC and in glycinamide in 50 mM Pipes buffer (pH 7.0) at 25 °C. Substrates were present in the reaction mixture as indicated. At regular time intervals, aliquots were withdrawn and assayed for dehydrogenase activity. The pseudo-first-order rate constants were calculated from the equation, $\ln E/E_0 = -kt$, where E/E_0 is the fraction of residual activity of the enzyme. Controls were always included to measure any denaturation of the enzyme and the rates have been corrected accordingly.

Effect of Ligands on the Inactivation Rate. Table II shows the effect on the inactivation rate of the addition of ligands to the reaction mixture at pH 7. The substrates isocitrate and α-ketoglutarate as well as the coenzyme DPN were added at concentrations high relative to their K_m values (Cohen and Colman, 1974; Shen and Colman, 1975). A small decrease in the rate constant was observed when 60 mM DL-isocitrate was added in the absence of metal ion (line 2). Since isocitrate itself is a tricarboxylic acid, it is possible that any decrease in the rate of inactivation may result from reaction of isocitrate with carbodiimide, leading to a decrease in the effective reagent concentration. That the decrease produced by isocitrate alone is probably nonspecific in nature is indicated by the observation that the same effect was also noticed in the presence of 60 mM 1,2,3-propanetricarboxylate (line 3), which is not known to be a substrate or competitive inhibitor of the enzyme. Similarly, addition of the other substrate α-ketoglutarate, or the coenzyme, DPN, did not afford any protection.

A divalent metal ion is essential for the isocitrate dehydrogenase reaction and it has been proposed that an enzyme-metal-dibasic isocitrate complex is formed during the course of the reaction (Cohen and Colman, 1974). The presence of manganous ion alone causes a twofold decrease in the rate of inactivation (Table II, line 1). However, the greatest decrease in the inactivation rate, about ninefold, is produced when 2 mM Mn^{2+} and 60 mM isocitrate are added together to the reaction mixture (Table II, line 2). The protection observed with other combinations of substrates and Mn^{2+} can be ascribed to the effect of metal ion alone.

The nucleotide ADP is an allosteric activator of the enzyme which decreases its K_m for Mn^{2+} and isocitrate (Cohen and Colman, 1972). As can be seen from Table III, the presence of ADP either by itself or combined with manganous ion did not affect appreciably the rate of inactivation (lines 2 and 3 as compared with line 1). However, the addition of ADP together with DL-isocitrate and Mn^{2+} decreased the concentration of substrate needed to protect against inactivation by the water soluble carbodiimide (line 6). In contrast, ADP, under similar conditions, did not enhance the protective effect of the carboxylic acids, 1,2,3-propanetricarboxylate or α-ketoglutarate (lines 4 and 5). Isocitrate dehydrogenase is known to be stereospecific, utilizing exclusively *threo*-D_s-isocitrate. Thus, it is of interest to note that the presence in the reaction mixture

TABLE III: Rate of Inactivation with Added Substrates in the Presence of ADP.[a]

Additions to Reaction Mixture	k ($10^3 \times$ min^{-1})
(1) None	14.2
(2) 1 mM ADP	14.1
(3) 1 mM ADP + 1 mM Mn^{2+}	9.1
(4) 1 mM ADP + 1 mM Mn^{2+} + 20 mM 1,2,3-propanetricarboxylate	7.0
(5) 1 mM ADP + 1 mM Mn^{2+} + 20 mM α-ketoglutaric acid	8.2
(6) 1 mM ADP + 1 mM Mn^{2+} + 20 mM DL-isocitrate	2.0
(7) 1 mM ADP + 1 mM Mn^{2+} + 20 mM L-isocitrate	7.1

[a] The reactions were conducted as described in Table II, except for the additions noted.

