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Isolation of γ-L-Glutaminyl 4-Hydroxybenzene and γ-L-Glutaminyl 3,4-Benzoquinone: a Natural Sulfhydryl Reagent, from Sporulating Gill Tissue of the Mushroom *Agaricus bisporus**

Robert F. Weaver†, K. V. Rajagopalan, Philip Handler, Peter Jeffs‡, William L. Byrne, and David Rosenthal

DEPARTMENT OF BIOCHEMISTRY, DUKE UNIVERSITY MEDICAL CENTER, DURHAM, NORTH CAROLINA 27706; P. M. GROSS CHEMICAL LABORATORY, DUKE UNIVERSITY, DURHAM, NORTH CAROLINA 27706; DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF TENNESSEE MEDICAL CENTER, MEMPHIS, TENNESSEE 38103; AND CHEMISTRY AND LIFE SCIENCES LABORATORY, DURHAM, NORTH CAROLINA 27706

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Abstract. Early in the development of spores, there appears in gill tissue of the mushroom *Agaricus bisporus* a red pigment that inhibits mitochondrial respiration. The inhibitor and its immediate precursor were isolated from the mushroom and identified as γ-L-glutaminyl 3,4-benzoquinone (I) and γ-L-glutaminyl 4-hydroxybenzene (II), respectively, neither of which had previously been described. II was synthesized chemically and the synthetic material was identical with isolated II in all regards. An enzyme that oxidizes II to I was isolated concurrently. I reacts unusually rapidly, completely, and at low concentration with the sulfhydryl groups of various mitochondrial enzymes, accounting for its originally observed properties. It may also prove of value as a general inhibitor of sulfhydryl-dependent enzymes.

Initiation of spore formation in the gill tissue of mushrooms is accompanied by a marked reduction in respiratory rate, apparently due to the formation of an inhibitor of mitochondrial respiration.[1] Since the synthesis of this inhibitor is a concomitant of sporulation, it was of interest to ascertain its structure and mechanism of action. Simple extracts of gill tissue proved inhibitory to the respiration of both mushrooms and rat liver mitochondria. Preliminary trials indicated that inhibitory activity was associated with a red pigment in extracts of sporulating gill tissue, but the material itself was unstable; activity was lost during a variety of attempted purification procedures.[2] Fortunately, it was observed that when homogenized gill tissue was incubated aerobically, there occurred an apparent increase in the concentration of the inhibitor of mitochondrial respiration proportional to the increase in concentration of a red pigment. This suggested the presence of a precursor of the actual inhibitor in such extracts as well as of an enzyme that oxidatively converted the precursor to red inhibitor.

Isolation of the precursor required for its assay at least partially purified enzyme that catalyzes the oxygen-dependent conversion to the red inhibitory

compound. Conversely, purification of the enzyme required a supply of substrate. The purification procedures were developed in parallel.

Isolation of the precursor: Gill tissue from fully opened mushrooms was homogenized for 1 min with a Sorvall Omnimixer at 0°C with 3 ml of 0.1 N HCl per gram of tissue. The supernatant obtained after centrifugation at 35,000 × *g* for 20 min was lyophilized, suspended in water, and recentrifuged. The clear supernatant was added to a 3 × 100 cm column of Sephadex G-25 and eluted with water at 4°C. Aliquots of the 10-ml fractions were assayed by addition of a small sample of partially purified enzyme (see below) at pH 6.8 and observing formation of a pink color. The positive fractions were combined, lyophilized, and the residue dissolved in a minimum volume of boiling water. The precursor crystallized in the cold as a white material, which melts with decomposition at 226–228°C. The yield was about 1 mg per g of gill tissue.

Isolation of the enzyme was achieved from gill tissue by the following procedure: Assay was conducted by adding material to be examined, in 0.05 ml, to 0.4 mg of precursor in 0.95 ml of 0.05 M sodium phosphate, pH 6.8 at 23°C; the reaction was monitored at 475 nm in a Cary model 14 recording spectrophotometer. The initial steps in enzyme purification were similar to those used by Kertesz and Zito[3] for purification of polyphenol oxidase.

