An Investigation of Imine Formation in the Isocitrate Dehydrogenase Reaction*

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SUMMARY

The occurrence of imine formation at the active site of acetoacetate decarboxylase and several aldolases prompted an attempt to demonstrate this mechanism in the formally similar isocitrate dehydrogenase reaction. Although treatment of isocitrate dehydrogenase with NaBH₄ in the presence of α-ketoglutarate resulted in inactivation of the enzyme, reduction in the presence of labeled α-ketoglutarate did not yield labeled protein, nor was inactivation dependent on NADPH which is required for the enzymic β-H exchange of α-ketoglutarate.

A study of the isocitrate dehydrogenase reaction in H₂¹⁸O was made possible by the observation that decarboxylation of α-ketoglutarate by H₂O₂ was faster than spontaneous exchange of the keto group with the medium. It was shown that succinate produced from enzymically formed α-ketoglutarate contained considerably less than 1 atom of ¹⁸O. It was concluded that this finding is not compatible with a mechanism of imine formation between keto intermediates (oxalosuccinate) and amino groups at the active site, since decarboxylation and hydrolysis of the imine to release α-ketoglutarate would have introduced 1 atom of ¹⁸O.

Studies with various aldolases have shown that imine formation between a keto substrate and an ε-amino group of a lysine residue of the enzyme is an obligatory step in enzyme-catalyzed aldol condensation reactions (1-5). Evidence has also been reported that in the enzymic decarboxylation of acetoacetate a Schiff base is formed between an ε-amino group of a lysine residue of acetoacetate decarboxylase and substrate (6-8). Isocitrate dehydrogenase shares with the aldolases the formation of a carbanion which exchanges stereospecifically with a proton from the medium (9-11). In addition it can act as β-keto acid decarboxylase for oxalosuccinate (12). It was therefore of interest to determine whether imine formation is involved in the over-all conversion of isocitrate to α-ketoglutarate. This paper reports a study of nicotinamide adenine dinucleotide phosphate-linked isocitrate dehydrogenase showing that imine formation with enzyme-bound oxalosuccinate is not obligatory to decarboxylation.

EXPERIMENTAL PROCEDURE

Materials—Two NADP-linked isocitrate dehydrogenase preparations were used. A freeze-dried pig heart preparation was obtained from Sigma and was used for most of the experiments. An NADP-linked isocitrate dehydrogenase was partially purified from an acetone powder of beef heart mitochondria as described by Chen and Plaut (13). The enzyme was purified through the first ammonium sulfate precipitation step. Both enzymes had a specific activity of 0.61 to 0.73 μmol per min per mg when assayed as described below. Unless otherwise stated the pig heart preparation was used.

Monopotassium three-α-isocitrate was a generous gift from Dr. H. B. Vickery. α-Ketoglutarate-5-¹⁴C (specific activity, 6.94 mC per mmole) was obtained from Calbiochem, and bovine liver catalase (two times crystallized) from Sigma. All other reagents and chemicals were commercial preparations and were used without further purification. Glass-distilled water was used throughout.

Methods—Isocitrate dehydrogenase was assayed in a 1.0-ml reaction mixture containing 30 mM triethanolamine-HCl buffer, pH 7.3, 8 mM MgSO₄, 4 mM dL-isocitrate, 0.2 mM NADP, 0.5 mM EDTA, and 1 mg of bovine serum albumin. NADP reduction was measured spectrophotometrically at 340 nm. Enzymic activity was expressed as micromoles of NADPH formed per min per mg of protein with the use of the extinction coefficient, 6.22 × 10⁶ M⁻¹ cm⁻¹.

For the determination of ¹⁸O in succinic acid, samples (6 mg) were pyrolyzed at 450° in sealed evacuated tubes with 150 mg of HgC₂ and 20 mg of Hg(CN)₂ according to the method of Rittenberg and Ponticorvo (14), and the resulting CO₂ was analyzed mass-spectrometrically. Aliquots of H₂¹⁸O (0.5 ml) were equilibrated with 1 ml of CO₂ in sealed tubes, heated at 100° for 2 to 3 hours (15), and the CO₂ was analyzed. Protein was determined by the method of Lowry et al. (16).

