III. \(\beta\)-KETOADIPATE CHLORINASE: A SOLUBLE ENZYME SYSTEM*

PAUL D. SHAW† AND LOWELL P. HAGER

From the Converse Memorial Chemical Laboratory, Harvard University, Cambridge, Massachusetts

(Received for publication, May 25, 1959)

In the preceding paper (1) it was established that acetone-dried mycelial powders of Caldariomyces fumago catalyze the conversion of inorganic chloride and \(\beta\)-ketoadipate to \(\delta\)-chlorolevulinic acid.

This communication reports the isolation of a soluble enzyme system from Caldariomyces fumago mycelial powders which catalyzes this chlorination reaction. A suitable system has been devised for the assay of the enzyme, and the various parameters with respect to enzyme and substrate concentration have been determined. Data on the stoichiometry of the reaction indicate that Equation 1

\[
\text{O} - \text{OOC-CH}_2-\text{CH}_2-\text{C-CH}_3-\text{COO}^- + \text{Cl}^- + 4\text{O}_2 \\
+ 2\text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O}
\]

adequately describes the over-all reaction. This equation undoubtedly represents the sum of two or more partial reactions since preliminary evidence indicates that the chlorination reaction requires more than one enzyme component. For this reason, the catalyst for the over-all chlorination reaction will be referred to as the \(\beta\)-ketoadipate chlorinase system to denote its multiplicity of function.

**EXPERIMENTAL**

**Preparation of Soluble Enzyme System**—The growth of C. fumago and the preparation of acetone-dried mycelial powder have been described previously (1). Although \(\beta\)-ketoadipate chlorinase activity could be directly extracted from the mycelial powders with dilute phosphate buffer, the enzyme yield was increased considerably by sonic oscillation. Routinely, 3 g of mycelial powder were suspended in 35 ml of 0.04 M potassium phosphate buffer, pH 6.5, and oscillated for 10 minutes in a 10-ke Raytheon sonic oscillator. After this treatment, mycelial debris was removed by centrifugation in the cold at 15,000 x g for 30 minutes in the Servall SS1 centrifuge. The resulting cell-free extract was dark brownish green in color and contained approximately 20 mg dry weight per ml. When stored in the freezer, these cell-free extracts appear to be quite stable.

**Assay for \(\beta\)-Ketoadipate Chlorinase Activity**—The chlorination reaction was routinely assayed by incubating a suitable amount of enzyme with 200 \(\mu\)moles of potassium phosphate buffer, pH 4.8, 10 \(\mu\)moles of radioactive potassium chloride (KCl\textsuperscript{3} of specific activity 9,000 to 11,000 c.p.m. per \(\mu\)mole), and 20 \(\mu\)moles of potassium \(\beta\)-ketoadipate in a total volume of 1 ml for 1 hour at 30° under aerobic conditions. The enzyme concentration was usually adjusted so that the rate of \(\delta\)-chlorolevulinic acid formation fell between the limits of 10 to 200 \(\mu\)moles per hour (100 to 2,000 total c.p.m.) for convenience in the determination of radioactivity. Actually the rate of \(\delta\)-chlorolevulinic acid synthesis was essentially linear over 100-fold concentrations of enzyme.

After incubation the reaction was stopped by the addition of 0.2 ml of 7 N sulfuric acid and extracted three times with 2 volumes of ether. The ether extracts were dried over anhydrous magnesium sulfate, concentrated to a small volume on the steam bath, plated, and counted in a gas flow counter with a thin end window. Considerable care was taken in the concentration and plating steps since \(\delta\)-chlorolevulinic acid has considerable volatility at atmospheric pressure and 100°.

**Definition of Units and Specific Activity**—A unit of \(\beta\)-ketoadipate chlorinase activity has been arbitrarily defined as that amount of enzyme which catalyzes the synthesis of 1 \(\mu\)mole of \(\delta\)-chlorolevulinic acid per hour under optimum conditions. Specific activity refers to the units of enzyme activity per mg of protein. The crude cell-free extracts usually contained between 100 and 200 \(\beta\)-ketoadipate chlorinase units per ml at a specific activity of 5 to 10.