An "acetone-powder" was prepared by treating 185 g of fully opened mushrooms (*Agaricus bisporus*) with 1800 ml acetone at −10°C for 1 min in a Waring Blendor. After it was dried with ether, the powder was extracted with 30% acetone, and enzyme precipitated from this supernatant by addition of 1.5 volumes of acetone. The precipitate was suspended in 60 ml H_2O, frozen, and thawed. This supernatant was treated with 3 ml of 10% calcium acetate, the precipitate discarded and the supernatant brought to 45% acetone. The resulting precipitate was suspended in water, dialyzed against 15 volumes of water and then against three changes of 15 volumes of 5 mM sodium phosphate, pH 6.8, and brought to 55% in ammonium sulfate. After the precipitate was redissolved in 20 mM sodium phosphate, the precipitation procedure was repeated stepwise, first to 55% and then 70% saturation with ammonium sulfate. The precipitates were redissolved in 20 mM sodium phosphate. Most of the tyrosinase was found in the 0–55% ammonium sulfate fraction while the desired enzyme was confined to the 55-70% fraction.

Further purification was achieved by successive adsorption and elution from a 1 × 5-cm column of DEAE-cellulose with 5 mM sodium phosphate, concentration with an Amicon ultrafiltration apparatus, and by absorption and elution from Sephadex G-200. A single major peak was obtained which exhibited the desired activity (conversion of "precursor" to red pigment) as well as only slight tyrosinase activity. When this preparation was examined by acrylamide gel electrophoresis, a major and a minor peak were obtained, both of which exhibited both enzyme activities. The material in the major peak was used in all subsequent studies.

Structure of the precursor: The elemental analysis was in good agreement with that calculated for $C_{11}H_{14}N_2O_4$: *Calculated*: C 55.46; H 5.92; N 11.80%. *Found*: C 55.15; H 5.88; N 11.66%. This analysis was substantiated by high-

resolution mass spectrometric measurement of the molecular ion at m/e 238 in the mass spectrum. The ultraviolet absorption spectrum, shown in Fig. 1 exhibits a maximum at 243 nm at pH 7.7, with the bathochromic shift to 260 nm at pH 11.6 typical of phenols. The infrared absorption spectrum shows bands that may be attributed to hydrogen-bonded hydroxyl (3270 cm^{-1}), $-NH_3^+$ (2400, 2760, 2680, 2610 cm^{-1}), amide carbonyl (1645 cm^{-1}), and carboxylate (1610 cm^{-1}). The precursor gave a rapid, positive ninhydrin reaction, even at 4°C,

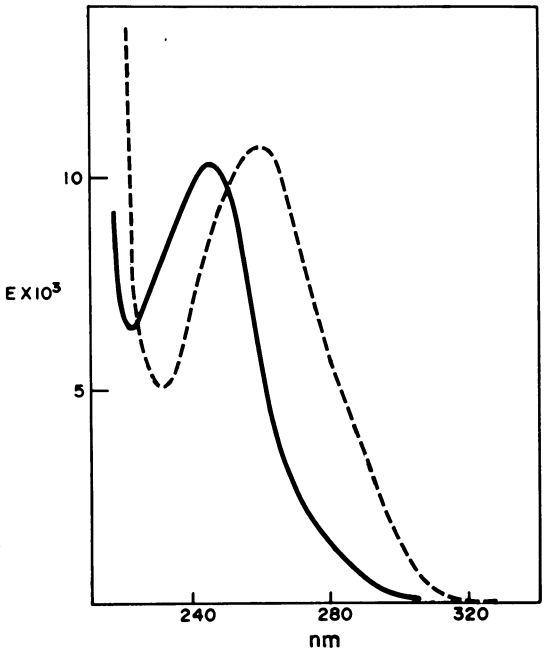
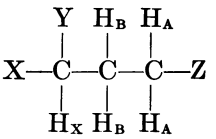
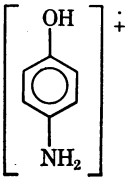


FIG. 1. Ultraviolet absorption spectrum of inhibitor precursor at pH 7.3 (solid line) and at pH 11.4 (broken line).

