ENZYMATIC MECHANISM OF OXIDATION OF TARTRATE*

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Mitochondria of all animal tissues, so far investigated, contain an enzyme system which catalyzes the oxidation of meso- and (-)-tartrate. Certain properties of this enzyme system, such as its extraction and coenzyme requirements as well as the isolation of oxidation products of tartrate, were described in a previous publication (1). The reconstruction of Reactions 1 to 3 was based on the chemical identification and rates of

1  Tartrate + DPN+ + Mg++ ⇌ DIHF (oxaloglycolate ⇌ dihydroxyfumarate) + DPNH + H+
2  DIHF + DPN+ ⇌ diketosuccinate + DPNH + H+  \[ CO_2 + \text{hydroxypyruvate} \]
3  2DIHF + Mg++  \[ \rightarrow \]
   2 glyoxylate

formation of these compounds. A more detailed knowledge of the individual steps of the sequence of reactions is a prerequisite of further work which may lead to the isolation of the enzyme components of this system. This paper deals with experiments which were designed to fulfill this requirement.

Methods

An aqueous solution of the enzyme system, prepared from acetone powder of beef heart mitochondria (1), was used throughout these experiments. The preparation of keto acids, their isolation as 2,4-dinitrophenylhydrazine derivatives, and their separation by means of paper chromatography have

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1 An equilibrium mixture of oxaloglycolate and dihydroxyfumarate is abbreviated in this paper as DIHF. It was previously suggested (1) that the first product of the enzymatic dehydrogenation of tartrate is the keto acid oxaloglycolate which rapidly attains equilibrium with its more stable dienol form, i.e. dihydroxyfumarate. The keto-enol equilibrium constant of this acid in aqueous solution is unknown. DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide, respectively; EDTA, ethylenediaminetetraacetate; A, absorbancy.
been described in a previous publication (1). Cytochrome c, DNP (90 per cent pure), DPNH, and triphosphopyridine nucleotide were obtained from the Sigma Chemical Company. All spectrophotometric measurements were carried out with the Beckman DU instrument, with 1 cm. quartz cuvettes of 1.4 ml. volume.

Results

Oxidation of Tartrate—The enzymatic reduction of DPN by tartrate (Reaction 1) was measured in 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.3. In agreement with previous results, obtained by manometric measurements (1), the addition of Mg++ increased the rate of DPN reduction (Fig. 1), although the initial rates were unaffected. Under identical conditions meso-tartrate was more rapidly oxidized than (-)-tartrate (Fig. 2).

The nature of the O2-consuming reaction which occurs during the aerobic oxidation of tartrate was studied in detail. A comparison of DPNH formation with O2 absorption suggested that the reoxidation of DPNH (either by the DPNH-oxidizing system of intact mitochondria or by the non-enzymatic oxidation by phenazine methonium sulfate) could account only in part for the amount of O2 consumed. It was, therefore, concluded that the further oxidation of DIHF must contribute to O2 consumption.

As shown in Fig. 3, cytochrome c is rapidly reduced during tartrate oxidation. The enzyme preparation used in this work contained only small amounts of DPNH cytochrome c reductase activity; therefore, the reduction of cytochrome c, which occurred during the enzymatic oxidation of tartrate, was due at least to the extent of 70 per cent to a reaction between DIHF and cytochrome c. It was found that DIHF similar to ascorbate directly reduces cytochrome c at pH 8.3 in the absence of an enzyme. This reduction is inhibited by EDTA. Certain dyes, such as 2,6-dichlorophenolindophenol, can also react non-enzymatically with DIHF. The reduced form of this dye is reoxidized by O2 only very slowly, while the reaction between reduced phenazine methonium sulfate and O2 is rapid. This property of phenazine methonium sulfate explains its role as a carrier in the aerobic oxidation of tartrate. In the course of the non-enzymatic aerobic oxidation of DIHF by a suitable dye, H2O2 is formed. Since H2O2 could conceivably decompose some products of the enzymatic oxidation of tartrate, this possibility was also investigated. With 2,6-dichlorophenolindophenol or phenazine methonium sulfate as an electron acceptor, the addition of crystalline catalase to a tartrate-oxidizing enzyme reaction mixture caused only a small diminution of hydroxypyruvate formation, suggesting that the decarboxylation of DIHF was but slightly increased by H2O2. This effect, however, was negligible in experiments of short duration.
(10 to 20 minutes). Under the experimental conditions used, catalase was not inhibited by the dyes.

