4-Alkyl-o-Quinone/2-Hydroxy-p-Quinone Methide Isomerase from the Larval Hemolymph of Sarcophaga bullata

I. PURIFICATION AND CHARACTERIZATION OF ENZYME-CATALYZED REACTION*

(Received for publication, January 24, 1990)

Steven J. Saul and Manickam Sugumarang

From the Department of Biology, University of Massachusetts at Boston, Harbor Campus, Boston, Massachusetts 02125

An enzyme which catalyzes the conversion of certain 4-alkyl-o-benzoquinones to 2-hydroxy-p-quinone methides has been purified to apparent homogeneity from the hemolymph of Sarcophaga bullata by employing conventional protein purification techniques. The purified enzyme migrated with an approximate molecular weight of 98,000 on gel filtration chromatography. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis, it migrated as a single band with a molecular weight of 46,000, indicating that it is made up of two identical subunits. It exhibited a pH optimum of 6.0 and readily converted chemically synthesized as well as enzymatically generated quinones derived from N-acetyl dopamine, N-β-alanyl dopamine, and 3,4-dihydroxyphenethyl alcohol to highly unstable 2-hydroxy-p-quinone methides. The quinone methides thus formed were rapidly and nonenzymatically hydrated to form side chain hydroxylated o-diphenols as the stable product. In support of this proposition, when the enzyme reaction with N-acetyl dopamine quinone was conducted in the presence of 10% methanol, racemic β-methoxy-N-acetyl dopamine was recovered as an additional product. The quinones of N-acetylnorepinephrine, N-β-alanylnorepinephrine, and 3,4-dihydroxyphenylglycol were also attacked by the isomerase, resulting in the formation of N-acetylarterenone, N-β-alanylarterenone and 2-hydroxy-3',4'-dihydroxyacetophenone, respectively as the stable products. The isomerase converted the dihydrocaffeyl methyl amide quinone to its quinone methide analog which rapidly tautomerized to yield caffeyl methyl amide. The importance of quinone isomerase in insect immunity and sclerotization of insect cuticle is discussed.

Phenolic compounds, which are the second most abundant compounds on earth, range from simple monobenzylic phenols to complex lignins and melanins, of which o-diphenols form an important group and serve such diverse functions as neurotransmitters (1), iron-sequestering siderophores (2), precursors for melanins (3, 4), substrates for oxidative browning of plant products (5) and tanning agents of insect cuticle (6–8). Central to the metabolism of o-diphenols is their oxidation and usually undergo Michael 1,4 nucleophilic addition reactions (8, 11).

Short-lived quinones such as dopaquinone and dopaminequinone undergo rapid cyclization and polymerization to melanin polymers (3, 4), while long-lived quinones like N-acetyl dopamine quinones react with available nucleophilic groups on surrounding proteins causing the formation of protein catechol adducts and protein-protein cross-links (6–8, 11–13). Therefore, quinones are highly toxic due to their notorious reactivity and their reactions are responsible for the reduction in nutritive value of plant proteins (5), cytotoxicity of dopamine derivatives (14), toxic effects of quinone antibiotics (15), encapsulation of foreign objects in insects (16), and sclerotization of insect cuticle (6–8). Hence, biological systems generating quinones must also possess appropriate detoxification mechanisms to protect self matter from destruction by quinone. Recently, we discovered one such enzyme system which detoxifies the long-lived quinones to short-lived quinone methides (17, 18). Quinone methides, being unstable, rapidly undergo nonenzymatic conversion to nontoxic side chain oxygenated compounds (17, 18). In this paper, we detail the purification and characterization of the reaction catalyzed by this enzyme.

MATERIALS AND METHODS AND RESULTS†

DISCUSSION

Using its ability to destroy phenoloxidase-generated quinones, an enzyme was purified from the hemolymph of Sarcophaga bullata larvae to apparent homogeneity. The purified enzyme on gel filtration migrated, with an approximate molecular weight of 98,000. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis it migrated as a single band with a molecular weight of 46,000. Thus the enzyme appears to be a dimer made up of two identical subunits. From the amino acid composition, the molecular weight of the protein was calculated to be about 98,000, which is close to the value determined on gel filtration. The quinone isomerase exhibited a pH optimum of 6.0 and lost its activity completely by heat treatment.

