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 μmole per min per mg when assayed as described below. Unless otherwise stated the pig heart preparation was used.

Monopotassium three-o-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 μm. Enzymic activity was expressed as micromoles of NADPH formed per min per mg of protein with the use of the extinction coefficient, 6.22 × 10⁴ 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 HgClt 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 μmole of α-ketoglutarate, 0.5 μmole of NADPH, 20 μmole of MgSO₄, 1 μmole of EDTA, 0.01 ml of octanol, and 100 μmole of buffer (potassium phosphate, pH 6.0, and triethanol-
amino-acetate, pH 7.0 and pH 8.0), in a final volume of 0.62 ml. Each solution was treated at 0°C, over a period of 15 min, with 0.3 ml of freshly prepared 2.0 M NaBH₄ in 0.05-ml portions; the pH was maintained by the addition of 2.0 M acetic acid. The final 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 0°C following the last addition of NaBH₄, and were dialyzed for 2 hours against 100 ml of a solution containing 0.1 mM KCl, 10 mM triethanolamine-HCl, pH 7.3, 2.0 mM a-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 the Addition Dehydrogenase with α-Ketoglutarate-δ¹⁴C.**—Reaction mixtures and incubation conditions were similar to those with unlabeled a-ketoglutarate at pH 8 (Table I, Experiment 2), except that 5 to 10 mg of enzyme were used and the amount of a-ketoglutarate was 0.2 µmole (0.8 x 10⁶ to 1.4 x 10⁶ 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 precipitate 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 µmole of α-ketoisocitrate, 1 µmole of Tris-HCl, pH 7.4, 0.3 µmole 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 µmoles of HzO₂ (final volume, 20 ml). After incubation for 3 hours at 30°C 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>
<thead>
<tr>
<th>Experiment</th>
<th>pH</th>
<th>Addition</th>
<th>Treatment with NaBH₄</th>
<th>Specific activity (µmoles/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>α-Ketoglutarate</td>
<td>+</td>
<td>0.32</td>
</tr>
<tr>
<td>7.0</td>
<td>α-Ketoglutarate</td>
<td>+</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>α-Ketoglutarate</td>
<td>+</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>α-Ketoglutarate</td>
<td>−</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>8.0</td>
<td>No substrate</td>
<td>−</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>8.0</td>
<td>No substrate</td>
<td>+</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**In the control reaction a solution containing 5 µmoles of Tris-HCl, pH 7.4, 0.3 µmole 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 µmoles of H₂O₂ (final volume, 20 ml). Solid α-ketoglutaric acid (0.6 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 N 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-δ¹⁴C a solution of 2.4 µmoles of disodium α-ketoglutarate and 5 µmoles of Tris-HCl, pH 7.4, was freeze-dried, and the residue was equilibrated with 40 ml of H₂O for 20 hours at room temperature. The solution was treated with 0.49 ml of 30% H₂O₂ (4.8 µmoles), and, after 1 hour at room temperature, with 5 µl of catalase. After the removal of H₂O₂ by freeze-drying, 148 µg (1.3 µmoles) of succinic acid were isolated as described below, and a sample was analyzed for ¹⁸O. A solution of 124 µg 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. Succinc acid (60 µc) 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 was eluted with 1.2 N 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 40-50°C in a rotary evaporator, and the product was purified by two sublimations at 190-210°C under vacuum, and by two to three crystallizations from tetrahydrofuran-benzene. The melting point of the succinic acid samples was 188-190°C (uncorrected).

**Decarboxylation of α-Ketoglutarate by H₂O₂.**—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 H₂O₂, in a final volume of 1 ml, were incubated at 30°C. 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-dinitrophenylhydrazine (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 acetoacetate 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 oxida-
tion, 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 m NaBH₄ completely in-
avtivated the enzyme in the presence of a-ketoglutarate, as
compared to a control without NaBH₄. Experiment 3 was done
with an isocitrate dehydrogenase preparation that was stable at
0° 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 a-ketoglutarate resulted in a 19% inactivation,
whereas the same treatment in the presence of a-ketoglutarate
resulted in a 58% inactivation. With both preparations of en-
zyme, inactivations obtained with NaBH₄ were variable. Treat-
ment of the enzyme with NaBH₄ in the presence of a-ketogluta-
tarate yielded inactivations 0 to 46% greater than those
obtained in the presence 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 a-ketoglutarate-dependent inactivation by NaBH₄.