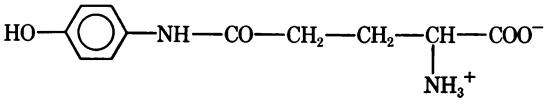
which could be prevented by prior addition of copper carbonate. The nuclear magnetic resonance spectrum was examined in D_2O at elevated pH. An AA^1BB^1 pattern, centered at 7.10 δ was found, corresponding to four aromatic hydrogens per molecule, as in a *p*-disubstituted benzene. Further, there were evident a one-proton triplet at 3.52 δ and a complex four-proton pattern centered at 2.38 δ. The signal at 3.52 δ was spin coupled to the two hydrogens giving rise to the upfield part of a multiplet at 2.38 δ. Five hydrogens gave rise to a triplet at 3.52 δ and a multiplet at 2.38 δ comprising an A_2B_2X spin system, thereby indicating a structural system of the following type in which X or Y must be a heteroatom or electronegative.



The remaining five hydrogens of the molecule do not appear in the spectrum and, hence, underwent exchange for deuterium in the medium, indicating attachment to heteroatoms. Since the mass spectrum showed a major fragment at m/e 109 with a composition of C_6H_7NO, the sum of these various observations strongly suggested the existence of the partial species



Acid hydrolysis yielded a material which was identical with L-glutamic acid in several paper chromatographic systems and in the conventional procedure with a Beckman model 120C amino acid analyzer. The only structure that reconciles all of this data is that of glutaminyl 4-hydroxybenzene.



This structure was confirmed by direct synthesis, utilizing the scheme shown in Fig. 2. The product was identical with "precursor" in all regards, including formation of the red inhibitor of respiration when incubated aerobically with the purified enzyme.

Structure of the inhibitor: Relatively pure preparations of natural inhibitor

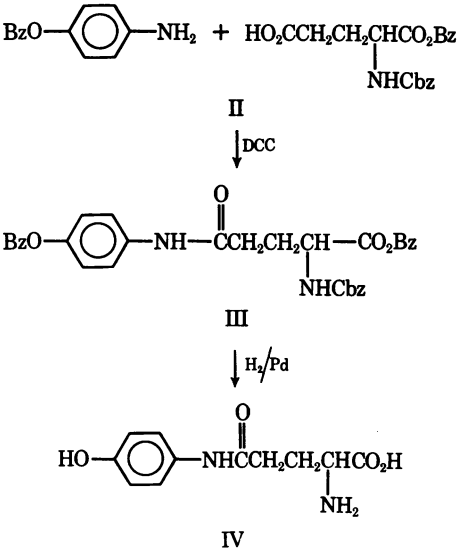


FIG. 2. Chemical synthesis of γ-L-glutaminyl 4-hydroxybenzene. I, *p*-Benzyloxyaniline; II, *N*-Carbobenzoxy-α-L-glutamic acid; III, product from coupling of I and II; IV, γ-L-glutaminyl 4-hydroxybenzene; DCC, dicyclohexylcarbodiimide.

can be easily isolated from mushroom gill tissue by homogenizing, centrifuging at 105,000 × *g* for 1 hr, discarding the residue, and placing the supernatant on a 3 × 90-cm column of Sephadex G-25 and eluting with water. Elution of inhibitor is monitored by its red color. Inhibitor was also prepared by incubation of 2 mg of either natural or synthetic glutaminyl 4-hydroxybenzene with enzyme in 5.0 ml of 50 mM sodium phosphate, pH 6.8 at 23°C for 10 min. Inhibitor was purified by adsorption and elution from a short column (1 × 15 cm) of Sephadex G-25. The ultraviolet absorption spectrum is shown in Fig. 3;

