Effect of Modification of a Methionyl Residue on the Kinetic and Molecular Properties of Isocitrate Dehydrogenase

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SUMMARY

Pig heart isocitrate dehydrogenase, which catalyzes the TPN-dependent dehydrogenation of isocitrate as well as the decarboxylation of oxalosuccinate, is inactivated by treatment with iodoacetate at pH 5.5 and 30°. Approximately 1 mole of 14C-labeled reagent is incorporated per mole of protein. The effect of alkylation on the isocitrate dehydrogenase activity is greater than that on the oxalosuccinate decarboxylase activity of the enzyme, and therefore the active sites for these two catalytic functions are distinguishable. Inactivation can be prevented by the inclusion of isocitrate in the reaction mixture, suggesting that iodoacetate causes a specific modification in the functional region of the enzyme. Vmax for both the native and the partially modified enzyme depends on the unprotonated form of an ionizable group of pK 5.4, which shifts to pK 6.3 when measured in 20% ethanol as solvent. A requirement for the dissociable form of a carboxyl group is suggested. It is concluded that iodoacetate alters an amino acid residue essential for the dehydrogenase reaction.

Iodoacetate treatment does not produce marked structural alterations in the enzyme, since no differences in molecular weight or in the ultraviolet absorption or optical rotatory dispersion spectra were detected. The pseudo-first order rate constant for inactivation of the enzyme by iodoacetate decreases from pH 7.8 to 7.2, is almost constant from pH 7.2 to 6.1, and increases markedly below pH 6.0. Enzyme alkylated at pH 5.6 exhibits the same sulphydryl content as the native enzyme, and an unaltered histidine, lysine, and tyrosine composition. Amino acid analysis indicates that the methionine content of the alkylated enzyme is decreased by 1 residue as compared to the native enzyme, and a new peak, that of carboxymethylhomocysteine, is observed. Paper chromatography of proteolytic digests of 14C-labeled alkylated enzyme confirms the conclusion that alklylation of 1 methionine residue is responsible for the altered activity of isocitrate dehydrogenase.

The rate of inactivation at pH 5.0 is independent of ionic strength. Modified enzyme exhibits a mobility identical with that of native enzyme in cellulose acetate electrophoresis from pH 6.2 to 9.5. These observations are consistent with attack of iodoacetate on an uncharged methionyl residue to yield dipolar carboxymethylmethionine sulfonium salt.

Two mechanistically distinct reactions, dehydrogenation and decarboxylation, are both catalyzed as intrinsic functions of isocitrate dehydrogenase (threo-β-isocitrate:NADP oxidoreductase (decarboxylating), EC 1.1.1.42) (1–3). The over-all reaction characteristically proceeds from isocitrate to α-ketoglutarate through an enzyme-bound oxalosuccinate intermediate (4). The two reactions thus appear to occur sequentially on the surface of the enzyme, but the precise relationship between these regions of the enzyme which contribute to dehydrogenase activity and those which accelerate the decarboxylase reaction is not understood.

The related acetoacetate decarboxylase reaction, in which a β-keto acid is also decarboxylated, has been found to involve a Schiff base intermediate formed by reaction of the substrate with the ε-amino group of an enzyme lysyl residue (5, 6). Major differences exist between the nonenzymatic decarboxylations of acetoacetate and oxalosuccinate (7); nevertheless, the Schiff base mechanism was considered for isocitrate dehydrogenase. Zalkin and Sprinson (8) reported that in the isocitrate dehydrogenase-catalyzed reaction the oxygen of the carbonyl group of α-ketoglutarate does not exchange with an H18O medium. In contrast, acetoacetate decarboxylase catalyzes exchange between this medium and acetoacetate labeled with 18O in the carbonyl group. Furthermore, exposure to sodium borohydride of isocitrate dehy-
dehydrogenase in the presence of 14C-labeled α-ketoglutarate failed to produce a radioactive catalytically inactive protein, as would be expected for a Schiff base intermediate. The enzymatic mechanisms for the decarboxylation of acetoacetate and oxalosuccinate are thus distinct, and different functional groups may be anticipated at the active sites of these enzymes.

The present investigation has revealed that treatment of isocitrate dehydrogenase with acetoacetate below pH 6 results in a differential loss of dehydrogenase as compared to decarboxylase activity. The active sites for these two reactions therefore cannot be identical. Protection against inactivation can be obtained by including the substrate isocitrate in the reaction mixture, suggesting that acetoacetate attacks a site involved in catalytic function of the enzyme. The physical characteristics of the enzyme are essentially unaltered by alkylation, and the changes in the reaction of the enzyme. The physical characteristics of the enzyme are essentially unaltered by alkylation, and the changes in the reaction of the enzyme. The physical characteristics of the enzyme are essentially unaltered by alkylation, and the changes in the reaction of the enzyme.

Experimental Procedure

Materials—Pig heart TPN-dependent isocitrate dehydrogenase was supplied as a solution in 50% glycerol by Boehringer and was further purified 10-fold as described below. All coenzymes and substrates, as well as iodoacetamide, were purchased from Sigma; Aldrich supplied 5-aminotetrazole monohydrate and 5,5'-dithiobis(2-nitrobenzoic acid).

Unlabeled iodoacetic acid obtained from Mann was recrystallized from water and benzene. Iodoacetic acid-14C was purchased from New England Nuclear. The radio purity of the reagent was established by preparation of 14C-labeled tricarboxymethylamine from glycine and the radioactive iodoacetate. The melting point of the recrystallized product was identical with the literature value (9). The specific activity of the original iodoacetic acid as measured directly was the same as that calculated from the radioactivity of tricarboxymethylamine.

Carboxymethylmethionine sulfoxonium salt was prepared by the method of Gundlach, Stein, and Moore (10) with 14C-labeled or nonradioactive iodoacetate. Homocysteine, tyrosine, histidine, and glutamic acid derivatives were synthesized as described previously (11). DL-Homoserine was purchased from Calbiochem, and DL-homocysteine and S-carboxymethyl cysteine from Nutritional Biochemicals.

Barium oxalosuccinate was obtained by acid hydrolysis of triethylxalosuccinate (K and K laboratories) as described by Ochoa (12). The barium salt was stored over calcium sulfate at 5°C. Solutions of oxalosuccinate were prepared prior to use (12).

