Salicylic acid acts as a reversible inhibitor of pig heart TPN-dependent isocitrate dehydrogenase competitive with manganous isocitrate ($K_i = 18.2 \text{ mM}$) but not with TPN. The salicylate derivative, 4-iodoacetamidosalicylic acid (0.5 to 4 mm), causes an irreversible time-dependent loss of both the isocitrate dehydrogenase and oxalosuccinate decarboxylase activities of the enzyme when incubated at pH 7.0 and 30 °C. The inactivation rate constant is linearly dependent on the 4-iodoacetamidosalicylic acid (ISA) concentration, yielding a second order rate constant of $10.7 \text{ min}^{-1} \text{ M}^{-1}$, a value 11 times greater than that for iodoacetamide under the same conditions; this result suggests that the salicylate moiety contributes to the effectiveness of ISA in inactivating this enzyme. Total protection against inactivation is provided by manganous ion and isocitrate, but not by either metal ion or isocitrate alone. When the incubation is conducted in the absence of ligands, total inactivation can be related to the incorporation of 4 mol of $[^1]CISA/mol of enzyme; however, when the reaction is carried out in the presence of Mn$^{2+}$ and isocitrate, the enzyme retains 100% activity, but still incorporates 2 mol of radioactive ISA/mol of enzyme. Carboxymethyl derivatives of lysine and cysteine are observed in acid hydrolysates derived from inactive enzyme prepared in the absence of ligands; whereas only carboxymethylcysteine is detected in samples derived from active modified enzyme prepared in the presence of Mn$^{2+}$ and isocitrate. Modification of a cysteine or partial modification of a cysteine and lysine by ISA appears to be responsible for inactivation. Enzyme reacted first with unlabeled ISA in the presence of protecting substrate plus metal ion, and then with radioactive $\epsilon$-dicarboxymethyllysine from the hydrolyzate of the inactive enzyme. It is concluded that in this case a lysine residue attacked by 4-iodoacetamidosalicylic acid is critical for the function of isocitrate dehydrogenase and is located within the metal-isocitrate binding site. A new method is also presented for the purification of TPN-dependent isocitrate dehydrogenase using fresh pig hearts as the starting material.

Specific modification of amino acid residues of the pig heart TPN-dependent isocitrate dehydrogenase (threo-β, isocitrate: NADP$^+$ oxidoreductase [decarboxylating], EC 1.1.1.42) has been aimed at identifying those in the catalytic site. Methionyl (1), glutamyl (2), arginyl (3), histidyl (4), and cysteinyl residues (6–9) have previously been implicated in the region of the substrate or coenzyme binding sites. Related studies of the more complex allosteric DPN-dependent isocitrate dehydrogenase from pig heart have revealed the involvement of a lysine residue in the binding site of the manganous-isocitrate complex (9); however, no comparable lysine has thus far been implicated in the case of the TPN-dependent isocitrate dehydrogenase.

Salicylate and its iodinated derivatives have been found to bind to the adenine binding sites of dehydrogenases and kinases (10–12). The reagent 4-iodoacetamidosalicylic acid was first proposed by Baker as an active site-directed reagent for glutamate dehydrogenase (13); and the compound has been found to react irreversibly within the substrate binding site of glutamate dehydrogenase, labeling different amino acid residues at pH 7.5 (14–16) and at pH 6.0 (17). 4-Iodoacetamidosalicylic acid was selected for evaluation as a possible affinity label for the TPN-dependent isocitrate dehydrogenase since it was considered to have the potential to react specifically at either the coenzyme or the isocitrate sites. The present study shows that this reagent inactivates isocitrate dehydrogenase with modification of a limited number of amino acids and provides the first evidence that a lysine residue may be involved in the metal-isocitrate site.

**EXPERIMENTAL PROCEDURES**

**Materials**—The 4-iodoacetamidosalicylic acid, obtained from Nutritional Biochemicals, Inc., was recrystallized from ethanol to yield a compound with a melting point of 207–208.5 °C. Aqueous solutions were prepared by careful titration with sodium hydroxide to pH 6.0, and the concentration was determined spectrophotometrically using extinction coefficients of $9.13 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $3.30 \times 10^2 \text{ M}^{-1} \text{ cm}^{-1}$ at 302 nm and 268 nm, respectively (17). Radioactive 4-iodoacetamidosalicylic acid was prepared by the method of Holbrook et al. (15) in which approximately 50 pCi of [$^1]Cl$odocetic acid (New England Nuclear Corp.) and 1.5 mmol of nonradioactive iodoacetic acid were converted to iodoacetylchloryde by reaction with 4.5 mmol of thionylchloride. The resultant [$^1]Cl$odocetic acid was added dropwise to a solution stirred at 0 °C of 1.5 mmol of sodium 4-aminosalicylate (Sigma Chemical Co.), and 8.4 mmol of NaHCO$_3$ in 8 ml of water, as previously described (15). The final radioactive product, obtained in 40% yield, exhibited a melting point of 208–209 °C, and a specific radioactivity of $9.17 \times 10^5 \text{ dpm/mol}$.