**Substrates**—The \(\beta\)-ketoadipic acid used in these experiments was obtained from the Sigma Chemical Company in the free acid form. It was neutralized to pH 6.0 with potassium hydroxide before use. The radioactive chloride was obtained from the Oak Ridge National Laboratories.

**Effect of Enzyme Concentration and Time on Synthesis of \(\delta\)-Chlorolevulinic Acid**—Assay curves showing the effect of time (Curve A) and enzyme concentration (Curve B) on the synthesis of \(\delta\)-chlorolevulinic acid as measured by the incorporation of Cl\textsuperscript{3} into an ether extractable form are given in Fig. 1. After a short lag period, the formation of \(\delta\)-chlorolevulinic acid is directly proportional to time. The chlorination reaction also is proportional to the amount of cell-free extract present except at very low concentrations so that the incorporation of Cl\textsuperscript{3} into \(\delta\)-chlorolevulinic acid adequately serves as an assay method for the enzyme.

**pH Optimum for \(\beta\)-Ketoadipate Chlorinase System**—The effect of hydrogen ion concentration on the rate of \(\delta\)-chlorolevulinic acid synthesis...
The pH optimum of 4.8 is quite acid but possibly not too surprising since the overall reaction (Equation 1) indicates the participation of 2 moles of hydrogen ion per mole of δ-chlorolevulinic acid formed.

**Effect of Chloride Ion and β-Ketoadipate Concentration on Rate of δ-Chlorolevulinic Acid Synthesis**—The effect of chloride ion and β-ketoadipate concentration on the rate of δ-chlorolevulinic acid formation is shown in Fig. 3. The effect of chloride ion concentration (with excess β-ketoadipate) on the rate of δ-chlorolevulinic acid synthesis shows typical first order reaction kinetics at low substrate concentrations shifting to zero order at high substrate concentrations. The β-ketoadipate chlorinase system showed maximum velocity at 0.01 M chloride ion concentration with a \( K_m \) as calculated from these data of approximately 2 \( \times 10^{-3} \) M.

In the case of the effect of β-ketoadipate concentration on the rate of the reaction, the substrate saturation curve is similar to that for chloride ion. The concentration of β-ketoadipate required for 1/4 of the maximum rate of δ-chlorolevulinic acid synthesis is 1.5 \( \times 10^{-4} \) M.
reaction is almost completely inhibited when carried out in a helium atmosphere.

Rate of β-Ketoadipate Decarboxylation and Oxygen Uptake Due to Enzymatic Chlorination—The curves given in Fig. 4A record the rate of oxygen uptake associated with the enzymatic chlorination and decarboxylation of β-ketoadipate together with the appropriate controls. The concentrated cell-free extract used in this experiment has a high rate of endogenous oxygen uptake (Curve 3) which apparently is due to the hydrolysis and oxidation of a polysaccharide present as a nondialyzable component of the cell-free extract. β-Ketoadipate also is slowly oxidized by the enzyme preparation in the absence of chloride ion (Curve 2). Since no CO₂ production is associated with this later oxidation (see Fig. 4B) it would appear probable that β-ketoadipate, in a side reaction, undergoes cleavage to acetate and succinate which are then further oxidized. In the presence of both chloride ion and β-ketoadipate, there is an increased rate of oxygen uptake associated with the formation of δ-chlorolevulinic acid (Curve 1). In the presence of chloride ion but in the absence of β-ketoadipate, the rate of oxygen uptake parallels the endogenous rate (Curve 4), and in the absence of enzyme there is no oxygen uptake (Curve 5).

The rates of enzymatic and nonenzymatic decarboxylation of β-ketoadipate are given in Fig. 4B. The endogenous rate of CO₂ production by the enzyme is very low (Curve 3), and although the nonenzymatic CO₂ formation from the spontaneous decarboxylation of β-ketoadipate is somewhat higher (Curve 5), it still amounts to less than 15% of the CO₂ formation in the complete system (Curve 1). Curve 2 in Fig. 4B shows that there is no enzymatic decarboxylation of β-ketoadipate in the absence of chloride ion, and Curve 4 shows that chloride ion alone does not affect the endogenous rate of CO₂ formation.