Kinetic Studies—Unless otherwise indicated, isocitrate dehydrogenase was measured at 25°C in 0.03 M triethanolamine chloride buffer, pH 7.4, with 1 × 10⁻⁴ M, 4 × 10⁻⁴ M, and 2 × 10⁻³ M TPN, 0.1 M isocitrate, and manganese sulfate, respectively, in a total volume of 1.0 ml. Initial velocities were determined spectrophotometrically at 340 nm with an expanded scale recorder (0.1 absorbance full scale). Specific activity is defined in terms of this assay as micromoles of TPN reduced per min per mg of protein. In determining individual Michaelis constants, each substrate was varied in turn, with the remaining substrates being maintained at the concentrations given in this section.

Reductive carboxylase activity was measured spectrophotometrically at 340 nm and 25°C in 0.04 M triethanolamine chloride buffer, pH 7.4, with 2 × 10⁻⁴ M, 2 × 10⁻³ M, 4 × 10⁻² M, and 2 × 10⁻¹ M TPNH, α-ketoglutarate, potassium bicarbonate, and manganese sulfate, respectively, in a total volume of 1.0 ml. Carbon dioxide was bubbled through the potassium bicarbonate stock solution prior to use.

Oxalosuccinate decarboxylase activity was measured in 0.2 M sodium acetate buffer, pH 5.6, at 15.5°C, or spectrophotometrically at 240 nm and 25°C, in accordance with Grafflin and Ochoa (13). In the manometric determinations, concentrations of 4 × 10⁻³ M and 4.6 × 10⁻⁴ M were used for manganese sulfate and oxalosuccinate, respectively, in a total volume of 1.0 ml. In the spectrophotometric determinations, 1.34 × 10⁻⁴ M, 2.3 × 10⁻⁴ M, and 2.3 × 10⁻⁵ M concentrations of KCl, oxalosuccinate, and manganese sulfate, respectively, were present in a total volume of 1.0 ml.

Enzyme Purification—Pig heart isocitrate dehydrogenase, obtained commercially, with a specific activity of approximately 2.7, was purified initially by chromatography on carboxymethyl cellulose (Bio-Rad) with a column measuring 2.5 × 32 cm at 5°C. The flow rate was 90 ml per hour. An inactive protein peak was eluted with the starting buffer (0.015 M triethanolamine chloride, pH 7.0, containing 0.9 mM EDTA and 10% glycerol). The eluting buffer was then changed to 0.1 M triethanolamine chloride, pH 7.7, containing 1 mM EDTA, 0.3 M Na₂SO₄, and 10% glycerol, and a second protein peak, with enzymatic activity, was obtained. The peak tubes, when pooled, exhibited a specific activity of 7.5 with an 80% yield. Glycerol was added to all buffers used in these purification procedures, since it was found to enhance the stability of the preparation significantly. Concentration of the protein solution was accomplished in the cold by covering a dialysis bag containing this solution with dry Sephadex G-100. The resultant solution was dialyzed against the pH 7.7 buffer.

Further purification was effected by gel filtration on Sephadex G-150, as shown in Fig. 1. Enzymatic activity appeared in the second protein peak, an observation that is consistent with a relatively low molecular weight. After combination of the peak tubes, concentration, and centrifugation to remove denatured protein, the specific activity was 29 μmoles of TPN reduced per min per mg of protein at pH 7.4 and 25°C. The over-all yield for these purification procedures was approximately 60%. Different batches of the commercial starting material occasionally varied in the relative amounts of the inactive proteins which were present. However, a homogeneous enzyme of the stated specific activity could always be obtained by these procedures. The enzyme was stored in the pH 7.7 buffer at 5°C.

FIG. 1. Gel filtration on Sephadex G-150. This purification procedure was conducted with a column, 2.4 × 34.5 cm, at 5°C, which was developed with a buffer at pH 7.7 containing 0.1 M triethanolamine chloride, 0.3 M Na₂SO₄, 1 mM EDTA, and 10% glycerol. The flow rate was 2 ml per hour. Isocitrate dehydrogenase activity was measured spectrophotometrically by the reduction of TPN, as described in "Experimental Procedure."
mg per ml and was dissolved in a buffer at pH 7.7 containing 0.1 M triethanolamine chloride, 0.3 M Na$_2$SO$_4$, and 1 mM EDTA. Equilibrium was established by sedimentation for 16 hours at 25,000 rpm and 7$^\circ$C. The density of the solvent was determined to be 1.0632, and the assumption was made that the partial specific volume was 0.74.

Before the protein was obtained in a homogeneous state the protein concentration was estimated by the method of Warburg and Christian (14). For homogeneous isocitrate dehydrogenase as well as its alkylated derivative, the $E_{427}^{1\%}$ was determined to be 9.10, based on the biuret reaction with bovine serum albumin as the standard.

Binding of $^{14}$C-Iodoacetate—Enzyme (approximately 1 mg per ml) was incubated with varying concentrations of $^{14}$C-labeled iodoacetate at pH 5.8 and 30$^\circ$. After a time interval sufficient to decrease the residual dehydrogenase activity to 0 to 10%, the enzyme was subjected to gel filtration at 5$^\circ$C on a column (1.2 × 25 cm) of Sephadex G-25, equilibrated with 0.1 M Tris acetate, pH 7.18, to stop the reaction and remove excess reagent. Aliquots of the resulting enzyme solutions were tested for enzymatic activity and protein concentration, and were dried on metal planchets for determination of radioactivity. A low background activity and protein concentration, and were dried on metal planchets for determination of radioactivity. A low background as well as its alkylated derivative, the was 0.14 mg per ml. The molecular weight calculated from these determinations was 58,000. This value compares with values of 61,000 and 64,000 calculated by Siebert et al. (3), and Moyle and Dixon (16), respectively, from sedimentation and diffusion data. In the preparation of Siebert et al., isocitrate dehydrogenase was thought to represent 97% of the total protein, whereas in that of Moyle and Dixon, 90 to 95% of the protein present was claimed to be the enzyme, although its specific activity was relatively low.

Purified isocitrate dehydrogenase yields a single band on disc electrophoresis at pH 9.5, and on cellulose acetate electrophoresis from pH 6.2 to pH 9.8. Fig. 3B illustrates an electrophoresis experiment conducted at pH 9.0. It is notable that even at this high pH the enzyme exhibits a net positive charge. The basic nature of this protein is also indicated by its strong binding to carboxymethyl cellulose during the purification procedures. The amino acid composition of the enzyme, reported earlier (11), indicates the presence of only 70 lysyl, histidyl, and arginyl residues per molecule, whereas 105 seryl plus glutamyl residues were found in the acid hydrolysate. This apparent discrepancy between the amino acid composition and the electrophoretic mobility of the enzyme may be attributable to the amide content. The observed ammonia content of the acid hydrolysate was 104 residues per molecule; however, the actual value is not considered as reliable as others obtained from amino acid analysis, because of the possible contribution of atmospheric ammonia. Earlier estimates of the electrophoretic mobility of isocitrate dehydrogenase are at variance with the results reported here. Siebert et al. (3), studying free electrophoresis in a Tiselius apparatus, suggested an isoelectric point of 7.8, and Moyle and Dixon (16), using the same method, stated that the electrophoretic mobility of the major component was small between pH 5.6 and 8.5. Both groups, however, commented on the instability of the enzyme during prolonged exposure to the conditions of low ionic strength used in these experiments; turbidity and heterogeneity were observed, which may invalidate their results.