Carboxymethyl derivatives were prepared by reaction of iodoacetate with the following amino acids: methionine, homocysteine, cysteine, histidine, lysine, and N-acetyllysine. For the first three amino acids, approximately 50 μmoles of amino acid were allowed to react for 24 hr at 40 °C with a 3.5-fold excess of iodoacetate in a total volume of 5 ml of water adjusted to pH 7.0. For the last three amino acids, reaction was conducted for 48 h at 40 °C using a 25-fold excess of iodoacetate at pH 8.0. At the end of the reaction period, the pH was adjusted to 2 with HCl, the excess iodoacetic acid was extracted with ether and the sample was evaporated to dryness. Where appropriate, the derivatized amino acids were hydrolyzed in 6 × HCl using the same conditions as those for modified isocitrate dehydrogenase.

Salicylic acid (Gold Label) was purchased from the Aldrich Chemical Co., manganous sulfate from Mallinckrodt, and anhydrous so-

The Reaction of 4-Iodoacetamidosalicylic Acid with TPN-dependent Isocitrate Dehydrogenase from Pig Heart*

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dium sulfate from Fisher Scientific Co. All coenzymes and substrates were purchased from Sigma Chemical Co., as were the iodoacetamide and triethanolamine hydrochloride.

Purification of Pig Heart TPN-dependent Isocitrate Dehydrogenase—The commercial (Boehringer Mannheim Corp.) partially purified TPN-dependent isocitrate dehydrogenase has previously been used in this laboratory as the starting material for the subsequent purification of the enzyme. However, because of the expense and the irregular availability of the commercial enzyme, as well as the limited lifetime of the NADP-agarose featured in the previous purification procedure (8), a new scheme of purification of the enzyme has been devised using fresh pig hearts as the starting material.

Pig hearts were obtained from a local abattoir. Excess fat and connective tissue were trimmed manually. The muscle tissue was cut into 1-inch cubes and frozen at −85 °C overnight. Batches (800 g) of semithawed tissue were homogenized at room temperature in a Waring blender with 2400 ml of Tris/O.01 M citrate buffer, pH 7.2, containing 10% glycerol and 2 mM MnSO₄. The homogenate was centrifuged at 8,000 rpm at 4 °C for 20 min in a Sorvall RC2-B refrigerated centrifuge with a GS-3 rotor. The combined supernatants (24,000 u.e./kg of trimmed hearts) were filtered through cheesecloth, and crystalline ammonium sulfate was added to 60% saturation at room temperature. After 1 to 2 h at 4 °C, the suspension was dialyzed for 45 min against 5% dialysis buffer or 5% ammonium sulfate at 4 °C. Unless specified otherwise, dialysis buffer was 0.1 M triethanolamine chloride, pH 7.7, containing 10% glycerol and 0.3 M Na₂SO₄ (Buffer A) by stirring overnight at 4 °C. The protein solution was then divided in aliquots and stored at −85 °C. As based on protein determination by the method of Warburg and Christian (18) and enzymatic activity by the standard assay, the enzyme solution typically exhibited a specific activity of about 2 u.e./mg. Approximately 66% of the units present in the initial homogenate were recovered. An additional 11% of the total initial units could be obtained upon re-fractionation of the "60% ammonium sulfate pellet" after the TPN-dependent isocitrate dehydrogenase was isolated.

The crude enzyme (2000 to 3000 units) was dialyzed for 18 h at 4 °C against three changes of 2 liters of 0.018 M triethanolamine chloride, pH 7.1, containing 10% glycerol and 1 mM MnSO₄ (Buffer B). The enzyme was diluted with Buffer B to a final volume of 30 ml prior to application to a column of carboxymethyl cellulose (0.5 × 30 cm, Whatman CM-52) equilibrated with Buffer B. After an inactive breakthrough peak was eluted by Buffer B, a linear gradient of Buffer B (300 ml) versus (240 ml of Buffer B + 60 ml of Buffer A) was applied. Fractions of 4 ml/tube were collected at a rate of 40 to 50 ml/h. The enzyme solution was passed through the column at a rate no greater than 20 ml/h. Fractions with specific activities greater than about 25 units/mg were pooled, leading to recovery of approximately 62% of the units applied to the column.

