INTRACELLULAR QUINONE REDUCTION IN *SPOROTRICHUM PULVERULENTUM* BY A NAD(P)H:QUINONE OXIDOREDUCTASE

Possible role in vanillic acid catabolism

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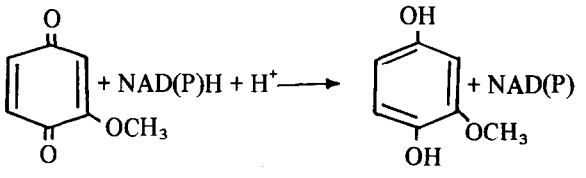
1. Introduction

Catabolism of vanillic acid by the lignin-degrading white-rot fungus, *Sporotrichum pulverulentum*, proceeds through an initial oxidative decarboxylation to yield methoxyhydroquinone [1,2]. Although the steps in the further conversion of methoxyhydroquinone have not yet been elucidated it is clear that cleavage of the aromatic nucleus occurs at some stage [3].

Phenolic substances produced during lignin degradation by *S. pulverulentum* and other white-rot fungi are converted to chromophoric structures as a result of the action of fungal phenol oxidases (laccase and peroxidase). Chromophore accumulation is prevented by cellobiose:quinone oxidoreductase, an extracellular enzyme which couples the oxidation of cellobiose with the reduction of a quinone to the corresponding phenol [4–6]. A function of this enzyme, which is produced by all white-rot fungi so far tested by us [7], may be to maintain any lignin-derived phenols in, or convert quinones to, a reduced state to ensure the further breakdown of the benzene ring [4]. We now report a second, intracellular, quinone oxidoreductase system from *S. pulverulentum* which reduces methoxyquinone to methoxyhydroquinone using reduced pyridine nucleotides as electron donors according to the scheme:



2. Materials and methods

Cultivation of *S. pulverulentum*, and the preparation of mycelial extracts, has been described [2]. NAD(P)H:quinone oxidoreductase was assayed by measuring the rate of decrease in A_{340} due to the oxidation of reduced pyridine nucleotide. Extracts were passed through a prepacked Sephadex PD-10 G-25 column (Pharmacia) before use to remove endogenous metabolites which interfered with the enyzme assay.

Toluquinone, *p*-benzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, menadione (2-methyl-1,4-naphthoquinone) and lawsone (2-hydroxy-1,4-naphthoquinone were obtained from Fluka, and 2,5-dihydroxy-1,4-benzoquinone from ICN Pharmaceut. All other quinones used in this study were kindly provided by Isa Norén and Drs H.-L. Hardell and G. Gellerstedt of this Department. Hydroquinone reaction products were identified as their trimethyl silyl derivatives by gas chromatography. Identification was based on comparison of retention times with those of authentic hydroquinones prepared by catalytic reduction of corresponding quinone using H_2/Pd.

Evolution of $^{14}CO_2$ from carboxyl-labelled vanillate by partially purified mycelial extracts was determined as in [2]. Protein was determined spectrophotometrically.

3. Results

Mycelial extracts of *S. pulverulentum* grown in

the presence of vanillate catalyzed the reduction of methoxyquinone to methoxyhydroquinone using NADH or NADPH as electron donor. Under assay conditions these coenzymes were about equally effective for the reduction of the quinone. Spectral changes associated with the formation of methoxyhydroquinone were observed during the course of NADH oxidation; i.e., the strong absorption peak at 255 nm decreased and a new peak at 285 nm appeared. Methoxyhydroquinone was confirmed as the reaction product by gas chromatography. The stoicheiometry of NADH consumption was difficult to establish unequivocally due to low rates of non-enzymic reduction of quinone by NADH, small increases in A_{340} due to product formation and to spontaneous reoxidation of methoxyhydroquinone. However, using assay conditions where non-enzymic oxidation of the hydroquinone was barely detectable (citrate buffer pH 5.6), higher concentrations of cell extract protein (100–150 μg) and 100–300 nmol methoxyquinone, reduction of 1 nmol quinone was accompanied by oxidation of 1.06 nmol NADH. Considerable oxidoreductase activity was detectable in extracts of mycelia grown on glucose but was increased 5-fold with vanillate present. The enzyme was active over a broad pH range (pH 4–8) with optimal activity near pH 5.6, and the rate of quinone reduction was doubled when citrate buffer replaced phosphate.

Of several quinones tested, highest oxidoreductase

Table 1
Specificity of quinone:NAD(P)H-oxidoreductase from *S. pulverulentum*

Quinone	Specific activity[a]	% Activity (methoxyquinone = 100%)
Methoxyquinone	12.99	100
Toluquinone	12.62	97
1,4-Benzoquinone	11.21	86
2-Methoxy-5-hydroxy-1,4-benzoquinone	n.d	0
3,5-Dimethoxy-1,4-benzoquinone	2.8[b]	22
2,5-Dihydroxy-1,4-benzoquinone	n.d	0
Methoxyquinone	11.83	100
2-Methoxy-5-*tert*-butyl-1,4-benzoquinone	5.84	49
1,4-Naphthoquinone	3.15	27
Lawsone	n.d	0
Menadione	2.19	19
4,5-Dimethoxy-1,2-benzoquinone	9.24	78

[a] Determined from the rate decrease in A_{340} and expressed as μmol NADH oxidized . min^{-1} . mg $protein^{-1}$. The rate of decrease was non-linear and values are calculated from the absorbance decrease observed after the initial 90 s of the assay period. Values are corrected for the non-enzymic reduction of quinones by NADH but are uncorrected for small increases in A_{340} due to hydroquinone formation