**Inactivation by Iodoacetate**

**Protection by Substrates**—Catalytic activity is lost as a result of treatment of isocitrate dehydrogenase with iodoacetate at pH 5.8 and 30$^\circ$. Under these conditions, the enzyme in the absence of reagent shows no decrease in activity. Table I shows the effect of substrates on the extent of inactivation by iodoacetate. Loss of activity can be almost completely prevented by including ni-isocitrate in the reaction mixture, suggesting that iodoacetate causes a specific modification in the active site of the enzyme. Manganese sulfate added together with isocitrate did not increase the enzyme activity, whereas it increased the activity of the carbamylated enzyme. This result indicates that the carbamylated enzyme has a different mechanism of inactivation than that of the native enzyme. The results of this experiment are shown in Table II.

![Fig. 3. Cellulose acetate electrophoresis of unmodified and alkylated isocitrate dehydrogenase. A, enzyme alkylated at pH 6.0, with 14% residual activity; B, unmodified enzyme. Electrophoresis was conducted in 0.02 M Tris chloride buffer, pH 9.0, at 200 volts, for 2 hours.](http://www.jbc.org/)

**Fig. 2.** Equilibrium sedimentation of isocitrate dehydrogenase In this experiment, the initial protein concentration was 0.14 mg per ml and was dissolved in a buffer at pH 7.7 containing 0.1 M triethanolamine chloride, 0.3 M Na$_2$SO$_4$, and 1 mM EDTA. Equilibrium was established by sedimentation for 16 hours at 25,000 rpm and 7$^\circ$. The density of the solvent was determined to be 1.0632, and the assumption was made that the partial specific volume was 0.74.

<table>
<thead>
<tr>
<th>pH</th>
<th>$E_{427}^{1%}$</th>
<th>$E_{427}^{270}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8</td>
<td>9.10</td>
<td>9.10</td>
</tr>
<tr>
<td>5.8</td>
<td>9.10</td>
<td>9.10</td>
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**Table I.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (as % of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>98</td>
</tr>
<tr>
<td>MnSO$_4$</td>
<td>80</td>
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**Table II.**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (as % of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
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<td>80</td>
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the protection afforded by the substrate alone. Furthermore, the addition to the reaction mixture with EDTA (which binds manganous ions tightly and totally blocks the enzymatic dehydrogenation of isocitrate) does not decrease the extent of protection obtained in the presence of isocitrate alone. The same results are obtained when EDTA is present in the reaction mixture along with isocitrate and added manganese sulfate. Therefore, the protective effect of isocitrate is independent of metal ion, and it appears that the binding of the substrate, at least in the vicinity of the enzyme amino acid residue modified by iodoacetate, is not affected by the metal ion. A lesser degree of protection is provided by α-ketoglutarate, while citrate, TPNH, TPN, and Mn++ alone exert little or no effect on the course of alkylation. Increases in the concentrations of substrates present in the reaction mixture do not change the extent of protection. The concentration of iodoacetate is in great excess over that of the enzyme, and therefore pseudo-first order kinetics is followed. In the last column of Table I, the presence of isocitrate can be seen to cause an 11-fold decrease in the observed rate constant for inactivation.

**Table I**

Effect of substrates on inactivation by iodoacetate

<table>
<thead>
<tr>
<th>Additions to reaction mixture</th>
<th>Residual activity %</th>
<th>kobs (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11</td>
<td>0.0402</td>
</tr>
<tr>
<td>8 X 10⁻³ M isocitrate</td>
<td>89</td>
<td>0.00339</td>
</tr>
<tr>
<td>8 X 10⁻³ M isocitrate + 2 X 10⁻² M MnSO₄</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>2 X 10⁻⁴ M MnSO₄</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>1 X 10⁻⁴ M α-ketoglutarate</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>1.0 X 10⁻⁴ M TPNH</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>3.0 X 10⁻⁴ M TPNH</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>1.0 X 10⁻⁴ M TPNH</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

*Measured spectrophotometrically at 240 μM.
*Measured manometrically.

A further indication of the differential effect of alkylation on these two functions of the enzyme is given in Fig. 4, which illustrates the residual isocitrate dehydrogenase and reductive carboxylase activities as a function of time of incubation with iodoacetate. It is apparent that, whether the alkylation reaction is conducted at pH 5.5 or 5.8, the pseudo-first order rate constant for inactivation is approximately 1.7 times as great when assessed by the isocitrate dehydrogenase (forward) as compared to the reductive carboxylation (reverse) reaction. The data of Siebert, Carioles, and Plaut (4) on the rates of the individual reactions imply that the rate-determining step in the forward reaction is the dehydrogenation of isocitrate, whereas the slow step in the reverse reaction is probably the carboxylation of α-ketoglutarate. If alkylation affects primarily the oxidation-reduction step, it is reasonable that the activity as determined by the over-all reaction would measure directly the disruption of that step, whereas the activity as determined by the over-all reverse reaction would be affected more slowly.

**Table II**

Effect of iodoacetate treatment on dehydrogenase and decarboxylase activities

<table>
<thead>
<tr>
<th>Inactivation process</th>
<th>Specific activity μmoles/min/mg</th>
<th>Residual activity %</th>
<th>Decarboxylase to dehydrogenase activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>29.3</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>None</td>
<td>2.4</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>29.3</td>
<td>32</td>
<td>9</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>2.4</td>
<td>36</td>
<td>12</td>
</tr>
<tr>
<td>Iodoacetate + 0.02 M α-ketoglutarate</td>
<td>1.9</td>
<td>79</td>
<td>42</td>
</tr>
<tr>
<td>4 M urea</td>
<td>2.4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Measured spectrophotometrically at 240 μM.