Studies of Isocitrate Dehydrogenase with NaBH₄—Pig heart isocitrate dehydrogenase (2.5 mg) was incubated at 0° for 5 min with 20 μmoles of α-ketoglutarate, 0.5 μmole of NADPH, 20 μmoles of MgSO₄, 1 μmole of EDTA, 0.01 ml of octanol, and 100 μmoles of buffer (potassium phosphate, pH 6.0, and triethanol-
amine-HCl, pH 7.0 and pH 8.0), in a final volume of 0.62 ml. Each solution was treated at 0⁰C, over a period of 15 min, with 0.3 ml of freshly prepared 2.0 M NaBH₄ in 0.05-nl portions; the pH was maintained by the addition of 2.0 M acetic acid. The initial NaBH₄ concentration was approximately 0.5 M. Similar incubations were performed with 3.5 mg of the beef heart isocitrate dehydrogenase preparation. Reaction mixtures were allowed to stand for 15 min at 0⁰C following the last addition of NaBH₄, and were dialyzed for 2 hours against 100 ml of a solution containing 0.1 M KCl, 10 mM triethanolamine-HCl, pH 7.3, 2.0 mM α-ketoglutarate, and 0.1 mM EDTA, with one change of the dialysis solution. Enzymic activity and protein content were then determined on appropriate aliquots.

**Incubation of Isocitric Dehydrogenase with α-Ketoglutarate-5-14C**—Reaction mixtures and incubation conditions were similar to those with unlabeled α-ketoglutarate at pH 8 (Table I, Experiment 2), except that 5 to 10 mg of enzyme were used and the amount of α-ketoglutarate was 0.2 μmole (0.8 x 10⁵ to 1.4 x 10⁷ cpm). Following treatment with NaBH₄, reaction mixtures were dialyzed against 1000 volumes of 1 mM EDTA solution, the latter being changed three times during a 20-hour period. Protein was precipitated with trichloroacetic acid, removed by centrifugation, and dissolved in NaOH. The precipitation and solubilization steps were repeated two to three times. Aliquots of the protein solution were plated on planchets, dried, and counted in a low background gas flow counter (Nuclear-Chicago).

**Isolation and Purification of Succinic Acid from Isocitrate Dehydrogenase Reaction in H₂¹⁸O**—A solution containing 0.6 mmole of three-nmol isocitrate, 1 mmole of Tris-HCl, pH 7.4, 0.3 mmole of MgSO₄, 2 μmoles of NADP, and 10 μmoles of EDTA was freeze-dried to remove H₂O. To a solution of the residue in 19.6 ml of H₂¹⁸O, were added 20 μmoles of phenazine methosulfate, 1.2 mmoles of H₂O₂ (0.12 ml of a 30% solution), and 30 mg of isocitrate dehydrogenase (final volume, 20 ml). After incubation for 3 hours at 30⁰C with shaking, 5 μl (0.1 mg) of catalase were added, the solution was kept at room temperature for 5 min, and the H₂¹⁸O was removed by freeze-drying. Succinic acid was isolated and purified as described below.

**Table I**

*Treatment of isocitrate dehydrogenase with NaBH₄*

The reaction components and conditions are given in the "Experimental Procedure." Experiments 1 and 2 were done with the pig heart enzyme, and Experiment 3 with the beef heart enzyme.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>pH</th>
<th>Addition</th>
<th>Treatment with NaBH₄</th>
<th>Specific activity (μmole/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>α-Ketoglutarate</td>
<td>+ 0.32</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>α-Ketoglutarate</td>
<td>+ 0.11</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.0</td>
<td>α-Ketoglutarate</td>
<td>+ 0.035</td>
<td></td>
</tr>
</tbody>
</table>

In the control reaction a solution containing 5 mmoles of Tris-HCl, pH 7.4, 0.3 mmole of MgSO₄, and 10 μmoles of EDTA was freeze-dried to remove H₂O. A solution of the residue in 19.5 ml of H₂¹⁸O was treated with 1.2 mmoles of H₂O₂ (final volume, 20 ml). Solid α-ketoglutaric acid (0.8 μmole) was added slowly over a 2-hour period at room temperature with rapid stirring, the pH being maintained at 7.4 by the addition of approximately 0.14 ml of 10 M NaOH. Catalase (5 μl) was added and the H₂¹⁸O was removed by freeze-drying. Succinic acid was isolated and purified as described below.