† Portions of this paper (including “Materials and Methods,” “Results,” Figs. 1, 16, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

* This work was supported by National Institutes of Health Grant 2ROI-AI-14753. This is the first paper in a series on quinone isomerase. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed.

1 Portions of this paper (including “Materials and Methods,” “Results,” Figs. 1, 16, and Tables 1 and 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.


Printed in U.S.A.
denaturation at 70 °C for 10 min, confirming the enzymic nature of the reactions.

Evidence that the isolated enzyme is a quinone isomerase comes from the substrate specificity studies. Thus, the purified enzyme, which is devoid of any o-diphenoloxidase activity, readily generated side chain-hydroxylated products from 4-alkyl-substituted quinones such as NADA\(^2\) quinone, NBAD quinone, and hydroxethyl-o-benzoquinone and converted the quinones of side chain hydroxylated products, viz. NANE, NBANE, and 3,4-dihydroxyphenylglycol into N-acetylarterenone, N-\(\beta\)-alanylarterenone, and 2-hydroxy-3',4'-dihydroxyacetophenone, respectively. These transformations can be accounted for by the intermediary formation of enzymatically generated quinone methides and subsequent nonenzymatic conversion of quinone methides to observed products as shown in Fig. 17. The quinone methides derived from NADA quinone, NBAD quinone, and hydroxyethyl-o-benzoquinone are hydrated rapidly to form NANE, NBANE, and 3,4-dihydroxyphenylglycol, respectively (Fig. 17, top line). The quinone methides derived from NANE, NBANE, and 3,4-dihydroxyphenylglycol will exhibit rapid keto-enol tautomerism rather than reacting with external nucleophiles. Such a tautomerization is unidirectional in favor of the keto form, as it results in the regeneration of the aromatic ring (Fig. 17, bottom line). These compounds accordingly generated N-acetylarterenone, N-\(\beta\)-alanylarterenone, and 2-hydroxy-3',4'-dihydroxyacetophenone as the respective end products.

Fully in accordance with this proposal is the finding that during NADA conversion to NANE, \(\beta\)-methoxy-NADA could be isolated as an additional product when the reaction was carried out in methanol. The structure of the methanol adduct could thus be resolved as a racemic mixture (24). The mechanism shown in Fig. 18 accounts for the observed reactions. Phenoloxidase-generated NADA quinone is converted by the quinone isomerase to tautomeric quinone methide. The NADA

\(^2\)The abbreviations used are: NADA, N-acetyldopamine; NANE, N-acetylnorepinephrine; NBAD, N-\(\beta\)-alanylarterenone; NBANE, N-\(\beta\)-alanylnorepinephrine; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
reaction mixture containing 1 mM NADA and 20 μg of mushroom tyrosinase in 50 mM sodium phosphate buffer, pH 6.0. If silver oxide was used in the place of tyrosinase to generate the quinone, the rate of NANE production was found to be about 2–5 nmol/min due to the higher influence of the silver ions on the isomerization of NADA quinone to its quinone methide. However, when quinone isomerase (1 unit) was included in the tyrosinase assay, the rate of NANE production increased dramatically to about 400 nmol/min. Since, the specific activity of quinone isomerase is 3981 units/mg, on a milligram basis quinone isomerase is able to accelerate the reaction nearly 2–20 × 10^3-fold over the nonenzymatic rate. Recently, Andersen (28) has reported the characterization of quinone isomerase activity from the cuticle of *Hyalophora cecropia*. His preparations generated at best only about 2–3 nmol of NANE/min, which in comparison with the above nonenzymatic rate and our enzyme-catalyzed rate, raises serious doubts about the presence of quinone isomerase in his samples.

Quinone methide formation was originally suggested to be a key process in sclerotization of insect cuticle by our group (29, 30). Although we initially proposed a direct two electron oxidation of catecholic precursors to be the route for the generation of quinone methides (6–8, 29–31), recently we have established that this reaction occurs in two steps involving oxidation of catechols to quinones by the cuticular phenoloxidase and subsequent enzyme-catalyzed isomerization of quinones to quinone methides (17–19, 32). Quinone methides derived from sclerotizing precursors such as NADA and NBAD react with cuticular nucleophiles forming catecholic cuticle adducts or react with surrounding water molecules to form NANE and NBANE accounting for quinone methide, which will be inactivated by water addition. Moreover, quinone methide formed can also be rapidly deposited on foreign matter to aid the encapsulation reaction. Therefore, quinone isomerase may be an important enzyme in the physiology and biochemistry of some insects. Currently, we are examining the molecular properties of quinone isomerase to unravel its mechanism of action.