Inactivation Rate as Function of Iodoacetate Concentration—In a strictly chemical displacement reaction, the pseudo-first order rate constant should increase linearly with the reagent concentration. If, on the other hand, an enzyme-inhibitor complex were formed initially prior to the alkylation reaction, one might expect to observe saturation of the rate at high concentrations of the reagent in accordance with the following equation (17):
with 0.12 were obtained by assays of aliquots of the same incubation mixture at 30° containing 2 mg of enzyme per ml and 0.6 × 10⁻² M iodoacetate in 0.05 M potassium phosphate buffer, pH 5.8. At the given times, aliquots were taken, immediately diluted 20-fold with 0.12 M triethanolamine chloride buffer, pH 7.4, at 0°, and assayed for isocitrate dehydrogenase (forward) or reductive carboxylase (reverse) activity as described in "Experimental Procedure." The rate constants that were calculated from the relationship

\[ \frac{-d(E)}{dt} = \frac{k(E)(I)}{K_+ + (I)} \]

where (E) and (I) are the free enzyme and inhibitor concentrations, respectively. Fig. 5 shows that the observed rate of inactivation upon alkylation at pH 5.8 is linearly proportional to iodoacetate concentrations up to 0.03 M. At higher concentrations, however, there is a slight negative deviation from the linear relationship. Application of the relationship

\[ \frac{1}{K_{	ext{cate}}} = \frac{K_+}{K(I)} + \frac{1}{k} \]

allows the calculation of an approximate dissociation constant of 0.59 M for the enzyme-iodoacetate complex. It may be that the reagent binds weakly to the substrate-binding site.

**Kinetic Parameters of Partially Alkylated Enzymes**—The observed loss of activity cannot be attributed to a weaker binding of substrates by an intrinsically active modified enzyme. In these experiments, isocitrate dehydrogenase was incubated with iodoacetate for varying time intervals to produce partially active preparations, the reagent was separated from the enzyme by gel filtration on Sephadex G-25, and the Michaelis constants were measured. Table III shows that the Michaelis constants for the substrates and coenzymes were not significantly altered by alkylation. The small differences observed are considered to represent the experimental errors and are far too small to account for the inactivation.

Similarly, the pH dependence of \( V_{\text{max}} \) is not altered in partially inactivated enzyme. Over the pH range from 5 to 8, the curve of maximum velocity with respect to pH can be described in terms of an ionizable group of pK = 5.42 for the native enzyme and pK = 5.49 for a 15% active alkylated enzyme.

**pH Dependence of Inactivation by Iodoacetate**—Fig. 6 shows that the rate of inactivation of isocitrate dehydrogenase by iodoacetate decreases from pH 7.7 and 7.2, is almost constant from pH 7.2 to 6.1, and increases markedly below pH 6.0. Iodoacetamide is a poor inhibitor of the enzyme at acidic pH, exhibiting a rate constant for inactivation one-third that of iodoacetate at pH 5.1, whereas it is approximately 5 times as effective as iodoacetate at pH 7.7. Electrostatic interaction with the reactant by an enzyme group which has a pK in this region may be an important factor in the enhanced reaction rates with iodoacetate at low pH. It is notable that the decrease in \( V_{\text{max}} \) with pH for the catalytic dehydrogenation of isocitrate, presumably reflecting ionization of the enzyme-substrate complex (Fig. 4A), corresponds reasonably well with the increase in the reaction rate of the free enzyme with iodoacetate. This correlation suggests that protonation of an amino acid residue, which is involved in its basic form in the activity of the enzyme-substrate complex, may increase the reactivity toward iodoacetate of a different amino acid in the active site. The modes of inactivation at high and low pH appear to be distinct. It has already been noted that

![Fig. 4. Effect of iodoacetate on isocitrate dehydrogenase and reductive carboxylase activities. The points on Lines A and B were obtained by assays of aliquots of the same incubation mixture at 30° containing 2 mg of enzyme per ml and 0.6 × 10⁻² M iodoacetate in 0.05 M potassium phosphate buffer, pH 5.8. At the given times, aliquots were taken, immediately diluted 20-fold with 0.12 M triethanolamine chloride buffer, pH 7.4, at 0°, and assayed for isocitrate dehydrogenase (forward) or reductive carboxylase (reverse) activity as described in "Experimental Procedure." The rate constants that were calculated from the relationship \( \frac{-d(E)}{dt} = \frac{k(E)(I)}{K_+ + (I)} \) where (E) and (I) are the free enzyme and inhibitor concentrations, respectively. Fig. 5 shows that the observed rate of inactivation upon alkylation at pH 5.8 is linearly proportional to iodoacetate concentrations up to 0.03 M. At higher concentrations, however, there is a slight negative deviation from the linear relationship. Application of the relationship \( \frac{1}{K_{	ext{cate}}} = \frac{K_+}{K(I)} + \frac{1}{k} \) allows the calculation of an approximate dissociation constant of 0.59 M for the enzyme-iodoacetate complex. It may be that the reagent binds weakly to the substrate-binding site.

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**Fig. 6. Effect of iodoacetate on isocitrate dehydrogenase activity.** The rate constants were calculated from the relationship \( \frac{-d(E)}{dt} = \frac{k(E)(I)}{K_+ + (I)} \) where (E) and (I) are the free enzyme and inhibitor concentrations, respectively. Fig. 5 shows that the observed rate of inactivation upon alkylation at pH 5.8 is linearly proportional to iodoacetate concentrations up to 0.03 M. At higher concentrations, however, there is a slight negative deviation from the linear relationship. Application of the relationship \( \frac{1}{K_{	ext{cate}}} = \frac{K_+}{K(I)} + \frac{1}{k} \) allows the calculation of an approximate dissociation constant of 0.59 M for the enzyme-iodoacetate complex. It may be that the reagent binds weakly to the substrate-binding site.