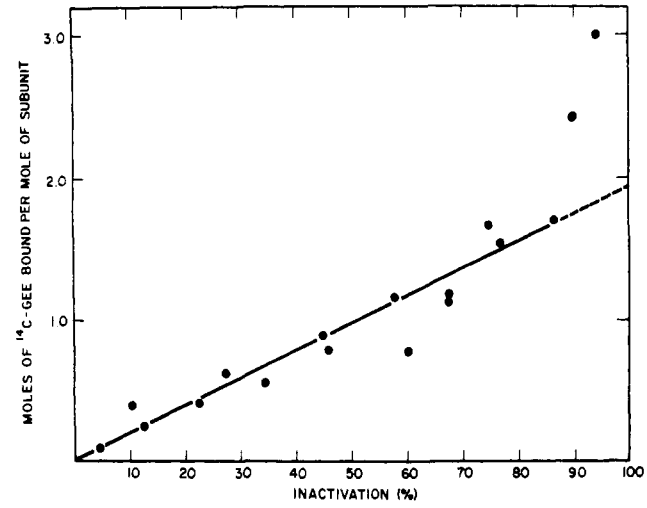

FIGURE 4: Incorporation of [1-^{14}C]glycine ethyl ester into isocitrate dehydrogenase as a function of inactivation. Enzyme (1 mg/mL) was incubated with 0.18 M [1-^{14}C]glycine ethyl ester (25 μCi) and 0.032 M CMC (experimental sample) or with 0.18 M [1-^{14}C]glycine ethyl ester (25 μCi) alone (control sample) in 50 mM Pipes buffer (pH 7.0) at 25 °C. At suitable time intervals, after determining the residual activity, 100-μL aliquots of the enzyme solution from both control and experimental samples were withdrawn and diluted to 0.6 mL with cold 50 mM Pipes buffer (pH 7.0) containing 20% glycerol and 1 mM Mn^{2+}. Dialysis was conducted and the number of moles of GEE bound per enzyme subunit was calculated as described in Experimental Procedure. The radioactivities associated with the control samples were subtracted from the corresponding experimental values to yield the net incorporation.

of L-isocitrate did not notably influence the rate of inactivation (line 7).

Incorporation of [1-^{14}C]Glycine Ethyl Ester. Isocitrate dehydrogenase when incubated with [1-^{14}C]glycine ethyl ester (GlyOEt) in the absence of carbodiimide did not lose any appreciable amount of enzymatic activity. However, even after the extensive dialysis procedure employed, the enzyme exposed to GlyOEt still retained approximately 0.5 to 0.8 mol of GlyOEt per subunit of the enzyme. Therefore, the radioactivity associated with the control samples was subtracted from the corresponding experimental values obtained when the enzyme was incubated with both GlyOEt and carbodiimide, to compute the net incorporation. As can be seen in Figure 4, the net incorporation is directly proportional to the extent of inactivation. Deviation from linearity is observed above 80% inactivation, presumably due to nonspecific modification of the already inactivated enzyme. Upon extrapolating the linear portion of the incorporation data, it is estimated that 2 mol of GlyOEt is incorporated per subunit for complete inactivation of the enzyme. The stoichiometry was found to be same whether the pH at which the enzyme was inactivated was either 7.0 or 6.5. Furthermore, the addition of 1 mM ADP to the reaction mixture did not alter the relationship between extent of inactivation and of incorporation of radioactive glycine ethyl ester.

Similar experiments were also carried out with enzyme under protected conditions in which the reaction mixture contained 20 mM DL-isocitrate, 1 mM Mn^{2+}, and 1 mM ADP. In most experiments the reaction was allowed to proceed until the enzyme had lost 40–50% of its initial activity and aliquots were withdrawn, as before, for analyses. The results indicated that approximately 0.8 mol of glycine ethyl ester was incorporated per subunit of the enzyme upon 40% inactivation.