The rate curves for oxygen uptake and CO₂ formation given in Fig. 4C are corrected rates of gas exchange and are due exclusively to the enzymatic chlorination reaction. These rate curves are given in A for oxygen uptake. The complete system as shown in Curve 1 (O—O) contained the identical components listed in Table I and as shown in Table I there were 10.3 μmoles of δ-chlorolevulinic acid synthesized in the complete system. The control flasks, in which there was no δ-chlorolevulinic acid synthesis (<0.1 μmole), contained the components of the complete system with the following omissions: Curve 2 (X—X), potassium chloride; Curve 3 (■—■), potassium chloride and β-ketoadipate; Curve 4 (△—△), β-ketoadipate; Curve 5 (■—■), enzyme. The CO₂ evolution rate measurements (B) were made by incubating a series of flasks identical to the oxygen uptake flasks but containing 750 μmoles of potassium hydroxide in the center well so that CO₂ evolution could be determined. Incubation time was 80 minutes at 30° either aerobically in air or anaerobically under a helium atmosphere.

### Table I

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Products</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂ uptake*</td>
<td>CO₂ production†</td>
<td>CPM incorporation</td>
<td>δ-Chlorolevulinic acid formed</td>
</tr>
<tr>
<td>Aerobic</td>
<td>4.7 μmole</td>
<td>10.4 μmole</td>
<td>112,000 c.p.m.</td>
<td>10.3 μmole</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0 μmole</td>
<td>0 μmole</td>
<td>1,740 c.p.m.</td>
<td>0.16 μmole</td>
</tr>
</tbody>
</table>

* Corrected for endogenous oxygen uptake and the oxygen uptake from β-ketoadipate in the absence of chloride ion.
† Corrected for the spontaneous decarboxylation of β-ketoadipate and endogenous CO₂ production.

FIG. 4. The rate of oxygen uptake and β-ketoadipate decarboxylation associated with the enzymatic formation of δ-chlorolevulinic acid. The rate curves given in A are for oxygen uptake. The complete system as shown in Curve 1 (O—O) contained the identical components listed in Table I and as shown in Table I there were 10.3 μmoles of δ-chlorolevulinic acid synthesized in the complete system. The control flasks, in which there was no δ-chlorolevulinic acid synthesis (<0.1 μmole), contained the components of the complete system with the following omissions: Curve 2 (X—X), potassium chloride; Curve 3 (■—■), potassium chloride and β-ketoadipate; Curve 4 (△—△), β-ketoadipate; Curve 5 (■—■), enzyme. The CO₂ evolution rate measurements (B) were made by incubating a series of flasks identical to the oxygen uptake flasks but containing 750 μmoles of potassium hydroxide in the center well. The rate of CO₂ evolution was determined by the difference in rate between each set of parallel flasks. The CO₂ evolution rate curves in B are numbered identically to those for oxygen uptake in A. The rate curves in C are corrected for endogenous oxygen uptake and CO₂ formation, for the nonenzymatic decarboxylation of β-ketoadipate, and for the oxidation of β-ketoadipate in the absence of chloride ion. Curve 6 (■—■) is the corrected rate of CO₂ formation due to the chlorination reaction, and Curve 7 (□—□) is the corrected rate of oxygen uptake.
curves have been corrected for the endogenous activity of the enzyme preparation, for the oxidation of β-ketoadipate in the absence of chloride ion, and for the nonenzymatic decarboxylation of β-ketoadipate. The short lag period for CO₂ evolution and oxygen uptake reflects the results obtained on the rate of Cl³ incorporation with the dilute enzyme preparations in the usual assay (see Fig. 1).

### Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>β-Ketoadipate chlorinase activity (total units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper half</td>
<td>128</td>
</tr>
<tr>
<td>Lower half</td>
<td>385</td>
</tr>
<tr>
<td>Pellet</td>
<td>38</td>
</tr>
</tbody>
</table>