Reduction of DIHF—It was reported (1) that DIHF is enzymatically reduced to tartrate when DPNH is present. As shown in Fig. 4, DPNH is rapidly oxidized by DIHF when a small amount of an extract of beef heart mitochondria is added (Reaction 1). A study of the conditions of this reaction led to the understanding of the rôle of Mg\(^{++}\) in the oxidation of tartrate. The following observations were made. A solution of DIHF exhibits a strong absorption band at 290 mµ, characteristic of its dienol structure (determined in a range pH 4.5 to 7.0). When Mg\(^{++}\) is added to this solution, the absorbance at 290 mµ diminishes at a linear rate (approximately equivalent to 1 to 2 mµoles of dienol disappearance per hour) at room temperature. Analyses of the DIHF solution (as the 2,4-dinitrophenylhydrazone of the keto form of this acid, i.e. as oxaloglycolate) after several hours of incubation with Mg\(^{++}\) revealed that the oxaloglycolate content had diminished and, among other products, hydroxypyruvate had appeared. It was found that EDTA inhibits this decarboxylation catalyzed by metal ion. When Mg\(^{++}\) is added to a solution of DIHF + EDTA, a very slow diminution of the absorbance at 290 mµ occurs. However,
the oxalolglycolate content of the solution remains unchanged under these conditions, as measured by keto acid analyses (1) of samples taken at 1 minute intervals for 10 minutes. As shown in Fig. 4, preincubation of DIHF with EDTA-Mg\(^{++}\) results in an increase in the enzymatic reoxidation of DPNH.

**Oxidation of DIHF**—The oxidation product of DIHF is diketosuccinate. This keto acid was isolated as the 2,4-dinitrophenylosazone when the enzymatic oxidation of tartrate was allowed to proceed for a short time (10 to 20 minutes). Diketosuccinate is very unstable and is rapidly decom-

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Fig. 3. Reduction of cytochrome \(c\) during tartrate oxidation (pH 8.3, 0.45 mg. of enzyme protein). The reaction mixture contains the same constituents as described in the legend of Fig. 1 and, in addition, 1.2 mg. of cytochrome \(c\).

Fig. 4. Enzymatic oxidation of DPNH by dihydroxyfumarate (pH 6.8). The enzymatic oxidation of DPNH (20 \(\gamma\)) is measured in 0.5 M phosphate buffer, pH 6.8, containing 4 mg. of DIHF per 5 ml. Preincubation with 5 \(\mu\)moles of Mg\(^{++}\) for 3 minutes. The reaction is started by addition of the enzyme (0.01 ml.). No reaction occurs without substrates. The final volume is 1.4 ml.