REFERENCES

Downloaded from http://www.jbc.org/ by guest on October 28, 2017
SUPPLEMENT TO
THE JOURNAL OF BIOCHEMISTRY
PHYSIOLOGY OF PROTEINS AND ENZYMES
Vol. 34, No. 1 (1956)

Materials and Methods

O-g-Tyroto tyrosine (specific activity 400 units/mg) [3H]- and 3-4-
hydroxyphenylalanine (specific activity 400 units/mg) [3H]-were
obtained from The New England Nuclear Corporation. The
methods given for the isolation of pig brain tyrosine hydroxylase from
DEAE fast flow Sepharose, HEPES (pH 7.0), and Sephadex G-25 and
G-100 were taken from the literature. The Sephadex column was
packed by LKB Biochemistry, Huddinge, Sweden. A.J.

Methods of Quinone Isomerization

Biological specimens were cut out at 0°C unless stated otherwise.
Tissue was homogenized in cold 0.1 M Tris HCl, pH 7.4, containing
0.5% glycerol. The homogenate was centrifuged at 4°C to remove
free fatty acids. The homogenate was centrifuged at 105,000 g
for 30 min in a Serva model L4-65 ultracentrifuge. The supernatant
was divided into 1 ml aliquots and the supernatant fractions were removed
and used for the experiments. The solutions were made up on a
1:1 (v/v) mixture with water and the protein content was measured.
The protein content was measured by the method of Lowry et al.

Preparation and characterization of the enzyme

The enzyme was obtained from pig brain at 0°C. The solution was
diluted with 0.05 M Tris HCl, pH 7.4, containing 0.01 M dithiothreitol.
The solution was then incubated at 37°C for 30 min. The solution was
then centrifuged at 105,000 g for 30 min. The supernatant was divided
into 1 ml aliquots and the supernatant fractions were removed
and used for the experiments. The solutions were made up on a
1:1 (v/v) mixture with water and the protein content was measured.

Methods of enzyme activity measurement

The enzyme activity was measured by a decrease in the optical
absorbance at 278 nm. The enzyme was diluted with 0.05 M Tris
HCl, pH 7.4, containing 0.01 M dithiothreitol. The solution was
then incubated at 37°C for 30 min. The solution was then centrifuged
at 105,000 g for 30 min. The supernatant was divided into 1 ml aliquots
and the supernatant fractions were removed and used for the experiments.
The solutions were made up on a 1:1 (v/v) mixture with water and the
protein content was measured.

Methods of enzyme isolation

The enzyme was obtained from pig brain at 0°C. The solution was
diluted with 0.05 M Tris HCl, pH 7.4, containing 0.01 M dithiothreitol.
The solution was then incubated at 37°C for 30 min. The solution was
then centrifuged at 105,000 g for 30 min. The supernatant was divided
into 1 ml aliquots and the supernatant fractions were removed
and used for the experiments. The solutions were made up on a
1:1 (v/v) mixture with water and the protein content was measured.

Methods of enzyme characterization

The enzyme was obtained from pig brain at 0°C. The solution was
diluted with 0.05 M Tris HCl, pH 7.4, containing 0.01 M dithiothreitol.
The solution was then incubated at 37°C for 30 min. The solution was
then centrifuged at 105,000 g for 30 min. The supernatant was divided
into 1 ml aliquots and the supernatant fractions were removed
and used for the experiments. The solutions were made up on a
1:1 (v/v) mixture with water and the protein content was measured.
Figure 1. Molecular weight estimation of quinone isomerase.

Purified quinone isomerase was chromatographed on a Sephadex G-200 column (1.5 x 150 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.5. The column was calibrated with the following molecular weight standards: 1. Cytochrome c (12,400), 2. Ovalbumin (42,700), 3. Bovine serum albumin monomer (66,200), and 4. Bovine serum albumin dimer (132,400). 5. Quinone isomerase.