Matrix Gel Red-A resin (Amicon Corp.) was prewashed with about 50 ml of 7 M urea in 0.1 M NaOH, followed by approximately 1 liter of water and was finally equilibrated with Buffer B. A column (1.0 × 32 cm) was loaded with the pooled enzyme and then eluted with 0.018 M triethanolamine chloride, pH 8.0, containing 10% glycerol and 0.15 mM Na₂SO₄ (Buffer C) for several hours. A linear gradient was established from Buffer C (125 ml) to 0.018 M triethanolamine chloride, pH 8.0, containing 10% glycerol and 0.65 mM Na₂SO₄ (125 ml) and 4 ml fractions were collected at a rate no greater than 20 ml/h. Fractions with specific activities greater than about 35 units/mg were pooled, yielding about 60% of the enzyme units applied to the column.

The enzyme was concentrated at 4 °C by ultrafiltration using a PM-10 membrane. The concentrated enzyme was adjusted to 0.17 to 2.0 mg/ml with a solution of 0.1 M sodium sulfate and 5% glycerol. Unless specified otherwise, the concentration of ISA was 2 mM. Substrates, metals, or coenzymes were added in the incubation mixture, where indicated, to test for their ability to protect against inactivation. At various times, aliquots were withdrawn and assayed for isocitrate dehydrogenase activity. In all cases, enzyme in which no change was obtained under the same conditions (i.e., in the presence of the appropriate ligands) but without ISA in order to correct for any minor activity changes. The rates of reaction of isocitrate dehydrogenase with ISA were determined from semilogarithmic plots of E/E₀ as a function of time, where E and E₀ represent activities at a given time for the experimental and control reaction, respectively.

Incorporation of Radioactive ISA into Isocitrate Dehydrogenase—In order to ascertain the stoichiometry of reaction of ISA with isocitrate dehydrogenase, enzyme was incubated with 2 mM [14C]ISA at 30 °C under the conditions indicated above. At specified times, dithiothreitol was added to yield a concentration of 20 mM in order to decompose the ISA and prevent further reaction. Measurements of enzymatic activity following the addition of dithiothreitol demonstrated that no further change occurred over at least a 30-min period. After 30 min, the reaction mixture was diluted with a solution of urea to yield a concentration of 5 M, and the enzyme sample was applied to a Sephadex G-25 column (1 × 28 cm) equilibrated with 0.05 M triethanolamine chloride buffer, pH 7.0, containing 5% glycerol, 0.15 mM Mn₂SO₄, and 6 mM urea. Enzyme was recovered in the void volume, completely separated from the excess ISA, which is adsorbed to Sephadex G-25. The radioactivity of the fractions was measured using a Packard Tri-Carb liquid scintillation counter, model 3330. The protein concentration was determined using the Bio-Rad Protein Assay which is based on the method of Bradford (22). Standard solutions were prepared by dilution of native isocitrate dehydrogenase with the same buffer used to equilibrate the Sephadex column.

Identification of Amino Acid Residues—In order to identify the modified amino acids, isocitrate dehydrogenase reacted with ISA under various conditions was separated from excess reagent by gel filtration as described above, dialyzed against distilled water for 24 h and then lyophilized. The protein samples were hydrolyzed with 6 N HCl under vacuum at 110 °C for 20 h after which they were taken to dryness. Each protein sample was applied to a Beckman model 120C amino acid analyzer and fractions of the effluent were collected at 1-min intervals (1.7 ml) after passage through the photometer. The fractions were assayed for radioactivity in the liquid scintillation counting and amino acid analysis were carried out on the fractions obtained from modified proteins were compared with those of standard carboxymethyl-amino acids chromatographed on the amino acid analyzer under the same conditions.  

RESULTS

Effect of Salicylic Acid on Catalytic Activity of Isocitrate Dehydrogenase—To obtain an initial indication as to what procedure was extended over more than 3 to 4 days, the final specific activity was 30 to 35 u.e./mg. However, these preparations were unstable, being susceptible to inactivation, kinetic properties from those of higher specific activity. The protein concentration of the purified enzyme was determined using a value of 10.8 for the E₅₀₀ (8). A molecular weight of 58,000 was used in all calculations (20).

Assay for Isocitrate Dehydrogenase Activity—Enzyme activity was measured at 25 °C using 0.1 mM TPN, 4 mM isocitrate, and 2 mM Mn₂SO₄ in 30 mM triethanolamine chloride, pH 7.4, in a total volume of 1.0 ml. Initial velocities were determined spectrophotometrically at 340 nm using a Gilford model 240 spectrophotometer with the sample set to 0.1 A units full scale. Specific activity is defined as micromoles of TPN" reduced per min per mg of protein.