An aqueous extract of acetone powder of beef heart mitochondria does not oxidize DIHF to diketosuccinate in the presence of O\(_2\). Similarly, the enzymatic reduction of DPN to DPNH could not be directly demonstrated with DIHF as substrate in a range from pH 7.5 to 9.0. The use of phenazine methonium sulfate or 2,6-dichlorophenolindophenol as an electron acceptor is complicated by the fact that these dyes oxidize DIHF non-enzymatically (see above). However, the DPN-linked enzymatic oxidation of DIHF (Reaction 2) can be demonstrated if correction is made for the non-
enzymatic reaction between the dye and DIHF. A solution of DIHF (4 mg. dissolved in 5 ml. of 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.3) reduces 2,6-dichlorophenolindophenol at a rate of 34 \( \gamma \) per 10 minutes at room temperature. This rate is not changed when the enzyme preparation (2 mg. of protein) is added; however, the addition of DPN + enzyme increases the rate of dye reduction to 42 \( \gamma \) per 10 minutes. Under given conditions DPN does not react with DIHF non-enzymatically, nor does DPNH with 2,6-dichlorophenolindophenol. This observation indicates that a DPN-linked enzymatic oxidation of DIHF can be shown only when one of the products, i.e. DPNH, is instantly reoxidized by another enzyme system (in this case diaphorase, present in the extract of mitochondria, which catalyzes the oxidation of DPNH by 2,6-dichlorophenolindophenol). This is in contrast to the enzymatic oxidation of tartrate by DPN which can be directly measured spectrophotometrically by the appearance of DPNH (see Figs. 1 and 2).

Triphenyltetrazolium chloride, a dye previously used for the determination of succinic dehydrogenase (2), does not react non-enzymatically with DIHF. However, this dye was not suitable for the demonstration of the DPN-linked enzymatic oxidation of DIHF. It should be mentioned that when triphenyltetrazolium chloride was used as an electron acceptor for the enzymatic oxidation of tartrate (in the presence of DPN under anaerobic conditions) the only products of the oxidation of tartrate were oxalosuglycolate and glyoxylate. This reaction was increased by Mg\(^{++}\).
Reduction of Diketosuccinate—Diketosuccinate does not oxidize DPNH non-enzymatically, but a rapid reaction occurs when a small amount of enzyme is added (Reaction 2). The product of the enzymatic reduction of diketosuccinate by DPNH is DIHF, which was identified by chromatography. The reduction of diketosuccinate under these conditions proceeds by way of DIHF partly to tartrate. As shown in Fig. 5, the enzyme preparation reduces both diketosuccinate and oxalacetate rapidly.

Other Reactions of DIHF (Reaction 3)—Hydroxypyruvate was found to be the product of an Mg++-catalyzed non-enzymatic decarboxylation. This decarboxylation of the keto acid oxaloglycolate, similar to that of oxalacetate as found by Krebs (3), proceeds very likely by the same mechanism described by Steinberger and Westheimer (4).

The rate of glyoxylate formation during the enzymatic oxidation of tartrate indicated that its precursor was the keto form of dihydroxyfumarate, i.e. oxaloglycolate (1). Incubation of DIHF with the enzyme preparation resulted in glyoxylate formation only when Mg++ + EDTA were added. The conversion of DIHF to glyoxylate occurred even in the absence of enzyme, provided Mg++ + EDTA were present. Since the rate of production of glyoxylate from DIHF, catalyzed by Mg++ + EDTA, was not increased upon the addition of the enzyme preparation, it was concluded that glyoxylate is formed non-enzymatically under these experimental conditions. A probable mechanism of this reaction will be discussed later.