Figure 2. Sephadex G-200 column chromatography on quinone isomerase.

Quinone isomerase obtained from the Sepharose column was dialyzed, concentrated, and chromatographed on a Sephacyl S-200 column (5.5 x 110 cm) using 25 mM Tris-Cl buffer, pH 8.0, containing 0.2 M NaCl. The flow rate was maintained at 8-10 ml/hr and fractions of 1.43 ml were collected. There was no absorbance at 280 nm.

Figure 3. Effect of temperature on quinone isomerase activity.

Each reaction mixture (1 ml) containing 0.2 M NADH and 40 mg tyrosine in 50 mM sodium phosphate buffer at specified pH was incubated for 1 min. Quinone isomerase (0.15 mg) was added to the mixture. After 1 min incubation, NADH formed was quantified by HPLC using system 1 as described in Materials and Methods.

Figure 4. Molecular weight estimation of quinone isomerase on SDS-PAGE.

The native molecular weight of quinone isomerase was determined by SDS-PAGE using a gelatin-membrane method. Molecular weight markers were (1) BSA (66,200), (2) Ovalbumin (42,700), (3) Cytochrome c (12,400), and (4) Lysozyme (14,300). The molecular weight standards were determined using the same method as for quinone isomerase.

Figure 5. Effect of temperature on quinone isomerase activity.

Quinone isomerase (1.0 unit) was incubated for 15 min at specified temperature in 1 ml of 50 mM sodium phosphate buffer, pH 7.0, and cooled on ice. It was then tested for enzyme activity at room temperature.

Figure 6. Substrate specificity of quinone isomerase.

Each substrate (1 mM final concentration) was incubated with enzyme for two minutes in 50 mM sodium phosphate buffer, pH 6.0 and filtered. The enzyme solution, 

A. NADH, B. NADPH, C. NADPH, D. 3,4-dihydroxyphenyl glycol, E. PMS, F. 3,4-dihydroxyphenyl glycol, G. ISG, H. 3,4-dihydroxyphenyl glycol.

Figure 7. Substrate specificity of quinone isomerase.

Each substrate (1 mM final concentration) was incubated with enzyme for two minutes in 50 mM sodium phosphate buffer, pH 6.0 and filtered. The enzyme solution was incubated with the substrate for 10 min at room temperature.
**o-Quinone/p-Quinone Methide Isomerase**

Figure 8: HPLC analysis of NADH quinone:quinone isomerase reaction.
- NADH (10 mM) was incubated with tyrosine (200 mM) in 50 mM sodium phosphate buffer, pH 6.0, for two min and filtered. The quinone formed was incubated with quinone isomerase (200 units) for 10 min. The reaction mixture was then analyzed by HPLC using System 1 at 290 nm.

Inset: The absorbance changes at 410 nm of the above two reaction mixtures over indicated time period.

Figure 9: Formation of NADH quinone methide-tyrosine adduct.
- Reaction mixture containing 2 mM NADH and 20 mg tyrosine were incubated for 30 min in 50 mM sodium phosphate buffer, pH 6.0. An aliquot (10 µl) was then submitted to HPLC analysis using HPLC System 1.
- A: Control reaction;
- B: A plus quinone isomerase (0.6 units);
- C: A with 1% methanol; and
- D: A plus quinone isomerase (0.6 units).

Figure 10: Ultraviolet spectra of A) NADH and B) β-methoxy-β-acetyl dopamine.
- Spectra were recorded in 0.2 M morphic acid (---) and 0.2 M sodium borate buffer (pH 8.5) (--).
- Inset: HPLC analysis of β-methoxy-NADH on cyclohexan column (see materials and methods for details).

Figure 11: NMR spectrum of β-methoxy-β-acetyl dopamine in A) DMSO-d_{6} and B) in the same solvent after D_{2}o exchange.
**o-Quinone/p-Quinone Methide Isomerase**

**Figure 13:** HPLC analysis of HNAD-tyrosinase-quinone isomerase reaction.

A reaction mixture containing HNAD (0.1 mM) and 10 μg tyrosinase in 30 mM sodium phosphate buffer, pH 6.0, was incubated for 20 min and then subjected to HPLC analysis using system II as described in Materials and Methods except the flow rate was maintained at 0.6 ml/min. The peaks at 2.8 and 3.9 min are due to HNAD and N\(^{2}\)-acetyl-tyrosine, respectively.