Identification of the Amino Acids Modified by Carbodiimide and Glycine Ethyl Ester. Water-soluble carbodiimide can potentially alter side chains of tyrosine, cysteine, and glutamic and aspartic acid residues in proteins (Carraway and Koshland, 1972). Carraway and Koshland (1972) have shown that tyrosine residues in protein can be regenerated from their corresponding *O*-acylisourea derivatives of carbodiimide by treatment with 0.5 M hydroxylamine at pH 7.0. Accordingly a control enzyme which was treated with glycine ethyl ester only and an experimental sample which was inactivated to 25% residual activity with glycine ethyl ester and carbodiimide were dialyzed overnight against 50 mM Pipes buffer, containing 1 mM Mn^{2+} and 20% glycerol, to remove excess reagents. Hydroxylamine was added to both enzyme samples at 25 °C to yield a final concentration of 0.5 M. The enzymatic activities of both the control and experimental samples were measured over a period of 2 h. Treatment of the enzyme with hydroxylamine itself was found to cause inactivation. However, the rates of inactivation of both control and experimental samples were identical, implying that the treatment does not lead to regeneration of free enzyme. Hence it is concluded that modification of tyrosine residues is probably not primarily responsible for the loss of activity of the enzyme upon reaction with carbodiimide.

To test whether cysteine residues are modified under the experimental conditions employed here, the -SH groups were determined at different extents of inactivation. The enzyme samples were extensively dialyzed to remove excess reagents and the number of -SH groups was measured by reaction with DTNB, as described in Experimental Procedure. The native enzyme contains six -SH groups (Stevens and Colman, unpublished results). However, with the experimental sample an initial sulfhydryl titer of only 3.3 was observed, immediately after the reaction mixture was prepared and before any inactivation had taken place. The sulfhydryl titer of this sample did not change further, despite a decline in the activity from 100 to 40% of its original value. The lack of correlation between inactivation and loss of free sulfhydryl groups seems to exclude the possibility that the modification of -SH groups by carbodiimide is responsible for the loss of activity of the enzyme.

The acidic amino acids, aspartic and glutamic acids, are thus the most probable sites of modification by CMC in the presence of glycine ethyl ester. These amino acids would be expected to yield β-aspartylglycine ethyl ester and γ-glutamylglycine ethyl ester, respectively, as the ultimate products of inactivation. Since these compounds are susceptible to acid hydrolysis, the unprotected, radioactive modified enzyme after

TABLE IV: Estimation of Glu-Gly and Asp-Gly in Protected and Unprotected Isocitrate Dehydrogenase.[a]

Enzyme Sample	Mol of [^{14}C]GlyOEt/ Mol of Enzyme Subunit	Inact. (%)	No. of Residues/ Subunit	
			Glu-Gly	Asp-Gly
Unprotected: no additions or 1 mM ADP added to reaction mixture	1.7	80	0.75	0.95
Protected: 20 mM DL-isocitrate, 1 mM Mn^{2+}, and 1 mM ADP added to reaction mixture	0.78	40	0.28	0.50

[a] The enzyme (1 mg) was inactivated in presence of 0.032 M CMC and [1-^{14}C]glycine ethyl ester under protected or unprotected conditions. The radioactively labeled enzyme samples were subjected to exhaustive proteolytic digestion and analyzed for the incorporation of label in the dipeptides as described in Experimental Procedure. In each experiment, the percentage of total radioactivity found in γ-glutamylglycine or β-aspartylglycine was determined and this percentage together with the measured moles of [^{14}C]GlyOEt incorporated were used to calculate the number of residues of each dipeptide formed per enzyme subunit. The results for the unprotected and protected enzymes represent average values obtained from several experiments.