Assays for Oxalosuccinate Decarboxylase Activity—The oxalosuccinate decarboxylase activity was measured spectrophotometrically at 240 nm in accordance with Graffin and Ochoa (21). A total volume of 1.0 ml contained 0.23 mM oxalosuccinate, 0.24 mM manganous sulfate, and 0.134 mM potassium chloride in 0.2 M sodium acetate buffer, pH 5.5.

Kinetics of Inactivation—Isocitrate dehydrogenase (0.17 to 2.0 mg/ml) was incubated with 4-iodoacetamidosalicylic acid at 30 °C in 0.05 M triethanolamine chloride buffer, pH 7.9, containing 0.15 mM Mn₃SO₄. The concentration of ISA was 2 mM. Substrates, metals, or coenzymes were added in the incubation mixture, where indicated, to test for their ability to protect against inactivation. At various times, aliquots were withdrawn and assayed for isocitrate dehydrogenase activity. In all cases, enzyme in which no change was obtained under the same conditions (i.e., in the presence of the appropriate ligands) but without ISA in order to correct for any minor activity changes. The rates of reaction of isocitrate dehydrogenase with ISA were determined from semilogarithmic plots of E/E₀ as a function of time, where E and E₀ represent activities at a given time for the experimental and control reaction, respectively.

The abbreviation used is: ISA, 4-iodoacetamidosalicylic acid.

1 E. V. Stevens and R. F. Colman, manuscript in preparation.
enzyme site might be attacked by 4-iodoacetamidosalicylic acid, the parent compound salicylic acid was tested as a competitive inhibitor with respect to isocitrate or TPN using the isocitrate dehydrogenase assay. While maintaining the concentrations of other ligands given under "Experimental Procedures," the $K_m$ for Dl-isocitrate was determined in the absence or presence of four constant concentrations of salicylic acid from 5 to 22 mM. The maximum velocity was not altered over this range of salicylic acid concentrations, but the apparent $K_m$ for total DL-isocitrate in the presence of 2 mM MnSO$_4$ increased with increasing salicylic acid concentrations. It has been proposed that manganous-isocitrate is the actual substrate for the TPN-dependent isocitrate dehydrogenase (23). Salicylic acid is known to form a chelate with manganous ion, with an association constant of $10^{12}$ M$^{-1}$ (24). In order to assess whether the apparent behavior of salicylic acid as a competitive inhibitor could be attributed to its depletion of the manganous ion or the manganous-isocitrate complex, the $K_m$ for substrate was calculated in terms of manganous-isocitrate using the equations and computer program described previously (25). The calculated $K_m$ for manganous-isocitrate increased up to 3-fold over the range of salicylic acid concentrations tested, yielding an average $K_m$ of 18.2 mM. A similar value of $K_m$ was derived from a comparison of the $K_m$ for manganous-isocitrate obtained using the higher total concentration of 10 mM MnSO$_4$ in the absence or presence of 15 mM salicylic acid. These results indicate that salicylic acid does not inhibit the enzyme merely by sequestering Mn$^{2+}$, but rather acts as a competitive inhibitor. In contrast, salicylic acid does not significantly affect the $K_m$ for TPN when measured at saturating concentrations of isocitrate (under the standard conditions given under "Experimental Procedures"), indicating that salicylic acid does not bind to the coenzyme binding site of isocitrate dehydrogenase.

**Inactivation of Isocitrate Dehydrogenase by 4-Iodoacetamidosalicylic Acid—Incubation of enzyme with 2.9 mM 4-iodoacetamidosalicylic acid at pH 7.0 leads to loss of both oxaloacetate dehydrogenase and isocitrate dehydrogenase activity, as shown in Fig. 1, suggesting that the group(s) attacked are essential for both functions of the enzyme. The reaction obeys pseudo-first order kinetics as far as 98% inactivation, yielding a rate constant of 0.0306 min$^{-1}$.