- A. Control reaction without tyrosinase;
- B. A plus tyrosinase;
- C. B plus quinone isomerase (0.3 units).

**Figure 14:** Ultraviolet spectral changes associated with the HNAD-tyrosinase-quinone isomerase reaction.

A reaction mixture containing 0.1 mM HNAD, 30 μg mushroom tyrosinase, and quinone isomerase (1.5 units) in 1 ml of 50 mM sodium phosphate buffer, pH 5.0, was incubated at room temperature and the spectral changes monitored at 360 nm. The absorbance was recorded at five min intervals.

- A. Control reaction; and at 17 min reaction
- B. 2.6 min and 4.9 min are due to HNAD and N\(^{2}\)-acetyl-tyrosine, respectively.

**Figure 15:** HPLC analysis of isomerase reaction with 1,4-dihydroxyphenylalanine-quinone isomerase reaction.

A reaction mixture containing 1.0 mM HNAD in 1 ml of 50 mM sodium phosphate buffer, pH 6.0, was incubated with 30 μg of tyrosinase and 0.4 mg of isomerase and subjected to HPLC analysis using System II as described in Materials and Methods. Reaction mixtures containing isomerase (0.3 unit) were processed similarly.

- A. Control reaction
- B. HNAD-tyrosinase 20 min reaction
- C. HNAD-tyrosinase 60 min reaction
- D. HNAD-tyrosinase-quinone isomerase 2 min reaction
- E. HNAD-tyrosinase-quinone isomerase 20 min reaction
- F. HNAD-tyrosinase-quinone isomerase 45 min reaction
- G. HNAD-tyrosinase-quinone isomerase 60 min reaction
- H. HNAD-tyrosinase-quinone isomerase 80 min reaction
- I. HNAD-tyrosinase-quinone isomerase 100 min reaction

The peaks at 2.6 min and 4.9 min are due to HNAD and N\(^{2}\)-acetyl-tyrosine, respectively.
Figure 16: Ultraviolet and visible spectral changes associated with the spontaneous redox oxidation of dihydrocaffeoyl methylamide A1 in the absence and B in the presence of quinone isomerase.

A reaction mixture containing 0.4 μM dihydrocaffeoyl methylamide, 10 μM mushroom tyrosinase in 1 ml of sodium phosphate buffer, pH 6.0 was incubated at room temperature for 5 min without and 1 min with quinone isomerase (10 units) and the absorbance changes accompanying the oxidation were recorded at indicated time intervals.

Traces: HPLC analysis of the above reaction.

The peaks at 8.7, 7.4 and 3.4 min are due to 3,5-dihydroxycaffeoyl methylamide, dihydrocaffeoyl methylamide and caffeoyl methylamide, respectively.

Table 1: Purification Chart for quinone isomerase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg)</th>
<th>Recovery %</th>
<th>Fold Enzyme (units/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Extract</td>
<td>410.0</td>
<td>1075</td>
<td>0.25</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solubilized</td>
<td>440.0</td>
<td>322.5</td>
<td>0.52</td>
<td>30</td>
<td>2.08</td>
</tr>
<tr>
<td>DEAE-</td>
<td>Sorbent</td>
<td>0.3</td>
<td>275</td>
<td>343.3</td>
<td>76.6</td>
</tr>
<tr>
<td>Sepharose</td>
<td>0.054</td>
<td>125</td>
<td>998.1</td>
<td>15923.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Amino acid composition of quinone isomerase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>g/mg protein</th>
<th>mg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>18.73</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>5.18</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>3.71</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>3.71</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>8.34</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>6.13</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>9.54</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>4.93</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>3.57</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.72</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>6.59</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.62</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.74</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>4.34</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>4.58</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>4.64</td>
<td></td>
</tr>
<tr>
<td>tryptophan</td>
<td>MD</td>
<td></td>
</tr>
</tbody>
</table>

MD = not determined
4-alkyl-o-quinone/2-hydroxy-p-quinone methide isomerase from the larval hemolymph of Sarcophaga bullata. I. Purification and characterization of enzyme-catalyzed reaction.
S J Saul and M Sugumaran