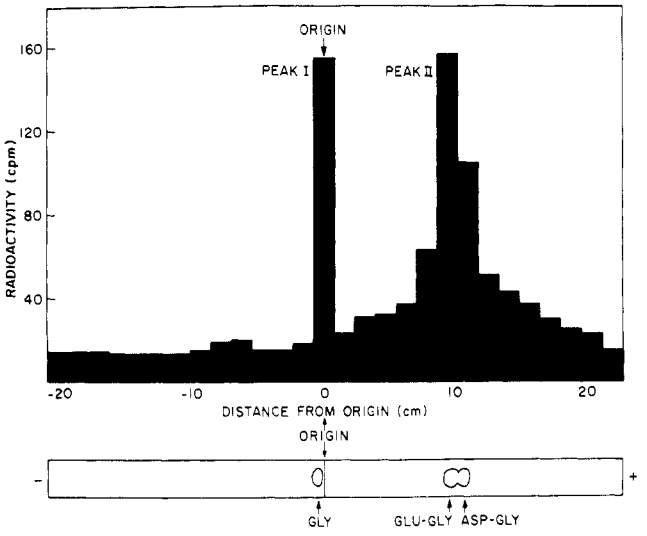

FIGURE 5: High voltage electrophoretic analysis at pH 6.4 of the proteolytic digest of ^{14}C-labeled, inactivated isocitrate dehydrogenase. The enzyme (~1 mg) incubated with CMC and [1-^{14}C]glycine ethyl ester, until it had reached 20% of its original activity, was subjected to exhaustive proteolytic digestion. The hydrolysate was then subjected to high voltage electrophoresis at pH 6.4 for 35 min at 2.5 kV along with standards. The longitudinal paper strip after staining with ninhydrin was cut into segments (2.0 cm × 1.2 cm) and assessed for radioactivity. The ninhydrin spots of the standards, glycine and the dipeptides, β-Asp-Gly and γ-Glu-Gly, are sketched at the bottom for reference. Peak I corresponds to glycine and peak II to the dipeptides.

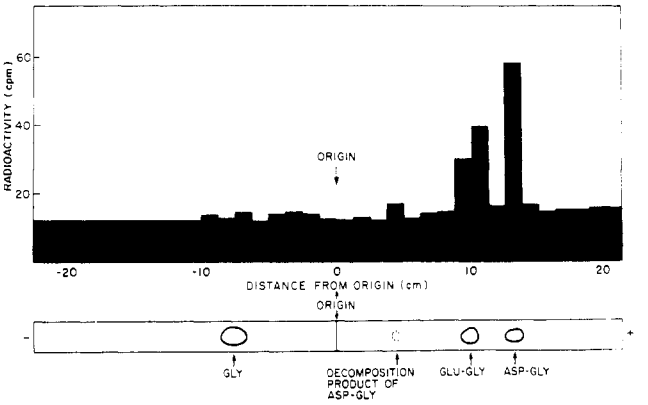

FIGURE 6: High voltage electrophoretic analysis of peak II at pH 3.5. Peak II from the unstained portion of the pH 6.4 electrophorogram was eluted with water and subjected to high voltage electrophoresis at pH 3.5 for 2 h at 2.5 kV along with standards. After the run, the paper was stained with ninhydrin and cut into segments as before, for determination of the radioactivity. The tracings of the ninhydrin positive spots corresponding to the standards are shown at the bottom. The dotted spot represents a ninhydrin positive spot routinely observed with β-aspartylglycine standard and is assumed to be a decomposition product of the dipeptide.

dialysis was subjected to extensive proteolytic digestion, as described in Experimental Procedure. Under the conditions of prolonged proteolytic digestion, the ethyl esters would be hydrolyzed to the corresponding free acids. The digested material was then analyzed by high voltage electrophoresis at pH 6.4. As shown, in Figure 5 only two major radioactive regions are detected. Peak I corresponds to glycine which is derived from the residual unreacted glycine ethyl ester not totally removed upon dialysis, since a similar peak was found in the case of the control sample as well. Peak II coincides with the region occupied by the standard dipeptides, β-aspartylglycine and γ-glutamylglycine, and under the conditions chosen here they do not resolve satisfactorily. Therefore, this peak was eluted and subjected to further high voltage electrophoretic analysis at pH 3.5, conditions which lead to separation of the two dipeptides.

As can be seen in Figure 6, most of the radioactivity is distributed between the two dipeptides. However, a small radioactive peak was also observed which has a slower mobility toward the anode than either of the standard dipeptides. Since a ninhydrin positive spot corresponding to this radioactive peak is regularly observed with the β-aspartylglycine standard also, it is assumed that the smaller radioactive peak is a decomposition product of this dipeptide. For purposes of calculation, the radioactivity observed in the small peak is added to the β-aspartylglycine peak. It can thus be estimated that, in the absence of substrates, inactivation is accompanied by formation of approximately equal amounts of β-aspartylglycine and γ-glutamylglycine, implying that one residue each of aspartic acid and glutamic acid is modified per enzyme subunit.