DISCUSSION

Mitochondrial preparations catalyze the following two reactions: (a) the reaction of tartrate and DPN to oxaloglycolate (the keto form of dihydroxyfumaric acid) + DPNH, and (b) the oxidation of oxaloglycolate by DPN to diketosuccinate + DPNH. However, this second reaction can be shown only when DPNH is instantly reoxidized (e.g. by diaphorase + 2,6-dichlorophenolindophenol). In the soluble enzyme system the demonstration of Reaction b is complicated by the fact that artificial electron acceptors such as phenazine methonium sulfate, cytochrome c, or 2,6-dichlorophenolindophenol oxidize DIHF non-enzymatically to diketosuccinate. In such a system DPNH produced in Reaction a reacts enzymatically with diketosuccinate to yield DIHF. This explains the accumulation of DIHF during the oxidation of tartrate. In intact mitochondria, in which an efficient DPNH cytochrome c-cytochrome oxidase system operates, the DPN-linked enzymatic oxidation of oxaloglycolate to diketosuccinate may easily occur. Further, oxaloglycolate can undergo either decarboxylation, catalyzed by Mg++ alone, or cleavage to glyoxylate, a reaction which occurs non-enzymatically in the presence of Mg++ + EDTA. Diketosuccinate is rapidly decomposed by traces of metal ions. The activating effect of Mg++ on the enzymatic oxidation of tartrate can be explained by the non-en-
zymatic removal of oxidation products. However, the fact that the re-
verse reaction, i.e. the reduction of DIHF to tartrate, is equally activated
by Mg$^{++}$ (in the presence of EDTA) points to a different rôle of Mg$^{++}$.
There is reason to believe that, in the presence of Mg$^{++}$ + EDTA, the
dienol, which is preponderant in aqueous solutions (5), is more readily con-
verted to the presumably reactive keto form (i.e. oxaloglycolate). This
keto acid is then enzymatically reduced by DPNH. Besides the enzymatic
evidence, the reactivity of DIHF with 2,4-dinitrophenylhydrazine can also
be used as a proof in favor of this explanation. DIHF reacts only slowly
with this carbonyl reagent and in dilute solutions it takes 10 to 12 hours
(at 4°C) before the crystalline derivative appears. Preincubation of DIHF
with Mg$^{++}$ + EDTA greatly accelerates the formation of the 2,4-dinitro-
phenylhydrazone which crystallizes in 10 to 20 minutes.

It is possible that Mg$^{++}$ may form an additional complex with the sub-
strate (four out of six coordination valences of Mg$^{++}$ are occupied by
EDTA while the remaining two participate in the complex with the car-
boxylic acid). Pedersen proposed a somewhat similar mechanism of the
Cu$^{++}$-catalyzed bromination of acetoacetic ester (6). According to this
author, the Cu$^{++}$ complex of the enolate ion of ethyl acetoacetate plays an
essential rôle in the bimolecular transfer of H$^+$ to a base.

Stafford, Magaldi, and Vennesland (7) reported that plant extracts con-
tain enzymes which oxidize DPNH by DIHF and diketosuccinate. These
reactions appear to be analogous to the ones catalyzed by the enzyme sys-
tem of animal mitochondria. Preliminary experiments with mitochondria
of lupine seedlings indicated that they also contain a tartaric acid oxidase
which attacks (-)- and meso-tartrate. As pointed out by Stafford et al.
(7) plants contain a DIHF oxidase, previously described by Banga and
Philippot (8). Further work is required to clarify the nature of this plant
enzyme.

SUMMARY

1. A soluble enzyme system, prepared from beef heart mitochondria, re-
duces DPN at pH 8.3 when (-)- or meso-tartrate is added as substrate.
Mg$^{++}$ activates this reaction. meso-Tartrate is oxidized more rapidly than
(-)-tartrate. The oxidation product appears to be oxaloglycolate which
is in equilibrium with its more stable dienol form, i.e. dihydroxyfumarate.

2. Dihydroxyfumarate (in equilibrium with oxaloglycolate) is enzymat-
ically reduced to tartrate by DPNH in phosphate buffer of pH 6.8 with
EDTA present. The rate of reaction is doubled when Mg$^{++}$ is also added.

3. An equilibrium mixture of dihydroxyfumarate and oxaloglycolate is
oxidized non-enzymatically to diketosuccinate by cytochrome c, phenazine
methionium sulfate, or 2,6-dichlorophenolindophenol.

4. The enzymatic oxidation of an equilibrium mixture of dihydroxyfuma-
rate and oxaloglycolate by DPN to diketosuccinate can be demonstrated only when the reaction is coupled to a DPNH-oxidizing enzyme system (diaphorase).

5. Diketosuccinate is enzymatically reduced to oxaloglycolate by DPNH.

6. Mg\(^{++}\) catalyzes the decarboxylation of oxaloglycolate to hydroxypyruvate, while Mg\(^{++} +\) EDTA catalyze the cleavage to glyoxylate.

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