Kinetic mechanism and specificity of bovine milk sulphydryl oxidase

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1. Sulphydryl oxidase is known to catalyse the synthesis *de novo* of disulphide bonds in a variety of thiol-containing compounds. Reduced glutathione is the best thiol substrate; however, D- and L-cysteine, cysteamine and N-acetyl-L-cysteine, as well as cysteine-containing peptides and proteins, are also effectively oxidized. In contrast, oxidation of the thiol groups of mercaptoethanol, mercaptopyridine, dithiothreitol, dithioerythritol, mercaptoacetate, mercaptopropionate or lipoic acid is not detectably catalysed. 2. In bovine milk, sulphydryl oxidase is closely associated with another glutathione-metabolizing enzyme, γ -glutamyltransferase. Covalent chromatography of crude preparations on cysteinylsuccinamidopropyl-glass resolves the oxidase from the transferase, thus permitting the kinetic characterization of glutathione oxidation. 3. Initial-rate data imply a Ter Bi substituted-enzyme mechanism, and the observed substrate inhibition by thiols suggest that O₂ binds first. Independent, non-kinetic, data, namely the immobilization of sulphydryl oxidase on cysteinyl-matrices, support formation of a mixed-disulphide intermediate between the thiol and enzyme, as predicted by the proposed mechanism. The enzyme-catalysed reaction appears not to be mediated via a superoxide intermediate, since O₂ consumption is not affected by the presence of Nitro Blue Tetrazolium. FAD, NAD⁺, NADP⁺ and Nitro Blue Tetrazolium are all inactive as electron acceptors for sulphydryl oxidase catalysis.

Bovine milk sulphydryl oxidase catalyses the aerobic oxidation of several thiol-containing compounds. The stoichiometry of the reaction (Janolino & Swaisgood, 1975) is:

The enzyme has been shown to be closely associated in skim-milk membranes with another glutathione-metabolizing enzyme, γ -glutamyltransferase (EC 2.3.2.2) (Sliwowski *et al.*, 1981). Resolution of the oxidase and transferase activities in preparations from kidney (Ashkar *et al.*, 1981; Schmelzer *et al.*, 1982) and milk (Sliwowski *et al.*, 1983) has been reported.

Abbreviations used: GSH and GSSG, reduced and oxidized forms of glutathione respectively.

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Availability of a method for preparation of γ -glutamyltransferase-free sulphydryl oxidase enabled the present kinetic characterization of the enzyme-catalysed oxidation of GSH without the competing hydrolytic action of the transferase.

Experimental

Sulphydryl oxidase was purified by covalent affinity chromatography on cysteinylsuccinamidopropyl-glass beads (Sliwowski *et al.*, 1983). For studies with substrates other than GSH, enzyme purified by the method of Janolino & Swaisgood (1975) was used. Glutathione reductase, catalase, coenzymes, phenazine methosulphate, Nitro Blue Tetrazolium, GSH and other thiol substrates were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); mercaptoethanol and mercaptopyridine were products of Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

The rate of O₂ consumption was measured with a Yellow Springs Instruments (Yellow Springs, OH, U.S.A.) oxygen monitor equipped with a

Clark electrode. The instrument was modified as described by Fridovich (1970) by increasing the available bucking potential, which allowed greater sensitivity at higher O_2 concentrations. Mixtures of air, O_2 and N_2 were prepared by using a flow-meter and were used to equilibrate solutions of 50mM-sodium phosphate buffer, pH 7.0, at 35°C. The concentration of dissolved O_2 was determined directly by using the phenazine methosulphate/NADH method of Robinson & Cooper (1970). Assays were initiated by addition of 40 μ l of concentrated GSH to 3.0ml of equilibrated buffer and enzyme solution.

Substrates other than GSH were assayed by following the depletion of thiol groups (Clare *et al.*, 1981; Sliwowski *et al.*, 1981).

Kinetic parameters were evaluated by computer analysis. Non-parametric estimates were calculated with a BASIC program by using the method of Cornish-Bowden & Eisenthal (1978). Initial-rate data were also analysed by using Wilkinson's (1961) weighted-regression method.

Protein concentration was determined by the dye-binding method of Bradford (1976) with the use of a Bio-Rad (Richmond, CA, U.S.A.) kit. In samples containing detergent, protein concentrations were estimated from absorbances at 280nm and 260nm (Layne, 1957), after correction for light-scattering (Leach & Scheraga, 1960).