In order to evaluate whether the modification of one or both of these residues is related to inactivation, the enzyme was allowed to react with carbodiimide under "protected" conditions, in which the reaction conditions were the same as in the "unprotected" case except for the addition of 20 mM DL-isocitrate, 1 mM Mn^{2+}, and 1 mM ADP. The resultant enzyme had lost only about 40% of its original activity. After dialysis and proteolysis, the digest was analyzed for the dipeptides as with the unprotected enzyme. Table IV summarizes the incorporation of radioactivity, the extent of inactivation, and the distribution between the two peptides for the "unprotected" and "protected" enzymes. When no additions were made to

the reaction mixture, the enzyme lost 80% of its activity in a period of 2 h with corresponding net incorporation of 1.7 mol of glycine ethyl ester per subunit of the enzyme. The radioactivity was about equally distributed between the two dipeptides with slightly greater formation of aspartylglycine. Under protected conditions during the same time interval, only 0.8 mol of glycine ethyl ester was incorporated; and, as with the unprotected sample, a somewhat greater incorporation was observed in aspartylglycine than in glutamylglycine. Comparison of the two types of experiments suggests that protection by substrates causes decreased labeling of both amino acid residues.

Discussion

This paper presents two types of evidence indicating the importance of carboxylic amino acid residues in the function of pig heart DPN-dependent isocitrate dehydrogenase: (1) kinetic evidence based on the interpretation of the pH dependence of the catalytic reaction; and (2) chemical modification evidence linking inactivation with the reaction of two acidic amino acids. The maximum velocity of the isocitrate dehydrogenase reaction has been shown to require the basic form of a functional group of the enzyme–substrate complex having a pK of 6.6. This pK [like that of the corresponding TPN-specific enzyme (Colman, 1973)] is independent of temperature in the range 10–33 °C, indicating a heat of ionization close to zero. This result is consistent with the characteristics of carboxyl ionization but not of imidazolium ionization (Cohn and Edsall, 1943). One must be extremely cautious in identifying a type of amino acid residue exclusively on the basis of kinetic data, for the apparent V_{max} might be a complex kinetic constant (Bruice and Schmir, 1959) and the pK of any particular residue may be considerably altered by its microenvironment (Schmidt and Westheimer, 1971). Nonetheless, the simplest interpretation of the data is that a carboxylate group in the enzyme–substrate complex participates in the catalytic reaction.

The possibility of the involvement of at least one carboxylic amino acid is further enhanced by the inactivation observed when the enzyme is incubated with a water-soluble carbodiimide and glycinamide. Carbodiimides are known to react with several functional groups of amino acids in proteins (Carraway and Koshland, 1972). The treatment of inactive enzyme with hydroxylamine does not regenerate the activity and hence it does not appear that modification of tyrosine residues is responsible for the loss of activity (Carraway and Koshland, 1972). In addition, no reduction in the number of sulfhydryl groups in the enzyme parallels the inactivation, which suggests that the modification of a critical sulfhydryl group by CMC is not responsible for the loss of activity. At room temperature, alcohols such as threonine and serine are relatively inert toward carbodiimide (Kurzer and Douraghi-Zadeh, 1967). Only the unprotonated forms of amines will react with carbodiimides to yield the corresponding substituted guanidines (Kurzer and Douraghi-Zadeh, 1967) and this reaction is not favored at the neutral pH employed for modification. The most likely candidates for reaction with the carbodiimide are thus the acidic amino acids. The inactivation of the enzyme with CMC and [^{14}C]GlyOEt concomitant with the incorporation of 2 mol of radioactive reagent, coupled with the identification of β-aspartylglycine and γ-glutamylglycine as the products of the reaction, indicate that the two carboxylic amino acids are the target of attack in isocitrate dehydrogenase by the carbodiimide. The specificity of the reaction with isocitrate dehydrogenase is indeed remarkable: despite the fact that the enzyme contains a large number of acidic amino acids (Shen et al., 1974), only one glutamyl and one aspartyl residue seem to react.