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**Table III**

<table>
<thead>
<tr>
<th>Residual dehydrogenase activity</th>
<th>( K_{\text{isoctrste}} ) ( \mu M )</th>
<th>( K_{\text{TTPM}} ) ( \mu M )</th>
<th>( K_{\text{ac}} ) ( \mu M )</th>
<th>( K_{\text{TPN}} ) ( \mu M )</th>
<th>( K_{\text{cat}} ) ( \text{glutamate} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 (native)</td>
<td>5.7</td>
<td>4.6</td>
<td>0.90</td>
<td>13.0</td>
<td>0.16</td>
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<tr>
<td>87</td>
<td>2.8</td>
<td>4.5</td>
<td>0.92</td>
<td>14.0</td>
<td>0.24</td>
</tr>
<tr>
<td>55</td>
<td>5.4</td>
<td>0.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>6.6</td>
<td>3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>26.0</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
iodoacetamide does not appreciably inactivate the enzyme under the acidic conditions, although it is effective at pH 7.7. As might be expected from the pK of mercaptoethanol and the high nucleophilicity of the mercaptide ion, 0.07 M mercaptoethanol when added to the alkylation mixture at pH 7.7 prevents inactivation of the enzyme, while it is without effect when added at pH 5.5. Similarly, when 0.07 M mercaptoethanol is added at a given time to an alkylation mixture at pH 7.7, it stops the reaction without reversing the effect on activity. These procedures cannot be used at acidic pH. It has further been noted that the pseudo-first order plots of inactivation by iodoacetate are linear from pH 4.8 to 7.15. However, at pH 7.7, the plot is biphasic and the rate constant given in Fig. 6 is the higher, predominating value. Analyses of proteolytic digests of enzymes alkylated below pH 6.5, which will be discussed later, yield evidence for modification of a single type of amino acid residue. However, similar analyses on enzymes alkylated at pH 7.7 suggest the presence of three classes of modified amino acid residues. Because of the complex nature of the reaction at pH 7.7, attention has been focused on inactivation of the enzyme by iodoacetate at low pH.

In an earlier study, Lotspeich and Peters (18) were unable to inactivate isocitrate dehydrogenase with 1 mM iodoacetate for 20 min at pH 7.2 and room temperature. These results are not surprising, since it can be estimated from the data of Fig. 6 that the half-life for the inactivation reaction would be greater than 1300 min under those conditions.

**V_max as Function of pH in Water and 20% Ethanol**

Observation of the pH dependence of inactivation by iodoacetate prompted a careful examination of the pH dependence of V_max for the isocitrate dehydrogenase reaction from pH 5.0 to 7.6, which is shown in Fig. 7A. The effects of pH on enzymatic activity were shown to be reversible throughout this range of pH, and, at the concentrations used, the enzyme remained saturated with substrate. By using the simplest possible model, which postulates that V_max depends upon the presence in the basic form of one ionizable group in the enzyme-substrate complex, it is possible to assign a value to the pK of this group by means of the equation

$$V_{max} = \frac{V_{max}}{1 + (H^+/K_{act})}$$

where V_{max} = observed maximum velocity at a given (H+); V_{max} = intrinsic maximum velocity, and K_{act} = dissociation constant of activity-dependent ionizable group in the enzyme-substrate complex. The pK calculated for this group is 5.42, which lies between the values generally thought to be typical for carboxyl and imidazolium groups in a protein (19). One method of distinguishing between two possible amino acids with similar ionization constants is to determine the pK of the ionizable group of the enzyme in water and in a solvent of lower dielectric constant, such as an ethanol-water mixture. Michaelis and Misutani (20, 21) showed that the pK values of uncharged acids, such as acetic acid, increase as the proportion of ethanol in a water-ethanol solvent is increased. On the other hand, the pK of cationic acids, such as imidazolium ion, decrease under the same conditions (22). In 20% ethanol, for example, the measured pK of acetic acid increases 0.17 logarithmic unit as compared to its value in water, whereas the pK of imidazolium ion decreases 0.20 logarithmic unit. This difference in the effect of ethanol on uncharged and cationic acids may be exploited to determine the charge type of the ionizable groups in the active site of an enzyme. Fig. 7 compares the pH-V_max profile of isocitrate dehydrogenase measured in 20% ethanol and in water. The intrinsic maximum velocity in the mixed solvent is only half
that found in water. However, this inhibition is independent of time of incubation in 20% ethanol and is completely reversible. The reaction rates are linear and independent of substrate. Denaturation does not, therefore, appear to be a factor in the inhibition. The pK for the activity-dependent ionizable group is 6.34 in 20% ethanol, an increase of 0.9 logarithmic unit over that found in water. The shift is in the direction to be expected for dissociation of a carboxyl group; however, the magnitude is considerably greater than that of model carboxylic acids. It must be remembered that the total effect of change of solvent on the ionization of any particular protein group may be a complex function of the charge type of the functional group involved, the effect on the ionizable residues in the immediate vicinity of the group under consideration, and the effect on the structure of the protein. Therefore, the experimental results obtained for an enzyme may exhibit departure from those of model compounds. 

**Optical Rotatory Dispersion and Molecular Weight**

In order to ascertain whether the conformation of the enzyme was extensively altered by alkylation, optical rotatory dispersion spectra were obtained for native and modified enzymes with a Cary model 60 spectropolarimeter in the wave length region from 350 to 220 m. Experiments were performed at 23° with the enzyme freed of excess reagent and dissolved in 0.1 M triethanolamine chloride buffer, pH 7.4. Alkylated enzymes with 28% residual activity exhibited spectra which were essentially superimposable on those of native enzyme preparations over this entire range of wave length. The specific rotation at 233 m was -7100°.

In separate experiments, native isocitrate dehydrogenase was treated with 2.4 x 10^{-4} M iodoacetate in 0.1 M potassium phosphate buffer, pH 5.6, at 23°. The specific rotation at 233 m of the reaction mixture was monitored as a function of time, concomitant with determinations of residual dehydrogenase activity. No change in the specific rotation was observed over the activity range from 100% to 15%. These results suggest that alkylation of isocitrate dehydrogenase does not produce changes in the protein structure extensive enough to be detected by optical rotatory dispersion.

Furthermore, the molecular weight of the alkylated enzyme was identical with that of native enzymes as determined by sedimentation equilibrium experiments, and the sedimentation constants of the two enzymes were similar.

**Binding of Iodoacetate-1-14C**

The binding of radioactive iodoacetate to isocitrate dehydrogenase was measured after appropriate incubation of the enzyme and reagent at pH 5.8 and 30°, followed by removal of excess reagent by gel filtration on Sephadex G-25 in the cold. Six determinations give an average value of 0.75 mole incorporated per mole of totally inactive enzyme, and therefore a single amino acid residue essential to activity has probably been modified in the enzyme. In addition to protecting against inactivation, isocitrate prevents binding of the reagent.

Explanations for the less than integral value obtained for iodoacetate binding will be considered under “Discussion.” When the enzyme is first denatured in 4 M urea and then incubated with 14C-labeled reagent, it binds 10 moles of radioactive iodoacetate per mole of enzyme. The limited reaction of the alkylating agent with isocitrate dehydrogenase thus requires the native configuration of the protein.