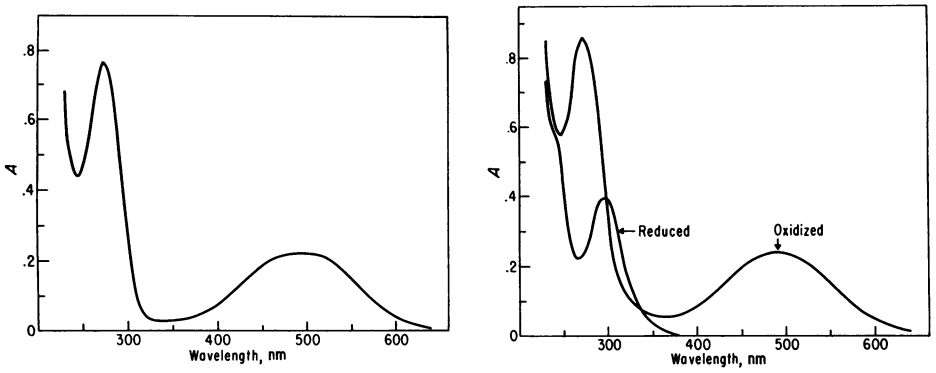


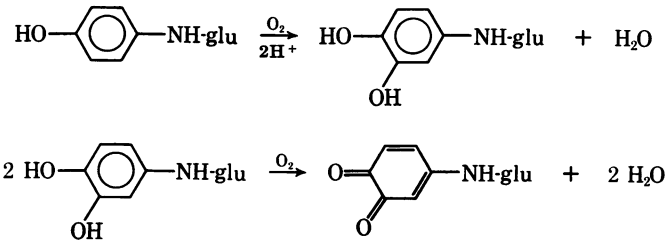
FIG. 3. Absorption spectrum of synthetic and natural samples of mushroom respiratory inhibitor. *Left*: a 5 μg/ml solution of synthetic γ-L-glutaminyl 4-hydroxybenzene was incubated with oxygenase and O_2 until no further increase in absorbance was observed at 490 nm. *Right*: Absorption spectrum of red inhibitor isolated from mushroom extracts. The 'reduced' spectrum was obtained by addition of a few crystals of $NaBH_4$ to the cuvette.

inhibitor prepared from natural or synthetic precursor yielded an essentially identical absorption spectrum which was bleached by addition of a few crystals of either sodium borohydride or sodium dithionite. This behavior, the structure of the precursor, and the tyrosinase activity of the enzyme combined to suggest that the inhibitor is a quinone.

This postulate was supported by adsorbing inhibitor on charcoal, dissolving it in pyridine, and reacting it with either acetic anhydride or benzoylchloride. When the acetylation mixture was flash-evaporated, dissolved in chloroform, and examined by thin-layer chromatography, two separable products, with infrared spectra characterized by stretching frequencies in the region 1690 to 1770 cm^{-1}, were obtained. These are expected[4] for an aromatic carbonyl and for an aliphatic ester carbonyl, respectively, suggesting that the two derivatives represent the half- and fully-acetylated compounds formed with the enols of the two carbonyls of a quinone. The stretching frequencies of the corresponding benzoyl derivatives were at lower frequencies (1690 and 1740 cm^{-1}), as expected. Natural inhibitor also gave glutamic acid after hydrolysis in 5 M HCl.

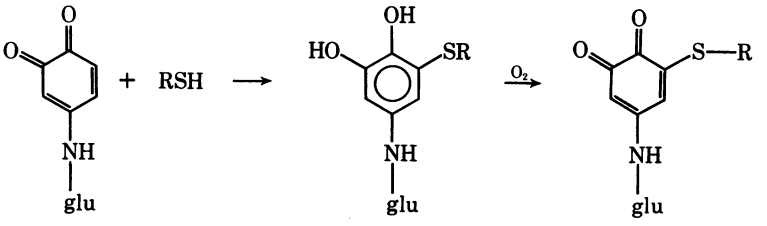
The stoichiometry of O_2 utilization in the enzymic formation of inhibitor was measured with a Gilson oxygraph. 2.7 atoms of oxygen were used per molecule of substrate oxidized. H_2O_2 did not accumulate, and the enzyme has no cata-

lytic activity. These observations indicate that the inhibitor must be γ-glutaminyl 3,4-benzoquinone, which is formed enzymatically as follows:



This compound is undoubtedly the pigment that was isolated by Jackson and Kendal in 1949 (ref. 5) but to which they assigned a structure in which the nitrogen is the pyrrolidine ring nitrogen of proline.