An important question that has to be answered is whether modification of both the residues or only one is associated with the inactivation process. Information relevant to these possibilities is obtained from the analyses of the radioactively modified enzyme samples in the absence ("unprotected") or presence ("protected") of substrates. If both the residues are essential, the incorporation of label will be related to the extent of inactivation and will be equally distributed between the two dipeptides under all conditions. In contrast, if one assumes that the integrity of only one of the two amino acid residues is required for the maintenance of activity, that essential amino acid might be expected to be protected against modification by the presence of substrates, whereas the second nonessential group would react equally well in the presence and absence of substrates. Therefore, the proportion of the two dipeptide products might be greatly altered when modification is conducted under protected conditions. As typified by the data given in Table IV, under unprotected conditions when 80% of the enzyme is inactivated with concomitant incorporation of 1.7 mol of GlyOEt per subunit, the radioactivity is about equally distributed between the two dipeptides γ-glutamylglycine and β-aspartylglycine; that is, approximately 0.8–1.0 mol of each is formed. The results of Table IV indicate that, in the presence of protecting substrates, the enzyme loses only 40% of its activity during the same time period. If both amino acid residues are essential for activity and are equally protected by the presence of substrates against reaction with carbodiimide, one might expect to observe 40% of 2.0 or 0.8 mol of [^{14}C]GlyOEt per subunit incorporated under these conditions. In contrast, with the assumption that only one residue is important for activity, one would anticipate a total incorporation of 1.2 mol of GlyOEt per subunit: 0.8 mol of GlyOEt, corresponding to the unchanged modification of the nonessential residue during that period of time, plus 0.4 mol of [^{14}C]-GlyOEt, corresponding to the 40% inactivation produced by the fractional modification of the essential amino acid residue, [0.8 + 0.4 = 1.2]. The actual data reported in Table IV show that only 0.8 mol of GlyOEt is incorporated for 40% inactivation under protected conditions, with significant reduction being observed in the labeling of both dipeptides. This result implies that isocitrate and manganous ion protect both the aspartic and glutamic acid residues against modification. A further interpretation that might be made is that both residues are essential for the activity of the enzyme.

Although the above conclusion seems to be the most direct, another plausible explanation which has not been excluded, and may in fact be more likely, warrants some consideration. It is possible that activity depends only on one acidic group and that once this group undergoes slow modification, reaction with the second amino acid ensues quite rapidly. Substrates which protect against modification of the slowly reacting group would necessarily also protect against reaction of the second group. This possibility is consistent with the observation that the loss of enzyme activity produced by carbodiimide obeys simple pseudo-first-order kinetics and can be characterized by a single rate constant (Figure 2). It is also consistent with the observation (Figure 3) that the reciprocal of the rate constant for activation is linearly related to the reciprocal of the hydrogen ion concentration, which suggests that the modification reaction requires the protonated form of a single ionizable group in the pH range examined with a pK of 6.7. [In contrast, if it were assumed that the modification of two groups of identical

pK is responsible for inactivation, the rate constant would be linearly related to the square of the hydrogen ion concentration.] The pH dependence of V_{max} of the reaction catalyzed by isocitrate dehydrogenase also reflects the ionization of only one group in the enzyme–substrate complex having a pK of about 6.6, although it has been estimated that the pK of the essential ionizable group is somewhat higher for the free enzyme (Cohen and Colman, 1974). It may be that the ionizable group essential for the catalytic reaction is one of the acidic amino acids which reacts with CMC and GlyOEt.