Determination of the pseudo-first order rate constant for inactivation of isocitrate dehydrogenase activity from 0.5 to 4.0 mM ISA reveals an apparently linear dependence on reagent concentration (Fig. 2), with a calculated second order rate constant of 10.7 min$^{-2}$ M$^{-1}$ for ISA. Saturation kinetics, which might be expected for an affinity label, are not observed; however, it may be notable that the highest ISA concentration used is far lower than the $K_m$ value of 18.2 mM calculated for salicylic acid. The salicylic acid moiety of 4-iodoacetamidosalicylic acid is important in determining the relatively high rate of inactivation of enzyme by 4-ISA since 2 mM iodoacetamide, the corresponding compound which lacks this group, inactivates the enzyme with a pseudo-first order rate constant of only 0.00193 min$^{-1}$ under the same conditions. A second order rate constant of 0.963 min$^{-2}$ M$^{-1}$ may be calculated for the inactivation of isocitrate dehydrogenase by iodoacetamide, a value less than one-tenth that observed for 4-iodoacetamidosalicylic acid.
Effect of Substrates on the Rate of Inactivation by 4-Iodoacetamidosalicylic Acid—As shown in Fig. 3, the addition of the substrates isocitrate (4 mM) and manganous ion (2 mM) to the incubation mixture totally protects the enzyme against loss of isocitrate dehydrogenase activity produced by 2 mM ISA. The activity measurements for the sample containing isocitrate and manganous ion together with ISA fall on the same line (line B) as those for the two control samples (i.e., enzyme in the absence or presence of the substrates but with no added ISA). These results suggest that reaction occurs in the region of the active site.

Table 1 records the effect of other ligands on the rate constant for inactivation of isocitrate dehydrogenase by 2 mM ISA. All ligands are present at concentrations high relative to their known dissociation or Michaelis constants. Although isocitrate is known to bind to the enzyme in the absence of metal ion (26), isocitrate in the absence of metal does not appreciably decrease the rate constant for inactivation (Table I, lines 3, 4); these results suggest that the manganese-isocitrate complex postulated to be the actual substrate of the TPN-dependent isocitrate dehydrogenase (23) is required to prevent reaction of ISA at the critical site. Several metals are known to interact with the enzyme: manganous, magnesium, and zinc ions serve as activators, whereas calcium ion functions as an inhibitor competitive with respect to Mn$^{2+}$ (23). The data of Table I (lines 5 to 8) indicate that none of these metals by themselves causes a marked decrease in the rate constant for inactivation by ISA. However, when present together with isocitrate, the activators Mn$^{2+}$ and Mg$^{2+}$, as well as the inhibitor Ca$^{2+}$, completely prevent measurable inactivation (Table I, lines 2, 9, 10). These results suggest that there are similarities in the binding sites of these three metal-isocitrate complexes. Since zinc acetate plus isocitrate (Table I, line 11) produces only a minimal decrease in the inactivation rate, the site occupied by the zinc-substrate complex must be distinguishable from those to which the other metal-chelates bind, despite the fact that zinc ion is also an activator of the isocitrate dehydrogenase reaction. The product of the reaction, a-ketoglutarate, fails to alter the inactivation rate in the absence or presence of metal ion and similar results are obtained for the oxidized coenzyme, TPN (lines 12 to 15). The reduced coenzyme yields some protection when present together with manganous ion (line 17, 18); however, the fact that the inactivation rate constant does not decrease more than 2.3-fold when the TPNH concentration is increased suggests that this small diminution in the inactivation rate is an indirect effect of binding to a site distinct from that attacked by ISA.

In contrast, the data of Table II demonstrate that in the presence of a constant total MnSO$_4$ concentration of 2 mM, the rate constant for inactivation by ISA decreases progressively with increasing isocitrate concentration, reaching an apparent $k_{obs}$ of zero at very high concentrations of isocitrate. If it is assumed that isocitrate [5] binds reversibly at the critical site attacked by ISA, the following equation can be used to describe the relationship between $k_{obs}$ (the pseudo-first order rate constant for inactivation by ISA in the presence of isocitrate), $k$, the inactivation rate constant in the absence of ligands, which is 0.021 min$^{-1}$ at 2 mM ISA), and $K_d$ (the dissociation constant for the enzyme-isocitrate complex):

$$k_{obs} = \frac{k_S}{1 + \frac{[S]}{K_d}}$$

Table II records the $K_d$ values obtained at several concentrations of total DL-isocitrate. An average $K_d$ of 20.7 µM can be calculated for total isocitrate. When the Michaelis constant for isocitrate in the isocitrate dehydrogenase assay was measured in the presence of 2 mM MnSO$_4$ at pH 7.0 in a buffer similar to that used for the ISA reaction, a value of 15.5 µM was obtained.