**Identification of Methionine as Site of Alkylation**

**Amino Acid Analysis**—Amino acid analysis, reported previously (11), indicates that the methionine content of enzyme alkylated at pH 5.6 is decreased by 1 residue as compared to native enzyme, and a new peak is observed, that of carboxymethylmethionine, the chief acid decomposition product of carboxymethylmethionine sulfonylum salt. No other differences were noted between the amino acid composition of the native and alkylated enzymes. In particular, the histidine, lysine, tyrosine, glutamate, and aspartate contents were essentially the same. Carboxymethylation of the dicarboxylic acids, a possible reaction at pH 5.5 (23), could not be determined in an acid hydrolysate, since any esters present would be hydrolyzed; however, the decrease in the methionine content can entirely account for the extent of incorporation of radioactive iodoacetate by the enzyme.

**Sulfhydryl and Aromatic Amino Acid Content**—The sulfhydryl content of the enzyme was measured by reaction with 5,5'-dithiobis(2 nitrobenzoic acid) (Ellman’s reagent) at pH 5.6 after denaturation in 0.2% sodium dodecyl sulfate. Enzyme alkylated at pH 5.5 and dialyzed to remove excess reagent was found to contain 12.5 sulfhydryl groups per molecule as compared to 11.9 for the native enzyme. Iodoacetate does not, therefore, appear to attack the cysteine residues under these conditions.

The tryptophan content was measured spectrophotometrically by the method of Beaven and Holiday (24), except that the solvent was 0.1 M NaOH-7 M urea. Use of the standards N-acetyltyrosilamine and N-acetyltryptophanamide led to values of 6.3 and 6.1 moles of tryptophan for the native and alkylated enzymes, respectively.

The absorption spectra of native and alkylated enzymes were compared at protein concentrations which were determined to be identical by means of the biuret reaction. No difference spectrum was observed from 240 to 350 m at neutral pH, indicating that the aromatic residues, in particular tyrosine and tryptophan, are not significantly altered by iodoacetate.

**Proteolytic Digests of Alkylated Enzyme**—Confirmation of the assignment of methionine as the site of carboxymethylation
Paper chromatography of proteolytic digests of 14C-alkylated enzyme

Paper chromatograms were developed by the ascending method for 18 hours in a solvent system consisting of phenol-water (4:1) with 0.3% NH₄OH in a separate beaker. Spots were located by reaction with ninhydrin or by the presence of radioactivity as detected by the Vanguard Autoscanner.

<table>
<thead>
<tr>
<th>Probable compound</th>
<th>14C-Alkylated enzyme hydrolysate</th>
<th>14C-Alkylated methionine</th>
<th>Nonradioactive standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radioactive spots, RF</td>
<td>Radioactivity, RF</td>
<td>Ninhydrin, RF</td>
</tr>
<tr>
<td>CH₃SCH₂COO—</td>
<td>0.20</td>
<td>0.21</td>
<td>0.26 (glutamic acid)</td>
</tr>
<tr>
<td>S-Methylthioglycolic acid</td>
<td></td>
<td></td>
<td>0.29 (carboxymethylcysteine)</td>
</tr>
<tr>
<td>S—CH₂COO—</td>
<td>0.37*</td>
<td>0.38*</td>
<td>0.34 (carboxymethylhomocysteine)</td>
</tr>
<tr>
<td>(CH₃)₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄CH COO—</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxymethylhomocysteine</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| OH
| (CH₃)₂ | 0.54                           | 0.53 (homoserine)        |
| NH₄CH COO—       |                                 |                          |                         |
| Homoserine        |                                 |                          |                         |
| CH₃S—CH₂COO—     | 0.68                            | 0.69                     | 0.69 (carboxymethylmethionine) |
| (CH₃)₂ |                                 |                          |                         |
| NH₄CH COO—       |                                 |                          |                         |
| Carboxymethylmethionine sulfonium salt | 0.76 (methionine) | 0.76 (methionine) |

* Minor component, less than 5% of the total.

comes from analyses of proteolytic digests of 14C-labeled alkylated enzyme. The instability of sulfonium salts makes very difficult the isolation of a single alkylated derivative of a sulfur-containing compound. Fig. 8 outlines the major decomposition products of carboxymethylmethionine as given by Gundlach, Moore, and Stein (25), with a designation of those which react with ninhydrin and those which are radioactive when the original compound was prepared with iodoacetate-1-14C.

Modified isocitrate dehydrogenase was exposed to Pronase for 48 hours at 40°, followed by treatment with carboxypeptidases A and B. Hydrolysis, as monitored by reaction of the digest with ninhydrin, was approximately 83% complete. Digests were desalted on Dowex 50, concentrated, and fractionated on a column of Sephadex G-10 (1 × 38 cm) by the procedures described previously (11). A single radioactive peak was found for the hydrolysate at 11.7 ml, whereas carboxymethyltyrosine, which is adsorbed by Sephadex, was eluted at 18.8 ml. The probability of direct modification by iodoacetate of tyrosyl residues of isocitrate dehydrogenase is thus extremely small.

Paper chromatography of the peak radioactive fractions was conducted with appropriate standards, as recorded in Table IV. The carboxymethylmethionine derivative yields three ninhydrin spots and three peaks of radioactivity. The major ninhydrin and radioactive peaks have RF values of 0.69 and undoubtedly represent carboxymethylmethionine sulfonium salt. The ninhydrin spot with an RF of 0.54 is identical in RF with authentic homoserine and, as expected, it is nonradioactive. It is impossible to identify this product in the chromatogram of the hydrolysate, since its RF is close to that of many amino acids which might still be present. The radioactive fragment which must be produced concomitantly with the generation of homoserine is S-methylthioglycolic acid, which is probably represented by the radioactive, ninhydrin-negative spot with an RF of 0.21. In addition, a minor radioactive, ninhydrin-positive species is found with an RF of 0.38, which corresponds reasonably well with 0.34 found for synthetic carboxymethylhomocysteine.

Electrophoresis of Alkylated Enzyme—Also consistent with methionine as the site of alkylation is the observation of identical mobilities of native and alkylated enzymes on cellulose acetate electrophoresis from pH 6.2 to 0.5 in phosphate, Tris, and glycine buffers. A single band without significant broadening was always noted for partially alkylated enzymes. The pattern of radioactivity obtained from the hydrolysate agrees well with that of the methionine derivative. The major peak, representing approximately 70% of the total radioactivity, is that with RF 0.68.
TABLE V

Effect of ionic strength on inactivation by iodoacetate at pH 6.5

In these experiments, 2 mg of isocitrate dehydrogenase per ml were incubated at 30° in potassium phosphate buffer, pH 6.58, with 9.6 × 10⁻⁶ M iodoacetate. The ionic strength could be established equivalently by varying the concentration of sodium chloride, sodium sulfate, or potassium phosphate buffer. The rate constants given are pseudo-first order constants.