One might consider that the inactivation of the enzyme observed here by CMC and a nucleophile results simply from the loss of the negative charge upon conversion of the carboxyl to an amide or ester as a result of reaction of the amino acid side chain with glycinamide or GlyOEt, respectively. That this is not the case is shown by the fact that the enzyme is also inactivated completely when glycine is used instead of the other nucleophiles. These experiments therefore emphasize the requirement of the precise location of the negative charges in the substrate binding regions of the enzyme.

Some insight into a possible function of these reactive carboxylic amino acids may be produced by an examination of the ligands which protect the enzyme from inactivation. No protection is produced by DPN or ADP, suggesting that the susceptible residues lie neither within the coenzyme nor the allosteric site. Similarly, no appreciable protection is imparted by DL-isocitrate or α-ketoglutaric acid alone which suggests either that the residues modified are not in the binding sites for these two substrates or, more likely, that these ligands do not bind correctly to the enzyme in the absence of Mn^{2+}. A marked decrease in the rate of inactivation is afforded only by isocitrate and Mn^{2+}, indicating that the target attacked by the carbodiimide is the manganous–isocitrate site. This postulate is supported by the observation that the tricarboxylic acid analogue of isocitrate (1,2,3-propanetricarboxylic acid) and the inert L stereoisomer of the natural substrate do not provide comparable protection, even when added together with metal ion. Furthermore, the concentration of manganous–isocitrate required to protect the enzyme is considerably reduced by the addition of ADP, an allosteric modifier of the enzyme which is known to lower the K_m for the substrates (Cohen and Colman, 1972). These experiments all point toward the conclusion that the substrate binding sites which undergo chemical modification are the same as those which function in the isocitrate dehydrogenase catalytic reaction. In the case of chemical modification by CMC, it is not possible to determine the dissociation constant (K_d) for isocitrate from the substrate concentration dependence of the inactivation rate constant, as has been done for previous chemical modification studies of isocitrate dehydrogenase (Mauck and Colman, 1976; Shen and Colman, 1975; Hayman and Colman, 1977). Isocitrate is itself a tricarboxylic acid and can react with CMC, thus causing a depletion of the effective concentration of both the reagent and substrate. Adequate controls have been performed to indicate that the protection observed is not merely due to the consumption of the reagent. However, the complication of the reaction of isocitrate with CMC makes it difficult to quantitate the K_d by the usual protection experiments.

The results of this paper indicate that the carboxylic amino acids are at the substrate binding site, but do not reveal precisely the role played by these amino acids. It is possible that the negative charges contributed by these carboxyl groups might be involved in binding Mn^{2+} during the catalytic reaction as has been suggested in the case of the TPN-dependent isocitrate dehydrogenase (Colman, 1973). The inactivation of the pig-heart DPN-dependent isocitrate dehydrogenase by CMC and nucleophile bears close resemblance to the reaction observed for the TPN-specific enzyme isolated from the same species (Colman, 1973). It has been shown with the TPN-dependent enzyme that modification of a single glutamic acid residue with CMC and glycinamide results in the loss of both TPNH and Mn–isocitrate binding capacity, with Mn^{2+} binding in the absence of isocitrate (Colman, 1973) and isocitrate binding in the absence of Mn^{2+} remaining unimpaired (Ehrlich and Colman, 1975). As is proposed for the TPN-dependent enzyme, the amino acids modified in the DPN-dependent enzyme may, in the presence of isocitrate, act as ligands for the essential divalent cation. Thus, the manganous ion may interact with the carboxylate group(s) of the essential amino acids and with both α- and β-carboxyl groups of isocitrate to reduce the repulsive force that might exist between the enzyme and substrates and facilitate binding. An evaluation of this hypothesis must await direct measurement of the binding of substrates, coenzymes, and metal ion by the native and chemically modified DPN-specific isocitrate dehydrogenase.