Incorporation of Radioactive ISA into Isocitrate Dehydrogenase—4-Iodoacetamidosalicylic acid reacts irreversibly and in a limited manner with TPN-dependent isocitrate dehydrogenase. The stoichiometry of the reaction was determined as described under "Experimental Procedures" by measurement of the incorporation of [14C]ISA as a function of time of incubation with 2 mM reagent in the absence of protecting ligands. A plot of residual enzymatic activity versus moles of reagent incorporated per mol of protein, shown in Fig. 4A,  

**TABLE I**

| Additions to reaction mixture | $k_{obs}$ (min$^{-1}$) x 10$^4$ |
|-----------------------------|--------------------------------|
| 1. None                     | 21.0                           |
| 2. 2 mM Mn$^{2+}$ + 4 mM isocitrate | No inactivation               |
| 3. 3 mM EDTA + 4 mM isocitrate | 11.0                           |
| 4. 3 mM EDTA                | 14.0                           |
| 5. 2 mM Mn$^{2+}$           | 14.0                           |
| 6. 2 mM Mg$^{2+}$           | 14.0                           |
| 7. 2 mM Ca$^{2+}$           | 7.2                            |
| 8. 2 mM Zn$^{2+}$           | 27.0                           |
| 9. 2 mM Mg$^{2+}$ + 4 mM isocitrate | No inactivation               |
| 10. 2 mM Ca$^{2+}$ + 4 mM isocitrate | No inactivation               |
| 11. 2 mM Zn$^{2+}$ + 4 mM isocitrate | 12.0                           |
| 12. 10 mM a-ketoglutarate   | 23.0                           |
| 13. 2 mM Mn$^{2+}$ + 10 mM a-ketoglutarate | 13.0                           |
| 14. 1 mM TPN                | 27.0                           |
| 15. 2 mM Mn$^{2+}$ + 1 mM TPN | 14.0                           |
| 16. 0.1 mM TPNH             | 24.0                           |
| 17. 2 mM Mn$^{2+}$ + 0.1 mM TPNH | 8.5                            |
| 18. 2 mM Mn$^{2+}$ + 2.0 mM TPNH | 9.5                            |

**FIG. 3.** Effect of isocitrate and MnSO$_4$ on inactivation of isocitrate dehydrogenase by 4-Iodoacetamidosalicylic acid. Enzyme (0.17 mg/ml) was incubated at pH 7.0 under the conditions given under "Experimental Procedures" in the absence (C, control) or presence (●) of 2 mM ISA. The isocitrate dehydrogenase activity was determined from aliquots of the incubation mixtures at the indicated times, to yield $E$. Line A is described by a pseudo-first order rate constant of 0.021 min$^{-1}$. Isocitrate (4 mM) and MnSO$_4$ (2 mM) were added as indicated in the absence (●, control) or presence (●) of 2 mM ISA.
Reactivity of ISA with TPN-Isocitrate Dehydrogenase

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Table II

Protection by different concentrations of isocitrate against inactivation by ISA

Enzyme (0.17 mg/ml) was incubated at pH 7.0 with 2 mM ISA under the conditions described under "Experimental Procedures." MnSO4 (2 mM) was added to the reaction mixture together with the indicated total concentrations of pL-isocitrate. The rate constants were determined as described in Table I.

| Additions to reaction mixture | kobs (min)\(^{-1}\) | Calculated Kα for total isocitrate |
|------------------------------|-------------------|----------------------------------|
| None                         | 21.0              | 0.53 μM                          |
| 2 mM Mn\(^{2+}\) + 16 μM isocitrate | 8.7               | 11.3 μM                          |
| 2 mM Mn\(^{2+}\) + 29 μM isocitrate | 6.2               | 8.4 μM                           |
| 2 mM Mn\(^{2+}\) + 60 μM isocitrate | 5.4               | 20.8 μM                          |
| 2 mM Mn\(^{2+}\) + 100 μM isocitrate | 4.5               | 27.3 μM                          |
| 2 mM Mn\(^{2+}\) + 160 μM isocitrate | 3.5               | 32.0 μM                          |
| 2 mM Mn\(^{2+}\) + 200 μM isocitrate | 2.3               | 24.6 μM                          |
| 2 mM Mn\(^{2+}\) + 4,000 μM isocitrate | 0                 | 0 μM                             |

Average Kα for total isocitrate = 20.7 μM

Fig. 4. Incorporation of radioactive ISA into isocitrate dehydrogenase. A, residual enzymatic activity as a function of mol of reagent incorporated in the absence of protecting ligands. B, time dependence of reagent incorporation (a) in the absence of protecting ligands; or (b) in the presence of 2 mM MnSO4 and 4 mM isocitrate. For line (a), residual activity was 100% for the entire time period. Incorporation of radioactive ISA was measured after removal of excess reagent by gel filtration, as described under "Experimental Procedures."