To study the decarboxylation of α-ketoglutarate-2-¹⁸O a solution of 2.4 mmoles of disodium α-ketoglutarate and 5 mmoles of Tris-HCl, pH 7.4, was freeze-dried, and the residue was equilibrated with 40 ml of H₂¹⁸O for 20 hours at room temperature. The solution was treated with 0.49 ml of 30% H₂O₂ (4.8 mmoles), and, after 1 hour at room temperature, with 5 μl of catalase. After the removal of H₂¹⁸O by freeze-drying, 148 mg (1.3 mmoles) of succinic acid were isolated as described below, and a sample was analyzed for H₂¹⁸O. A solution of 124 mg of this product in 5 ml of H₂O was neutralized with NaOH, and added to 20 ml of 0.25 M Tris-HCl, pH 7.4. Succinic acid (60 mg) was reisolated and repurified.

For isolation of succinic acid the residue obtained after freeze-drying was treated with 20 ml of H₂O, insoluble protein was removed by centrifugation, and the supernatant solution was applied to a column (1.8 X 11 cm) of Dowex 1-X8 (formate form, 200 to 400 mesh). The column was washed with 20 ml of H₂O, and eluted with 1.2 M formic acid; 10-ml fractions were collected. Succinic acid usually appeared in Fractions 6 to 10. The pooled succinic acid fractions were taken to dryness at 40-50⁰C in a rotary evaporator, and the product was purified by two crystallizations from tetrahydrofuran-benzene. The melting point of the succinic acid samples was 188-190⁰C (uncorrected).

**Decarboxylation of α-Ketoglutarate by H₂¹⁸O**—Reaction mixtures containing 10 μmoles of α-ketoglutarate, 50 μmoles of Tris-HCl buffer, pH 7.4, and 10, 20, 30, and 40 μmoles, respectively, of H₂¹⁸O, in a final volume of 1 ml, were incubated at 30⁰C. Aliquots were removed at 30-sec intervals and added to 0.01 ml of catalase (diluted 1:10). Residual α-ketoglutarate was determined with 2,4-dinitrophenyldrazine (17), and rate constants for the decarboxylation were calculated.

**RESULTS**

*Treatment of Isocitrate Dehydrogenase with NaBH₄*—Inactivation by treatment with NaBH₄ in the presence of appropriate substrates has been demonstrated for several aldolases (1-5) and acetocetate decarboxylase (7), and has been attributed to reduction of an enzyme-substrate Schiff base at the active site. Optimal conditions were sought for a similar inactivation of isocitrate dehydrogenase in the presence of α-ketoglutarate, NADPH, and Mg++. It may be seen from Table I, Experiment 1, that at pH 6.0 treatment with NaBH₄ had relatively little effect on enzyme activity whereas at pH 7.0 and pH 8.0 inactivation was observed. In the absence of NaBH₄ the enzyme was stable at pH 6 to 8. Further experiments were performed at pH 7.0 and pH 8.0, and attempts were made to demonstrate an α-ketoglutarate-dependent inactivation of isocitrate dehydrogenase by NaBH₄. Since the pig heart isocitrate dehydrogenase preparation was unstable without substrate, control experiments with NADPH and α-ketoglutarate were always included.
The forward reaction, which required NADP for isocitrate oxidation, could not be studied with NaBH₄ since NADP is rapidly reduced by this reagent. Representative results at pH 8 (Table I, Experiment 2) indicated that 0.5 mM NaBH₄ completely inactivated the enzyme in the presence of α-ketoglutarate, as compared to a control without NaBH₄. Experiment 3 was done with an isocitrate dehydrogenase preparation that was stable at 0°C in the absence of substrate, thus providing a control for the effect of NaBH₄ alone on the enzyme. Treatment with NaBH₄ in the absence of α-ketoglutarate resulted in a 19% inactivation, whereas the same treatment in the presence of α-ketoglutarate resulted in a 58% inactivation. With both preparations of enzyme, inactivations obtained with NaBH₄ were variable. Treatment of the enzyme with NaBH₄ in the presence of α-ketoglutarate yielded inactivations 0 to 48% greater than those obtained in the absence of isocitrate or in the absence of any substrate. Variations in the concentration of NaBH₄, the type of buffer, or changes in pH did not improve the reproducibility. Furthermore, it was found that NADPH was not required for the apparent α-ketoglutarate-dependent inactivation by NaBH₄.