[b] This substrate was not completely soluble under the conditions used in the preparation of quinone solutions and the concentration added to the reaction mixture could not therefore be accurately determined

The reaction mixture contained in 3 ml: 250 μmol citrate buffer (pH 5.6); 14–18 μg cell extract protein; 0.5 μmol NADH and 0.3 μmol quinone substrate added as 0.03 ml of a methanolic solution freshly prepared at room temp. $T = 22°C$. Reaction initiated by addition of cell extract protein. Control cuvettes were without NADH. Assays were typically carried out in two groups due to enzyme lability in dilute solution. Crude extracts (containing 8–12 mg protein) were passed through a Sephadex G-25 column and diluted immediately prior to each group of assays. n.d., no activity detected

activity was observed with methoxyquinone, toluquinone and 1,4-benzoquinone (table 1). The *ortho*-quinone 4,5-dimethoxy-1,2-benzoquinone was also rapidly reduced but naphthoquinones of the vitamin K series were less effective substrates. In all cases the product of oxidoreductase activity was identified as the corresponding hydroquinone.

4. Discussion

Levels of NAD(P)H:quinone oxidoreductase in *S. pulverulentum* are increased by growth in the presence of vanillate. Vanillate undergoes an initial oxidative decarboxylation and, while only methoxyhydroquinone can be detected, it is conceivable that methoxyquinone is the intermediate product which is then reduced by the oxidoreductase. However, this appears incompatible with a NADH consumption of ~1.0 mol NADH/mol vanillate converted to methoxyhydroquinone observed under conditions where the rate of product autoxidation is low. Oxidative decarboxylation is catalysed by a monooxygenase (unpublished results) and a mechanism which involved methoxyquinone as an intermediate would thus require 2 mol NADH/mol vanillate.

From studies using ^{14}C-ring-labelled methoxyhydroquinone it is now clear that this compound is a true intermediate in vanillate degradation by *S. pulverulentum* [3]. Subsequent catabolism may involve a hydroxylation step to yield a trihydroxy-methoxylated compound, followed by cleavage of the aromatic ring [3]. A dioxygenase which cleaved the aromatic nucleus of hydroxyquinol, but which did not attack catechol or protocatechuate, has been described in crude extracts of *S. pulverulentum* [8]. Therefore, one likely function of the oxidoreductase would be to reverse any enzymic or non-enzymic conversion of methoxyhydroquinone to methoxyquinone within the fungal cytoplasm and thus ensure its further conversion to Krebs cycle intermediates. Hydroxylation of methoxyquinone, followed by reduction to a trihydroxylated ring-fission substrate, appears unlikely. Hydroxyl substitution is known to stabilize quinones and none of the hydroxylated quinones tested served as substrate for NAD(P)H:quinone oxidoreductase or for cellobiose:quinone oxidoreductase (table 1).

Quinones are readily formed through the action of phenol oxidases induced during the growth of white-rot fungi on both low molecular weight phenolic compounds and lignin. Although certain quinones are normal components of cellular electron-transport systems, quinones generally are highly reactive and re known to inhibit a wide range of metabolic processes. Reduction of quinoid intermediates would thus be essential. The key enzymes is the catabolism of aromatic substances by many fungi, protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase, are depressed by *o*, and *p*-benzoquinone [9], and by 1,2-naphthoquinone [10]. We have been unable to develop an assay to examine the effect of methoxyquinone on the dioxygenase in *S. pulverulentum* due to substrate–inhibitor interaction; however the decarboxylating enzyme is inhibited by very low quinone concentrations (fig.1).

It has been suggested that pyridine nucleotide:quinone reductase systems participate in electron transfer between respiratory substrates and polyphenol oxidases [11]. A similar role was advanced for cellobiose:quinone oxidoreductase in which an

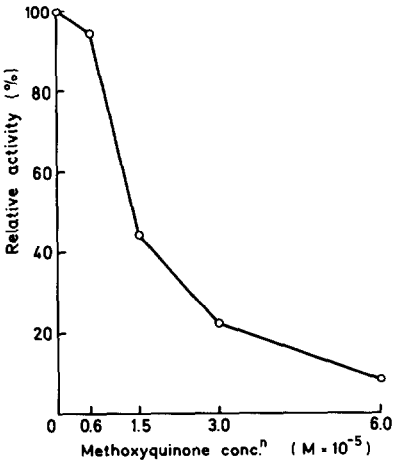


Fig.1. Inhibition of vanillate decarboxylase activity by methoxyquinone. Reaction mixtures contained in 3 ml: 250 μmol K-phosphate buffer (pH 7.4); 1.0 μmol NADH; 96 μg partially purified cell extract protein; methoxyquinone as indicated. Enzyme was incubated with methoxyquinone for 10 min before addition of NADH and 20 μl [*carboxy*-^{14}C]vanillate (1.43×10^5 dpm) to initiate reaction. Evolution of $^{14}CO_2$ was measured after 1 h incubation at 30°C.

extracellular 'electron-transport chain' involving a quinone acceptor and laccase was visualized [5].

Although the NAD(P)H:quinone oxidoreductase described here shares some properties with cellobiose: quinone oxidoreductase (pH dependence, lower activity in phosphate buffer, lack of specificity for quinone substrate), the electron donors for the two enzymes were not interchangeable. Glucose-dependent quinone reduction was also undetectable; thus, NAD(P)H:quinone oxidoreductase is distinct from the proposed glucose:quinone oxidoreductase system [12] which does not appear to operate in *S. pulverulentum.*

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