Fig. 5. Amino acid analyses of acid hydrolysates of modified isocitrate dehydrogenase. Radioactivity in effluent fractions from amino acid analyzer was measured as described under "Experimental Procedures." Acid hydrolysates from three types of enzyme samples are shown: A, from 86% inactive enzyme, prepared by reaction with \([^{14}C]\)ISA in absence of ligands. Sample had 2.1 mol of radioactive reagent incorporated/mol of enzyme. B, from 100% active enzyme, prepared by reaction with \([^{14}C]\)ISA in the presence of 2 mM MnSO4 and 4 mM isocitrate. Sample had 2.2 total mol of reagent incorporated/mol of enzyme. C, from 86% inactive enzyme prepared by reaction with nonradioactive ISA in the presence of MnSO4 and isocitrate, followed by removal of unlabeled ISA and substrates and inactivation with \([^{14}C]\)ISA in the absence of ligands. Sample had 2.2 mol of radioactive reagent incorporated per mol of enzyme. The amounts of protein applied to the amino acid analyzer were not the same in the three cases; only the distribution of radioactivity among the major labeled peaks can properly be compared. The elution position of standard carboxymethyl-amino acids is indicated along with the peak positions of other standards.
Radioactivity was detected in the region where carboxymethyllysine, glycolic acid, and carboxymethylcysteine. No large following glutamic acid) or where carboxymethylhistidine derivatives are expected to elute (immediately (27) of lysine, edicarboxymethyllysine; since this is a disubstituted lysine derivative, the radioactive incorporation represents 0.35 mol of modified lysine/mol of protein.

This is lost during the decarboxylation reaction) is required for the protection by isocitrate, as it is for the enzyme-catalyzed oxidative decarboxylation. The metal ion requirements for isocitrate protection against ISA inactivation can be fulfilled by manganous and magnesium ions, which support the overall catalytic reaction and (in the presence of isocitrate) strengthen the binding of a coenzyme analogue (30), as well as by calcium ion, which acts as an inhibitor competitive with respect to Mn$^{2+}$ and (in the presence of isocitrate) weakens the binding of the coenzyme analogue. Although these metals may have different effects on the function and/or conformation of the enzyme, they appear to occupy similar sites at least with respect to their protection of the critical residue(s) which react with ISA. In contrast to the other metal activators, zinc ion has been found to facilitate the enzyme-catalyzed dehydrogenation of isocitrate, but not the enzyme-dependent decarboxylation of oxalosuccinate (23). Thus, it is not surprising that zinc ion is also distinguished from the other divalent metal ions tested in yielding only a small decrease in the ISA inactivation rate when added with isocitrate. Zinc must bind to a site which is not identical with that at which other metals are bound.

While isocitrate dehydrogenase catalyzes both the pyridine nucleotide-dependent dehydrogenation of isocitrate to form oxalosuccinate and the subsequent decarboxylation of the $\beta$-keto acid to yield $\alpha$-ketoglutarate, it might be anticipated that certain amino acid residues would participate in only one of these reactions. For example, alkylation of a methionyl residue by iodoacetate causes a profound disruption of the dehydrogenase activity with a relatively minor effect on the decarboxylase activity (1, 31). Furthermore, incubation of isocitrate dehydrogenase with ethyoxymiform anhydride causes a 9-fold greater loss of dehydrogenase than of decarboxylase activity and it has been postulated that this results from modification of a critical histidine in the nucleotide binding site (30). In contrast, an amino acid residue involved in binding the $\beta$-carboxylate group of isocitrate might well be expected to be involved in binding the analogous groups of oxalosuccinate. The observation that 4-idoacetamidosaliclyic acid causes an equal loss of the dehydrogenase and decarboxylase activities of isocitrate dehydrogenase indicates that the group(s) which is modified is important for both phases of the overall oxidative decarboxylation reaction of the enzyme; this observation is consistent with the postulate that the ISA-susceptible group(s) has as its normal function interaction with the $\beta$-carboxylate moiety of the substrate.

Although 4 mol of radioactive 4-idoacetamidosalicylic acid
are incorporated into isocitrate dehydrogenase concomitant with complete inactivation, the observation that two groups still react covalently in the presence of isocitrate and Mn²⁺ when no inactivation occurs suggests that the requirement for inactivation is modification of an average of only 1 to 2 amino acid residues. The prime candidates for the critical residues are a cysteine and a lysine, since these are the extra amino acids which are labeled in the presence of ligands but not in the presence of isocitrate. Johanson and Colman have presented evidence for a cysteine residue in the manganous-isocitrate binding site which is attacked by 5,5'-dithiobis(2-nitrobenzoic acid) and have isolated a critical cysteine-containing peptide (8). Inactivation by ISA in the absence of ligands (unprotected enzyme) may be caused by alteration of the same cysteine. However, it is also possible that modification of a combination of cysteine and lysine are responsible for inactivation in this case. It is notable that after two cysteines have reacted with ISA in the presence of isocitrate and Mn²⁺ and the protectants are removed, ISA still inactivates isocitrate dehydrogenase, but with modification of only a lysine residue. For this protected then unprotected enzyme, reaction with about 1 lysine appears to be responsible for the inactivation by 4-iodoacetamidosalicylic acid.