In order to determine if the α-ketoglutarate-dependent inactivation by NaBH₄ was due to reduction of a Schiff base formed between enzyme and substrate, experiments were performed with α-ketoglutarate-5-¹⁸O. Following NaBH₄ treatment, dialysis, and precipitation with acid, the amount of radioactivity bound to protein was no greater than in control experiments where NaBH₄ was omitted. In both cases approximately 0.005% of the added radioactivity remained with the enzyme. This corresponds to less than 0.01 mole of α-ketoglutarate per mole of enzyme.

Isocitrate Dehydrogenase Reaction in H₂¹⁸O—Formation of a Schiff base between an amino group of isocitrate dehydrogenase and enzyme-bound oxalosuccinate resulting from isocitrate, and its subsequent decarboxylation, requires that the α-ketoglutarate liberated by hydrolysis from the enzyme contain ¹⁸O in the α-keto group when the reaction takes place in H₂¹⁸O. Since ketones and aldehydes undergo a rapid nonenzymic exchange with H₂¹⁸O (18) it is necessary to remove the α-ketoglutarate as soon as it is formed in the reaction mixture. Two methods were tried: reduction with NaBH₄ to yield α-hydroxyglutaric acid; and oxidation with H₂O₂ to give succinic acid. In the first method, destruction of borate esters with methanol, and removal of Na⁺ with Dowex 50 failed to yield crystalline α-hydroxyglutaric acid. Similar difficulties have been reported in the isolation of lactic acid from the reduction of pyruvate by NaBH₄ (19). However, oxidation of α-ketoglutarate with H₂O₂ proceeded smoothly and gave crystalline succinic acid. The rate of α-ketoglutarate oxidation (see “Experimental Procedure”) was proportional to the molar ratio of H₂O₂ to keto acid. For H₂O₂ to α-ketoglutarate ratios of 1:1, 2:1, 3:1, and 4:1 rate constants of 0.18 min⁻¹, 0.37 min⁻¹, 0.51 min⁻¹, and 0.69 min⁻¹ were observed. The presence of 0.06 M H₂O₂ did not interfere with the activity of isocitrate dehydrogenase, although some inhibition was observed with 0.12 M H₂O₂.

The results of studies of the isocitrate dehydrogenase reaction in H₂¹⁸O are summarized in Table II. In the first experiment the H₂O₂ concentration was 0.06 M and the initial H₂O₂ to isocitrate ratio was 2:1. In the presence of phenazine methosulfate (PMS) both NADP and H₂O₂ were regenerated according to the following reactions:

$$\text{NADPH} + \text{H}^+ + \text{PMS} \rightarrow \text{NADP}^+ + \text{PMSH}_2$$