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Binding Rates, O–S Substitution Effects, and the pH Dependence of Chymotrypsin Reactions†

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ABSTRACT: The pH dependence for acylation of α-chymotrypsin by *N*-acetyltryptophan *p*-nitrophenyl-, *p*-nitrothiophenyl-, ethyl-, and thiolethyl esters has been studied by the stopped-flow technique. Values for the acylation rate constant, k_2, and the binding constant, K_S, were obtained by using measurements of phenolate release, for the *p*-nitrophenyl esters, and proflavin displacement, for the ethyl esters. The oxygen esters tested have slightly higher k_2 values, and substantially higher K_S values relative to the analogous thiol esters. Whereas k_2/K_S for the thiolethyl ester is higher than that for the analogous oxygen ester, the k_2/K_S values for oxy- and thio-*p*-nitrophenyl esters are nearly identical. These data are interpreted to indicate rate-determining formation of a tetrahedral intermediate in acylation of α-chymotrypsin by *p*-nitrophenyl esters, and rate-determining breakdown of such an intermediate in the case of the ethyl esters. It is also concluded that the oxygen to sulfur substitution causes a substantial increase in the proportion of nonproductive binding in these substrates. pH dependent k_2 and K_S values were used to calculate values for k_1 and k_{-1}, the binding and debinding rate constants for the two *p*-nitrophenyl compounds. This is the first such calculation based on experimentally determined acylation rate constants.

The mechanistic description of the action of chymotrypsin requires the observation of individual steps of the enzymatic reactions and a knowledge of all the intermediates on the pathway. It has been well established that the pathway of α-chymotrypsin-catalyzed hydrolysis reactions includes the formation and decomposition of an acyl-enzyme intermediate. This can be described in terms of eq 1 as a minimal requirement (Bender and Kezdy, 1965; Bruice and Benkovic, 1966; Bender, 1971):

$$E + S \overset{K_S}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_2} \underset{P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (1)$$

where E·S is the enzyme-substrate complex, ES′ is the acyl-enzyme, and P_1 and P_2 are the alcohol and acid portions of an ester substrate, respectively. Both acylation and deacylation may be clearly categorized as examples of acyl-transfer reactions at a carbonyl carbon atom. Since the occurrence of a tetrahedral intermediate was demonstrated in acyl-transfer reactions of nonenzymatic hydrolysis of esters and amides (Bender, 1960), whether or not a similar intermediate is formed in the enzymatic reaction pathway has been a subject of great interest. Comparisons of the kinetic behavior between oxygen esters and their sulfur counterparts of nonspecific (Frankfater and Kezdy, 1971) and specific substrates (Hirohara et al., 1974) have presented kinetic evidence for the occurrence of a tetrahedral intermediate in the acylation process of α-chymotrypsin-catalyzed reactions. Thus, our data are discussed in terms of a mechanism involving this intermediate and may be summarized by

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \rightleftharpoons ET \rightarrow \underset{P_1}{ES'} \xrightarrow{k_3} E + P_2 \qquad (2)$$

where ET is the tetrahedral intermediate.

In the case of the α-chymotrypsin-catalyzed hydrolysis of specific substrates, the rate constant of the acylation step, k_2 (in eq 1), was found to be roughly identical for both thiol esters and their oxygen counterparts (Hirohara et al., 1974). Since, in an SN2 type reaction, an -SR group should be displaced at least 250 times faster than an -OR group (Connors and Bender, 1961; Martin and Hedrick, 1962), and since in the breakdown of similar tetrahedral compounds such as hemithioacetals (Jencks, 1969), acetaldehyde hydrate (Jencks, 1969), and ketene *O,S* acetals (Hershfield and Schmir, 1972) the -SR group has shown a greater leaving ability compared with an -OR group, it was concluded that a one-step transfer does not occur. The results were explained by postulating a metastable tetrahedral intermediate, the formation of which is the rate-determining step in acylation. Proposals for the formation of such an intermediate prior to acylation have been recently made based on a series of kinetic studies on amide substrates (Caplow, 1969; Lucas and Caplow, 1972; Lucas et al., 1973; Fersht and Requena, 1971a; Fastrez and Fersht, 1973; Philipp et al., 1973).

It was suggested in the study of specific ester substrates at two pHs (Hirohara et al., 1974) that: (a) the greater values of k_2/K_S of thiol esters over the corresponding oxygen esters

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