Carbamoylation of 1 lysine per average subunit was reported to irreversibly inactivate the allosteric pig heart DPN-dependent isocitrate dehydrogenase (9) and it was postulated that the reaction locus was in the binding site of the manganous-isocitrate complex. Cyanate failed to inactivate the TPN-dependent isocitrate dehydrogenase under similar conditions, which suggested that the substrate binding sites were not identical in the two isocitrate dehydrogenases from the same species and tissue. In the present study, the effect of the salicylic acid moiety in directing the reaction of 4-iodoacetamidosalicylic acid may facilitate alkylation of a relatively inaccessible, yet critical lysine residue. The protonated lysine residue may participate in an electrostatic interaction with the β-carboxylate group of isocitrate which strengthens and properly orient the binding of the isocitrate-metal complex.

REFERENCES
1. Colman, R. F. (1968) J. Biol. Chem. 243, 2454-2464
2. Colman, R. F. (1973) J. Biol. Chem. 248, 8137-8143
3. Ehrlich, R. S., and Colman, R. F. (1977) Biochemistry 16, 3378-3383
4. Ehrlich, R. S., and Colman, R. F. (1978) Eur. J. Biochem. 89, 575-587
5. Colman, R. F. (1969) Biochemistry 8, 888-898
6. Colman, R. F., and Chu, R. (1970) J. Biol. Chem. 245, 601-607
7. Colman, R. F., and Chu, R. (1970) J. Biol. Chem. 245, 608-615
8. Johanson, R. A., and Colman, R. F. (1981) Arch. Biochem. Biophys. 207, 9-20; 21-31
9. Shen, W.-C., and Colman, R. F. (1975) J. Biol. Chem. 250, 2973-2978
10. Einarsson, R., Eklund, H., Zeppuzauer, E., Boiwe, T., and Branden, C.-I. (1974) Eur. J. Biochem. 49, 41-47
11. Pai, E. F., Sachsenheimer, W., Schirmer, R. H., and Schulz, G. E. (1977) J. Mol. Biol. 114, 37-46
12. Subramanian, S., and Ross, P. D. (1978) Biochemistry 17, 2193-2197
13. Baker, B. R. (1967) Design of Active-Site-Directed Irreversible Enzyme Inhibitors, pp. 165-172, John Wiley & Sons, Inc., New York
14. Malcolm, A., and Radda, G. (1970) Eur. J. Biochem. 15, 555-561
15. Holbrook, J. J., Roberts, A., and Wallis, R. B. (1973) Biochem. J. 133, 165-171
16. Wallis, R. B., and Holbrook, J. J. (1973) Biochem. J. 133, 173-182
17. Rosen, N. L., Bishop, L. Burnett, J. B., Biabop, M., and Colman R. F. (1973) J. Biol. Chem. 248, 7359-7369
18. Warburg, O., and Christian, W. (1941) Biochem. Z. 310, 384
19. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) Biochemistry 10, 2606-2617
20. Colman, R. F. (1972) J. Biol. Chem. 247, 6727-6729
21. Grafflin, A. L., and Ochoa, S. (1950) Biochim. Biophys. Acta 4, 205-210
22. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
23. Colman, R. F. (1972) J. Biol. Chem. 247, 215-223
24. Dawson, R. M. C., Elliott, D. C., Elliott, W. H., and Jones, K. M. (1969) Data for Biochemical Research, 2nd Ed, Oxford University Press, NY
25. Cohen, H. F., and Colman, R. F. (1972) Biochemistry 11, 1501-1508
26. Colman, R. F. (1969) Biochim. Biophys. Acta 191, 469-472
27. Gundlach, H. G., Stein, W. H., and Moore, S. (1969) J. Biol. Chem. 234, 1754-1760
28. Gundlach, H. G., Moore, S., and Stein, W. H. (1969) J. Biol. Chem. 234, 1761-1764
29. Szwczuk, A., and Connell, G. E. (1965) Biochim. Biophys. Acta 105, 352-367
30. Ehrlich, R. S., and Colman, R. F. (1978) Eur. J. Biochem. 89, 575-587
31. Seelig, G. F., and Colman, R. F. (1979) J. Biol. Chem. 254, 1191-1195