$$\text{PMSH}_2 + \text{O}_2 \rightarrow \text{PMS} + \text{H}_2\text{O}_2$$

The 0.06 M H₂O₂ concentration should therefore remain relatively constant during the incubation. The ratio of H₂O₂ to enzymically formed α-ketoglutarate would be considerably higher than 2:1, and would depend on the steady state concentration of α-ketoglutarate. Succinic acid derived from this α-ketoglutarate contained 0.20 atom % excess ¹⁸O, and the medium water, 1.76 atom % excess; this is equivalent to an average incorporation of (4 x 0.0020) = 0.008 atom % ¹⁸O. Since, a maximum of only 45% of the α-ketoglutarate exchanged with H₂¹⁸O, whereas isotope formation and hydrolysis would require 100% exchange. Even the 45% labeling can be accounted for by spontaneous exchange of the keto group of α-ketoglutarate with H₂¹⁸O, as shown by the control for Experiment 1. Solid α-ketoglutaric acid was added to a reaction mixture similar to the one containing isocitrate dehydrogenase, and 0.58 atom of ¹⁸O was found in the succinate. In the control experiment the H₂O₂ concentration was initially 0.06 M and was 0.08 M at completion because a regenerating system could not be employed.

Experiment 2 of Table II was carried out with 0.12 M H₂O₂ and a ratio of H₂O₂ to isocitrate of 4:1. In the control experiment the initial H₂O₂ concentration was 0.15 M and the final concentration was 0.12 M. It is clear that only 0.29 to 0.28 atom of oxygen were exchanged in both the enzymic reaction and the control when higher relative concentrations of H₂O₂ were used, thus minimizing the time available for spontaneous exchange of α-ketoglutarate.

The ¹⁸O found in succinic acid was not introduced from the medium during decarboxylation of the α-keto acid, as shown by Experiment 3. α-Ketoglutarate was allowed to equilibrate completely with H₂¹⁸O, and was decarboxylated with H₂O₂ as in

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>H₂¹⁸O found in</th>
<th>%O in α-ketoglutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>atom % excess</td>
<td>atoms</td>
</tr>
<tr>
<td></td>
<td>Succinic acid</td>
<td>Succinic acid</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1.76</td>
<td>0.20</td>
</tr>
<tr>
<td>With enzyme</td>
<td>1.81</td>
<td>0.26</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.58</td>
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<tr>
<td>Experiment 2</td>
<td>1.43</td>
<td>0.10</td>
</tr>
<tr>
<td>With enzyme</td>
<td>1.40</td>
<td>0.08</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>1.41</td>
<td>0.37</td>
</tr>
<tr>
<td>Decarboxylation of α-ketoglutarate-2-¹⁸O</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>Succinic acid (from above) repurified</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.03</td>
<td></td>
</tr>
</tbody>
</table>

We are grateful to Dr. K. R. Hanson for suggesting the use of this reagent (20).
the previous experiments. If $^{18}O$ had entered the succinate during decarboxylation, more than 1 oxygen atom per molecule of succinic acid would have been introduced. However, only 1 atom of $^{18}O$ was found per molecule of succinic acid, as expected from incorporation of 1 atom of oxygen by exchange into $\alpha$-ketoglutarate, and 1 atom from $H_2O_2$ during formation of the new carboxyl group. The results in Experiment 3 also show that there is no loss of $^{18}O$ from succinic acid during purification. Therefore, the low $^{18}O$ content observed for this compound in Experiments 1 and 2 cannot be attributed to this possibility.

**DISCUSSION**

Several observations suggested that the enzymic decarboxylation of oxalosuccinate might involve amine catalysis. (a) Catalysis by primary amines has been found in the nonenzymic decarboxylation of $\beta$-keto acids, including oxalosuccinic acid (21, cf. Reference 22). (b) Schiff base formation between enzyme and $\alpha$-ketoglutarate has been reported in the decarboxylation reaction catalyzed by $\alpha$-ketoglutaric dehydrogenase (6-8). (c) The mechanism of several aldolase reactions has been shown to involve Schiff base formation between a lysine group of the enzyme and keto group of a substrate (1-5). Carboxylations are analogous to aldol condensations in which the electrophilic carbonyl component is carbon dioxide, and decarboxylations are the reverse (29). (d) The stereochemistry of the isocitrate dehydrogenase reaction (10, 23) and of the aldolase reaction (24) is similar. In both, carbon-carbon bond cleavage is followed by protonation with retention of configuration. (e) Both isocitrate dehydrogenase and aldolase catalyze stereospecific proton exchange between the appropriate keto substrate and the solvent (9-11).