### Table III

**Effect of various inhibitors on β-ketoadipate chlorinase system**

The enzyme assays were carried out according to the procedure described in the text under "Experimental" with the inhibitor included at the concentration indicated. Unless otherwise noted, all incubations were carried out at the optimum pH for enzymatic chlorination (pH 4.8).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium azide</td>
<td>1 × 10⁻⁴</td>
<td>86%</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>1 × 10⁻¹</td>
<td>39%</td>
</tr>
<tr>
<td>Potassium fluoride</td>
<td>1 × 10⁻³</td>
<td>0%</td>
</tr>
<tr>
<td>Potassium bromide</td>
<td>1 × 10⁻¹</td>
<td>81%</td>
</tr>
<tr>
<td>Potassium iodide</td>
<td>1 × 10⁻¹</td>
<td>82%</td>
</tr>
<tr>
<td>Potassium arsenite</td>
<td>1 × 10⁻⁴</td>
<td>40%</td>
</tr>
<tr>
<td>Ferrous sulfate</td>
<td>1 × 10⁻¹</td>
<td>85%</td>
</tr>
<tr>
<td>Potassium ferrocyanide</td>
<td>1 × 10⁻²</td>
<td>51%</td>
</tr>
<tr>
<td>Cysteine</td>
<td>1 × 10⁻⁴</td>
<td>88%</td>
</tr>
<tr>
<td>Glutathione</td>
<td>1 × 10⁻⁴</td>
<td>77%</td>
</tr>
<tr>
<td>Mercaptoethanolamine</td>
<td>1 × 10⁻¹</td>
<td>66%</td>
</tr>
<tr>
<td>Dihydrolipoic acid</td>
<td>1 × 10⁻³</td>
<td>80%</td>
</tr>
<tr>
<td>Thiourea</td>
<td>1 × 10⁻⁴</td>
<td>56%</td>
</tr>
<tr>
<td>Thiouracil</td>
<td>1 × 10⁻⁴</td>
<td>66%</td>
</tr>
<tr>
<td>6-Methylthiouracil</td>
<td>1 × 10⁻⁴</td>
<td>68%</td>
</tr>
<tr>
<td>Potassium arsenate (replacing the phosphate buffer)</td>
<td>2 × 10⁻¹</td>
<td>15%</td>
</tr>
</tbody>
</table>

* In incubated at pH 6.0.
† In incubated at pH 6.9.

**DISCUSSION**

The isolation of the β-ketoadipate chlorinase system in a soluble form appears to offer an ideal system for a detailed study of the biological mechanisms involved in the enzymatic formation of the carbon-chlorine bond. The incorporation of radioactive chloride ion into an ether extractable organic form affords a convenient and sensitive assay reaction. The enzyme can be assayed at widely varied enzyme concentrations, and the assay is unusual only in respect to the short lag period before the estab-
lishment of a linear rate of δ-chlorolevulinic acid synthesis. This lag period was observed both with dilute and concentrated en-
zyme preparations and in three different types of assays for measuring the rate of enzymatic chlorination. Thus it would
appear that the lag period represents more than an analytical artifact and may in fact reflect the time required for the accumu-
lation or build-up of an intermediate.

There is no evidence to indicate the requirement for an energy source such as adenosine triphosphate or related nucleotides, which has been postulated for biological chlorination (2). How-
ever, in view of the relatively high rate of endogenous oxygen uptake, any comment on the energy requirement must be made
with caution. As mentioned previously, this endogenous respira-
tion is undoubtedly caused by the enzymatic hydrolysis and
oxidation of a polysaccharide which is present in all the active
enzymes preparations. Although the chemical nature of this
polysaccharide is not known, it comprises 10% of the dry weight
of the cell free extract and is resistant to attack by common
amylolytic enzymes.1

By analogy to chemical halogenation in general (3) and to
the chemical chlorination of β-keto acids in particular (4), one
would predict that the mechanism for the enzymatic chlorina-
tion reaction would involve the oxidation of chloride ion to the
oxidation state of chlorinium ion with oxygen serving as the
electron acceptor. The chlorinium ion or potential chlorinium
ion, formed by the oxidative reaction and stabilized by combina-
tion with the enzyme or an enzyme prothetic group, could then
serve as the active chlorinating agent. Since the over-all reac-
tion starting with chloride ion and ending with the carbon-chlo-
rine bond represents a two electron oxidation step, the observed
uptake of ½ mole of oxygen per mole of chloride ion incorporated
would be expected on the assumption that oxygen serves as the
terminal electron acceptor. While the inhibition of the chlori-
nation reaction by low concentrations of azide would tend to
implicate a copper or heme oxidase in the oxidation reaction, the
insensitivity of the chlorination reaction toward cyanide would
not support this inference. A possible explanation for
the inhibition of the enzymatic chlorination by reducing agents
would be the reaction of the reducing agent with the positively
charged chlorine atom thereby converting it back to chloride
ion.