Enzyme inhibition by γ-glutaminyl 3,4-benzoquinone: Since we were interested in this compound because of its inhibition of mitochondrial respiration, the mechanism of this action was of some moment. This has been studied in some detail but only a few aspects will be presented here. The inhibitor was found to inhibit purified pyruvic-, α-ketoglutaric-, and succinic-dehydrogenases at concentrations low enough to account for the observed respiratory failure of live gill tissue. Since quinones are known to react with sulfhydryl compounds, it was suspected that this is the mechanism in the present instance. Indeed, cysteine was found to react with the inhibitor, rapidly bleaching it; when O_2 is present, this reaction maybe followed by the appearance of a purple color. The solution exhibits a broad absorption band at 550 nm and shows two isosbestic points with the absorption spectrum of the inhibitor when a 1:1 adduct is formed (Fig. 4). Presumably this observation signifies that reaction proceeds by the usual nucleophilic attack on a quinone by a sulfhydryl group[6] followed by reoxidation:



In confirmation of this concept, the inhibitor was also found to inhibit chicken-liver xanthine dehydrogenase, dihydroorotic dehydrogenase, and triosephosphate dehydrogenase, all of which are known to be sulfhydryl dependent enzymes.[2] Of particular interest were the following observations: Xanthine dehydrogenase inhibition was entirely dependent upon the presence of xanthine, i.e., reduction of the enzyme by substrate exposed the reactive sulfhydryl group whereas DPNH, the alternative substrate, was without effect and, even in the presence of glutaminyl benzoquinone, the reaction DPNH→methylene blue was unaffected. A parallel situation was observed with dihydroorotic dehydrogenase.

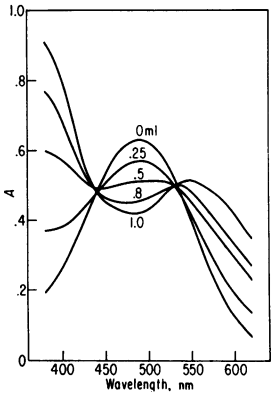


FIG. 4 Aerobic titration of inhibitor with cysteine in 50 mM phosphate, pH 7.8. 1.0 ml of cysteine corresponds to 1:1 stoichiometry with inhibitor. Further addition of cysteine bleached the purple color of the adduct.

Earlier observations[7] had indicated that, whereas mercuribenzoate inhibited the reaction dihydroorotate→O_2 or a dye by 85%, the reaction DPNH→O_2 was inhibited only 15–25%. Glutaminyl benzoquinone, however, inhibited the the reaction with O_2 totally while it was completely without any effect on the reaction with dye. At low concentrations (14 μM) this quinone totally inhibited triosephosphate dehydrogenase in a few minutes. When followed spectrophotometrically, this reaction closely resembled that of the quinone with cysteine. Thus, γ-glutaminyl 3,4-benzoquinone is a superlative laboratory reagent for use as an inhibitor of enzymes which are "sulfhydryl-dependent." And it appears highly likely that it is this property that accounts for its natural behavior as a respiratory inhibitor in the sporulation of *Agaricus bisporus*.

Both the precursor and the inhibitory quinone are previously undescribed derivatives of glutamic acid; no evidence was obtained for equivalent derivatives of any other amino acid. Despite the appearance of relatively large quantities of the precursor in early sporulation, little quinone, if any, can be detected as such; quinone formation occurs rapidly after the tissue is macerated, indicating that the phenolic precursor and the oxidizing enzyme are physically segregated in the gill cells and that as quinone is formed *in vivo* it is bound by enzymic sulfhydryl groups.

Remaining to be established are the pathway for biosynthesis of γ-glutaminyl 4-hydroxybenzene, the relationship between the observed properties of the quinone and the process of spore formation, and the nature of the signal which derepresses that portion of the mushroom genome responsible for synthesis of the enzymes that participate in the synthesis of γ-glutaminyl 3,4-benzoquinone.

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† Present address: Department of Biochemistry and Biophysics, University of California Medical Center, San Francisco.

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