<table>
<thead>
<tr>
<th>Ionic strength</th>
<th>k (10⁻⁶ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0270</td>
<td>11.1</td>
</tr>
<tr>
<td>0.0552</td>
<td>11.9</td>
</tr>
<tr>
<td>0.130</td>
<td>10.4</td>
</tr>
<tr>
<td>0.205</td>
<td>11.1</td>
</tr>
<tr>
<td>0.325</td>
<td>10.8</td>
</tr>
<tr>
<td>0.505</td>
<td>11.1</td>
</tr>
</tbody>
</table>

TABLE VI

Histidine and tyrosine content of native and alkylated enzymes

In these experiments, enzyme aliquots were diluted with 1 M KHCO₃, pH 8.8, to 0.8 ml. In accordance with Sokolovsky and Vallee (29), 0.2 ml of diazonium-1H-tetrazole (0.14 M), generated immediately before use by diazotization of 5-amino 1H-tetrazole with sodium nitrite, was added at zero time, and a second 0.2 ml of reagent solution was added after 10 min. The absorbances at 480 and 550 nm were measured after the reaction mixture had been allowed to stand 90 min at room temperature. The final protein concentrations were approximately 8 × 10⁻⁶ M. Alkali denaturation, used where indicated, involved prior incubation of the enzyme in 0.5 M KOH for 30 min at room temperature. Automatic amino acid analysis was conducted on enzyme samples hydrolyzed in 6 N HCl in sealed, evacuated tubes at 110° for 18 hours.

<table>
<thead>
<tr>
<th>Method</th>
<th>Histidine</th>
<th>Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>Alkylated</td>
</tr>
<tr>
<td></td>
<td>residues/molecule</td>
<td>residues/molecule</td>
</tr>
<tr>
<td>Diazo reaction (no denaturation)</td>
<td>6.49</td>
<td>6.28</td>
</tr>
<tr>
<td>Diazo reaction (after alkali denaturation)</td>
<td>7.60</td>
<td>7.65</td>
</tr>
<tr>
<td>Amino acid analysis</td>
<td>12.2</td>
<td>12.2</td>
</tr>
</tbody>
</table>

whereas carboxymethylation of methionine produces the dipolar sulfonium salt with no resultant alteration in the net charge. The question may always be raised as to whether it would be possible to detect a change of 1 charge by these methods. However, examples of such distinctions abound in the literature; e.g. hemoglobins S and A, which differ by only 2 charges per molecule, are readily distinguished by electrophoresis (26), as are native and acetylated glutamate dehydrogenase, which differ by 1 charged group per peptide chain (27).

Effect of Ionic Strength on Alkylation—The effect of ionic strength on the rate of inactivation of isocitrate dehydrogenase at pH 5.58 further supports the designation of methionine as the site of attack. If the negatively charged iodoacetate reacts with a negatively charged functional group on the enzyme, the rate constant should increase with ionic strength; if reaction is with a positively charged group, the rate constant should decrease with ionic strength, and if reaction is with an uncharged species, no change with ionic strength would be expected (28). The results shown in Table V indicate that the rate of reaction of enzyme with iodoacetate is independent of ionic strength at this pH, strongly implicating an uncharged reactant, such as methionine.

Histidine and Tyrosyl Reactivity—The reactivity of the histidyl and tyrosyl residues of native and alkylated enzymes toward diazonium-1H-tetrazole was investigated in accordance with the methods of Sokolovsky and Vallee (29). Since carboxymethyl-histidine does not participate in this diazo coupling reaction, this reaction may be used as a measure of the extent of modification of these residues. The content of reacting histidine residues is the same for native and alkylated enzymes, as shown in Table VI, again indicating that these groups are not carboxymethylated by iodoacetate. These values, however, are considerably lower than the total histidine content of 12.2 moles per mole of enzyme observed on the amino acid analyzer. The implication is that diazonium-1H-tetrazole can react with only a certain fraction of the histidyl residues, perhaps determined by the protein conformation.

Differences are observed in the reactivity of tyrosyl residues of native and alkylated enzymes. Table VI records a decrease upon alkylation of 1 to 2 tyrosyl residues per molecule of enzyme which react with the diazonium salt. It is notable that no more than half of the total tyrosyl residues of the enzyme combine with the diazonium salt, even in the native enzyme, and it appears, as in the case of histidine, that only certain residues are available for reaction with diazonium-1H-tetrazole. In view of the overwhelming evidence against direct carboxymethylation of tyrosyl groups by iodoacetate (amino acid analysis, ultraviolet absorption spectrum, analysis of proteolytic digests) and the indication that the diazo coupling reaction is sensitive to protein conformation, the decrease in reactivity of 1 to 2 tyrosyl residues in the alkylated enzyme can most reasonably be ascribed to steric hindrance introduced by carboxymethylation of a proximal residue, or to local conformational changes (too slight to be observed by optical rotatory dispersion) which are caused by carboxymethylation at another site.

DISCUSSION

Both the oxidation of isocitrate and the decarboxylation of oxalosuccinate have long been recognized as dual catalytic aspects of the intrinsic activity of isocitrate dehydrogenase. However, controlled treatment of the enzyme with iodoacetate, which produces alkylation of a single amino acid residue, results in a greater decrease in the dehydrogenase activity than in the decarboxylase activity. The active sites for these two reactions must therefore be at least partially separable. Since the dehydrogenase activity falls to zero, the specific residue that has been modified must participate directly in the dehydrogenase reaction. Alkylated enzyme, which lacks dehydrogenase activity, still exhibits considerable ability to catalyze decarboxylation. A reasonable explanation is that the affected functional group does not lie within the active site for decarboxylation, but that its alteration causes an indirect effect on the decarboxylase reaction. Perhaps this phenomenon is mediated through a local change in the conformation of the enzyme or through the influence of the increased ionic character of the alkylated residue on the reactivity of functional groups which are essential for decarboxylation. It is apparent that, under conditions of assay which measure the
dehydrogenation of isocitrate as the rate-determining step, the observed loss of activity will be directly proportional to the extent of alkylation. Under conditions in which the oxidation-reduction step is relatively fast, as in the measurement of the rate of reductive carboxylation, the extent of inactivation will appear to be less, since this enzymatic rate reflects initially the indirect effect on carboxylation. Only as a result of extensive alkylation could one expect a change in the rate-limiting step of the over-all reductive carboxylation reaction.