In order to determine if the a-ketoglutarate-dependent in-
avtivation by NaBH₄ was due to reduction of a Schiff base
formed between enzyme and substrate, experiments were
performed with a-ketoglutarate-5-¹⁴C. Following NaBH₄ treat-
ment, dialysis, and precipitation with acid, the amount of radio-
activity bound to protein was no greater than in control experi-
ments 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 a-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 a-ketoglutarate
liberated by hydrolysis from the enzyme contain ¹⁸O in the
a-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 a-ketoglutarate as
soon as it is formed in the reaction mixture. Two methods
were tried: reduction with NaBH₄ to yield α-hydroxysuccinic
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-hy-
droxysuccinic 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 isoc-
itrate ratio was 2:1. In the presence of phenazine metho-
sulfate (PMS)¹ both NADP and H₂O₂ were regenerated accord-

¹ We are grateful to Dr. K. R. Hanson for suggesting the use of
this reagent (20).

| Experiment | %O found in | %O in α-keto-
<table>
<thead>
<tr>
<th></th>
<th>H₂O</th>
<th>Succinic acid</th>
<th>glutarate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With enzyme</td>
<td>1.76</td>
<td>0.20</td>
<td>0.45</td>
</tr>
<tr>
<td>Control</td>
<td>1.81</td>
<td>0.96</td>
<td>0.58</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With enzyme</td>
<td>1.43</td>
<td>0.10</td>
<td>0.28</td>
</tr>
<tr>
<td>Control</td>
<td>1.40</td>
<td>0.08</td>
<td>0.23</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decarboxylation of α-ketoglutarate-2-¹⁸O</td>
<td>1.41</td>
<td>0.37</td>
<td>1.06</td>
</tr>
<tr>
<td>Succinic acid (from above) repurified</td>
<td></td>
<td>0.30</td>
<td>1.03</td>
</tr>
</tbody>
</table>

The conditions for Experiment 1 (with enzyme, and control) are
described in the "Experimental Procedure." Experiment 2 was
similar except that in the enzyme reaction 2.4 mmoles of H₂O₂
were used, and after 14 hours of incubation an additional 30 mg of
isocitrate dehydrogenase were added; in the control 3 mmoles of
H₂O₂ were used. Experiment 3 is described in the "Experimental
Procedure."

The 0.06 M H₂O₂ concentration should therefore remain relatively
constant during the incubation. The ratio of H₂O₂ to enzy-
mically 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-keto-
glutарате contained 0.20 atom % excess ¹⁸O, and the medium
water, 1.76 atom % excess; this is equivalent to an average
incorporation of (4 × 0.0020)/0.0176 = 0.45 atom of ¹⁸O. Hence,
a maximum of only 45% of the α-ketoglutarate exchanged with
H₂¹⁸O, whereas imine formation and hydrolysis would re-
quire 100% exchange. Even the 45% labeling can be accounted
for by spontaneous exchange of the keto group of α-ketoglutarate
with H₂¹⁸O, whereas imine formation and hydrolysis would re-
quire 100% exchange. 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 com-
pletely with H₂¹⁸O, and was decarboxylated with H₂O₂ as in

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

\[ \text{PMSH}_2 + \text{O}_2 \rightarrow \text{PMS} + \text{H}_2\text{O}_2 \]
the 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 acetoacetate has been reported in the decarboxylation reaction catalyzed by acetoacetate decarboxylase (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 (22). (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 irreversible (25), and a partial reaction, stereospecific exchange of a \(\beta\)-II 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 \(NADPH\), which is required for \(\beta\)-II 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.

Univocal 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 \(H_2O_2\) 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, acetoacetic and oxalosuccinic. The nonenzymic decarboxylation of both acids is catalyzed by primary amines but only the decarboxylation of oxalosuccinic acid is catalyzed by metals (26). Preliminary evidence suggests that the enzymic decarboxylation of acetoacetate 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 obligatorily Schiff base formation. In the aldolases as well, yeast aklasone, which requires a metal ion for activity, functions without Schiff base formation (cf. Reference 4).

![Scheme 1](image-url)
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-$d_3$-isocitrate.

REFERENCES