In the present study on the mechanism of isocitrate dehydrogenase, we therefore wished to inquire if the NADP-dependent oxidation of isocitrate gave rise to oxalosuccinate bound to enzyme by an amino group in Schiff base linkage. Since the isocitrate dehydrogenase reaction is reversible (25), and a partial reaction, stereospecific exchange of a $\beta$-H atom of $\alpha$-ketoglutarate, has been studied in detail (9, 10), attempts were made to reduce enzyme-substrate complexes of isocitrate dehydrogenase and $\alpha$-ketoglutarate. $NaBH_4$ treatment of isocitrate dehydrogenase resulted in greater inactivation of enzymatic activity in the presence of $\alpha$-ketoglutarate than in its absence. However, inactivation by $NaBH_4$ was not dependent on $NADH$, which is required for $\beta$-H exchange of $\alpha$-ketoglutarate (9), and did not occur at pH 6 as with the aldolases (1-5). Furthermore, $NaBH_4$ treatment of the enzyme in the presence of radioactive $\alpha$-ketoglutarate did not yield a labeled enzyme. The reason for the apparent substrate-dependent inactivation by $NaBH_4$ is not known.

Unequivocal evidence that Schiff base formation is not obligatory in the isocitrate dehydrogenase reaction was obtained by studying the forward reaction in $H_2^{18}O$. As shown in Scheme 1, imine formation requires that each molecule of $\alpha$-ketoglutarate contain 1 atom of $^{18}O$ from the $H_2^{18}O$. Since ketonic oxygen undergoes a rapid nonenzymic exchange with $H_2^{18}O$ of the medium, $H_2O_2$ was included in the reaction mixture and $\alpha$-ketoglutarate was decarboxylated to succinate, the keto oxygen atom of $\alpha$-ketoglutarate becoming 1 of the 4 carboxyl oxygen atoms of succinate. It is clear from the results summarized in Table II that the oxidation by $H_2^{18}O$ of enzymically generated $\alpha$-ketoglutarate is faster than the $\alpha$-ketoglutarate-$H_2^{18}O$ exchange, and that oxidation proceeds without incorporating $^{18}O$ from the medium into succinate. Since succinic acid derived from $\alpha$-ketoglutarate contained considerably less than 1 oxygen atom from the solvent it is concluded that formation of a Schiff base does not occur as written in Scheme 1.

It is of interest to compare the mechanisms for the decarboxylation of two $\beta$-keto acids, acetocacetate and oxaloacetic. The nonenzymic decarboxylation of both acids is catalyzed by primary amines but only the decarboxylation of oxaloacetic acid is catalyzed by metals (26). Preliminary evidence suggests that the enzymic decarboxylation of acetocacetate involves imine catalysis (6-8). On the other hand, given a choice between imine and metal catalysis, isocitrate dehydrogenase catalyzes the decarboxylation of enzyme-bound oxalosuccinate without obligatory Schiff base formation. In the aldolases as well, yeast aildolase, which requires a metal ion for activity, functions without Schiff base formation (cf. Reference 4).

![Scheme 1](image-url)

Scheme 1. Reaction sequence showing the result of Schiff base formation in the isocitrate dehydrogenase reaction in an $H_2^{18}O$ medium. Each molecule of succinate formed by decarboxylation of $\alpha$-ketoglutarate with $H_2O_2$ would contain 1 atom of $^{18}O$. The manner of attachment of isocitrate to the enzyme (RNH$_2$) is not specified.
Acknowledgments—We are greatly indebted to Professor D. Rittenberg and to Miss L. Ponticorvo for help and advice with $^{18}O$ analyses, and to Dr. H. B. Vickery for a generous gift of potassium threo-3,4-isocitrate.

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