The observed loss of activity upon alkylation cannot be attributed to a shift in the pH dependence of the dehydrogenase reaction, since the pH-maximum rate profile is essentially identical for native and partially modified enzymes. Similarly, the inactivation cannot be described in terms of weaker binding of substrates by an intrinsically active modified enzyme, since the Michaelis constants for the substrates are unaltered in partially active, alkylated enzymes. It is likely, in fact, that the measurable activity in incompletely alkylated preparations is attributable to unmodified enzyme.

It should be re-emphasized that the changes observed on alkylation are the result of a specific modification reaction rather than generalized denaturation. Enzyme in the presence of iodoacetate is inactivated under conditions in which enzyme in the absence of reagent remains fully functional. Furthermore, substrates protect against inactivation when added to the enzyme together with iodoacetate. Extensive structural alterations in the modified enzyme are excluded by the lack of change in the optical rotatory dispersion spectrum and molecular weight, and by the similarity of the ultraviolet absorption spectra of native and alkylated enzymes.

Significant protection against loss of activity by iodoacetate treatment is provided by isocitrate, indicating that reaction takes place at the active site of the enzyme. It is notable in this regard that α-ketoglutarate partially prevents inactivation by alkylation. Binding of these two substrates at similar sites on the enzyme is suggested, with the implication that the β-carboxylate group of isocitrate is not necessarily involved in binding to the site altered by iodoacetate. These observations also constitute evidence that the carboxylic acid substrates bind to the enzyme in the absence of coenzyme. Ramakrishna and Krishnaswamy (30) have presented data which suggest the formation of an isocitrate dehydrogenase-carbon dioxide complex, and Fisher and Cross (31) have proposed the existence of an enzyme-TPNH complex in the absence of substrates. In these cases, it is not clear whether the binding constants are comparable to those observed kinetically; however, the sum of these data suggests a mechanism for isocitrate dehydrogenase involving random order of addition of substrates to the enzyme. The kinetics of the TPN dependent isocitrate dehydrogenase appears to conform to this mechanism (32).

The pH dependence of Vmax for isocitrate dehydrogenase yields a pK of 5.4, which increases when the reaction is conducted in 20% ethanol. Extreme caution must be exercised in attempting to identify any measured ionization constant with a particular amino acid residue. The range of pH within which these groups dissociate may be significantly altered by other functional groups within the protein environment; furthermore, in a complex reaction involving more than one enzyme-substrate intermediate, the apparent ionization constant may include terms for any equilibrium reaction prior to the rate-determining step (33). In addition, the effect of a mixed solvent on enzymatic activity may be subject to several interpretations; for example, it may promote a structural change, or it may differentially influence individual steps in the reaction sequence. In the case of isocitrate dehydrogenase, irreversible structural changes have been excluded, and any solvent-induced changes in the binding constants for substrates are not pertinent, since the reaction was examined only under conditions of substrate saturation. The simplest interpretation of the data for isocitrate dehydrogenase is that a protein carboxylate ion is involved in the enzymatic reaction. It may be the same carboxylate ion which causes electrostatic repulsion of the negatively charged iodoacetate in its reaction at an adjacent site, and which is responsible for the enhanced rate of inactivation below pH 6.0, as it is protonated.

In considering the site of alkylation of the enzyme, the only amino acid residue consistent with all the data is methionine. The conversion of an uncharged thioether group to dipolar carboxyethylmethionine accounts for the lack of alteration in the electrophoretic mobility, as well as for the lack of dependence of the inactivation rate on ionic strength. A decrease of 1 methionine residue per molecule of protein and the appearance of carboxyethylhomocysteine were noted in amino acid analyses of alkylated enzyme, and carboxyethylmethionine and its degradation products were identified in paper chromatograms of proteolytic digests of alkylated enzymes. The instability of the sulfonium salts is probably responsible for the low value obtained for moles of 14C-labeled iodoacetate bound per mole of protein (0.75). Approximately 70% of the recovered radioactivity was found in the major spot of the paper chromatograms of the alkylated enzyme digests, carboxyethylmethionine sulfonium salt (Table IV). This major product would be enzyme-bound in the undegraded, alkylated enzyme and presumably is responsible for the observed value of 0.75 mole of 14C incorporated per mole of protein. The radioactive degradation products (Fig. 8), S-methylthioglycolic acid and glycolic acid, are small molecules and were undoubtedly removed from the enzyme by gel filtration on Sephadex G-25 in the 14C-iodoacetate-binding experiments.

Methionine has frequently been thought to fulfill a structural role in proteins; however, a direct role in the catalytic process has been assigned to this amino acid in but a few enzymes. In ribonuclease, for example, alkylation of a methionine causes an inability of the enzyme or of its S-peptide to refold into native conformation (34, 35); in chymotrypsin, modification of a methionyl residue results in increased Michaelis constants for the substrates without altering the ability of the enzyme to catalyze the hydrolysis of bound substrates (36, 37); and in Pseudomonas cytochrome c, methionine has been implicated in the coordination of the heme iron (38). Some, although not conclusive, evidence exists for the participation of methionine in the catalytic function of phosphoglucomutase (39), myokinase (40), and peroxidase (41). The data presented in this paper show that 1 methionine residue in isocitrate dehydrogenase is essential for activity and that its alkylation leads to loss of activity without marked structural change in the enzyme. However, any detailed mechanism would be premature at this level of our knowledge of the active site of the enzyme.

The specific nature of the reaction between isocitrate dehydrogenase and the usually nonspecific reagent iodoacetate deserves comment. In selecting particular reagents which might react with amino acid residues involved in the catalytic function of an enzyme it is, in general, preferable to use reagents which are most
Alkylation of Methionyl Residue of Isocitrate Dehydrogenase

Vol. 243, No. 10

specific for the particular functional group under scrutiny. However, highly reactive compounds can be rendered more specific by appropriate choice of pH or by conducting the reaction in a buffer which competes with the protein for the reagent (27, 42). In the case of isocitrate dehydrogenase, the specificity of reaction with iodosuccinate can probably be attributed to the combination of low pH, which effectively excludes reaction with typical sulfhydryl and amino groups, and to the native conformation of the enzyme, which must mask other groups normally reactive at this pH.

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REFERENCES

14. Warburg, O., and Christian, W., Biochim. Z., 310, 384 (1941).
42. Disbratou, C., Biochemistry, 4, 2988 (1965).
Effect of Modification of a Methionyl Residue on the Kinetic and Molecular Properties of Isocitrate Dehydrogenase
Roberta F. Colman


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