It is clear from the high degree of specificity of the enzymatic
chlorination for β-ketoadipate (1) and from the rates of decar-
boxylation of β-ketoadipate in the complete and control systems
that this substrate serves a precise role in the enzymatic chlori-
ation and does not serve merely as a chemical trap for “active
chlorine.” In both the acid and base catalyzed halogenation of
carbonyl compounds, it has been established that the enol or
the enolate anion is the intermediate which reacts with the posi-
tively charged halogen to yield the halogenated product (5).
Furthermore, it has been shown that the acid catalyzed rate of
α,α-dimethylacetonecetate decarboxylation is unaffected by the
presence of bromine although the product of the decarboxylation
in the presence of bromine is 2-bromoisopropyl methyl ketone
(6). The conclusion drawn from the chemical analogy is that

1 H. Meltaer and L. P. Hager, unpublished results.

the rate determining step in the chemical reaction is the decar-
boxylation of the β-keto acid to yield the enol form of the prod-
uct. The enol may then react with a proton to yield isopropyl-
methyl ketone, or in the presence of bromine, with brominium
ion to yield the bromine-containing product. If such were the
case in the enzymatic chlorination reaction, the decarboxylation
of β-ketoadipate would yield the enol form of levulinic acid (or
the enolate anion) which could serve as the trap for enzymatically
generated chlorinium ion. However, in this instance, the sponta-
naneous rate of β-ketoadipate decarboxylation would necessarily
have to be equal to or greater than the rate of δ-chlorolevulinic
acid formation. Since it has been shown that the rate of enzy-
matic chlorination may exceed the spontaneous rate of β-
ketoadipate decarboxylation by at least 5-fold, this clearly cannot
be the case. The results also show that the enzyme system does
not catalyze the decarboxylation of β-ketoadipate in the absence
of chloride ion so that an enzymatically generated, free enol
form of levulinic acid could not be the chlorine acceptor. Thus
it would appear most probable that either the enzymatic chlorina-
tion leads to the synchronous decarboxylation of β-ketoadipate,
or alternatively, that β-ketoadipate is specifically decarboxylated
by the enzyme system to yield a “bound enol” which in turn
could be chlorinated to yield δ-chlorolevulinic acid.

SUMMARY

A soluble enzyme system has been extracted from acetone
powders of Caldariomyces fumago which catalyzes the conversion
of chloride ion and β-ketoadipate to δ-chlorolevulinic acid. A
suitable assay system with radioactive chloride as a tracer has
been developed, and optimum conditions with respect to enzyme
and substrate concentrations have been determined.

The stoichiometry of the enzymatic chlorination indicates that
1 mole of CO₂ is produced and ½ mole of oxygen is consumed per
mole of δ-chlorolevulinic acid synthesized from chloride ion and
β-ketoadipic acid. By analogy to chemical halogenation, it is
suggested that chloride ion is oxidized to the oxidation state of
a stabilized chlorinium ion with oxygen serving as the electron
acceptor. Furthermore, from the rates of chlorination, oxygen
uptake, and CO₂ formation, it is concluded that either the enzy-
matic chlorination leads to the synchronous decarboxylation of
β-ketoadipate or that β-ketoadipate is specifically decarboxylated
to yield an enzyme-bound enol of levulinic acid which is then
chlorinated.

The enzymatic chlorination system is sensitive to a number
of inhibitors, notably: cyanide at high concentrations, azide at
low concentrations, all the other halogen anions and reducing
agents.

REFERENCES

3. INGOLD, C. K., Structure and mechanism in organic chemistry,
4. HENNEK, H., Chemie der beta-dicarbonylverbindungen, Springer-
Verlag, Berlin, 1950, p. 41.
Biological Chlorination: III. \(\beta\)-KETOADIPATE CHLORINASE: A SOLUBLE ENZYME SYSTEM
Paul D. Shaw and Lowell P. Hager


Access the most updated version of this article at
http://www.jbc.org/content/234/10/2565.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/234/10/2565.citation.full.html#ref-list-1