Results

The reaction of sulphhydryl oxidase with GSH, at ambient O_2 concentration, approximates Michaelis-Menten kinetics at concentrations of GSH up to 5mM, but at higher GSH concentrations

substrate inhibition was observed. Such substrate inhibition was also observed with L-cysteine, D-cysteine, *N*-acetyl-L-cysteine and cysteamine. For kinetic studies with GSH as substrate, glutathione reductase, NADPH and catalase were included in the reaction mixture to ensure that product formation did not influence the initial reaction rates. Consequently, O_2 was reduced at the expense of NADPH. It can be shown that the net stoichiometry of the reactions within the Oxygraph cell under these conditions is:

Primary plots of initial-rate data ($[S]/v$ as a function of $[S]$) are presented in Fig. 1. The common intersection of each family of lines on the ordinate axis is a characteristic of substituted-enzyme mechanisms, since $K_{m(app.)}/V_{max(app.)}$ is unaffected by $[S]$ (Cornish-Bowden, 1979). Accordingly, calculation of the apparent kinetic constants by the method of Cornish-Bowden & Eisenthal (1978) revealed that the ratio $V_{max(app.)}/K_{m(app.)}$ remains constant over a wide range of substrate concentrations, as occurs for enzyme-catalysed reactions that employ a Bi Bi substituted-enzyme mechanism. A diagnostic method has been developed by Eisenthal & Cornish-Bowden (1974) to discriminate between substituted-enzyme and ternary-complex mechanisms for two-substrate kinetics. Thus a plot of $K_{m(app.)}$ versus $V_{max(app.)}$ follows the relationship $K_{m(app.)} = (K_m/V_{max}) V_{max(app.)}$ for two-substrate substituted-enzyme mechanisms. Such plots for both O_2 and GSH are shown in Fig. 2. The relationships are linear ($r^2 = 0.999$), and each intercept is near zero (0.000 to 0.004 ± 0.009 mM), indicating Bi Bi substituted-

Fig. 1. Initial-rate data for sulphhydryl oxidase plotted as $[S]/v$ versus $[S]$

Sulphydryl oxidase (0.75 μ g) was assayed by adding 40 μ l of concentrated GSH solution to 3.0ml of equilibrated buffer and enzyme solution. Lines were generated by non-parametric regression analysis of the kinetic data. Symbols: (a) ■, 0.74mM-GSH; ○, 0.99mM-GSH; ●, 1.48mM-GSH; □, 2.97mM-GSH; (b) ■, 0.095mM- O_2 ; ○, 0.196mM- O_2 ; ●, 0.415mM- O_2 ; □, 0.842mM- O_2 .

Fig. 2. Eisenthal–Cornish-Bowden plots of $K_{m(app)}$ as a function of $V_{max(app)}$ for sulphydryl oxidase
This represents a diagnostic plot for two-substrate mechanisms; only substituted-enzyme mechanisms give data that pass through the origin.

Table 1. Summary of kinetic constants for bovine milk sulphydryl oxidase
Constants were calculated from secondary plots by using Wilkinson regression analysis.

Parameter	Value
$V_{max}^{O_2(app)}/K_{m}^{O_2(app)}$	78.9 ± 1.1
$V_{max}^{GSH(app)}/K_{m}^{GSH(app)}$	18.6 ± 0.4
K_m^{GSH}	$3.16 \pm 0.48 \text{ mM}$
$K_m^{O_2}$	$0.77 \pm 0.04 \text{ mM}$
V_{max}	$61.1 \pm 5.9 \text{ units/mg}$

* $K_m^{GSH} \equiv K_m^{GSH(1)} + K_m^{GSH(2)}$.

Table 2. Apparent kinetic constants for various thiol substrates of sulphydryl oxidase
Initial rates were calculated from progress curves determined by monitoring the depletion of thiol groups by reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) as previously described (Clare *et al.*, 1981; Sliwowski *et al.*, 1981). All assays were performed in sodium phosphate, pH 7.0 and 10.1, at ambient O_2 concentrations.

Substrate	$K_{m(app)}$ (mM)	Relative specific activity ($V_{max(app)}/K_{m(app)}$ relative to that of GSH)
L-Cysteine	0.805	0.62
D-Cysteine	0.966	0.62
N-Acetyl-L-cysteine	1.13	0.34
Cysteamine	1.25	0.68

enzyme mechanism kinetics, even though the reaction is Ter Bi in the forward direction. The kinetic constants for sulphydryl oxidase obtained from secondary plots of the initial-rate data with GSH as the thiol substrate are summarized in Table 1.

The apparent kinetic constants for several other

thiol substrates determined at ambient O_2 concentrations are listed in Table 2. Several other thiol compounds examined, including dithiothreitol, dithioerythritol, mercaptoethanol, mercaptoacetate, mercaptopropionate, 2-mercaptopyridine and lipoic acid, did not result in detectable activity.

The possibility of enzymic production of superoxide was also tested. The addition of Nitro Blue Tetrazolium (0.275 mM or 2.59 mM) did not significantly inhibit the rate of O_2 consumption in the sulphydryl oxidase-catalysed oxidation of 0.8 mM-GSH at pH 7.0. By contrast, control experiments with bis-*o*-phenanthroline- Cu^{2+} complexes, which act as a non-enzymic catalyst for GSH oxidation (Kobashi, 1968), displayed marked inhibition of O_2 consumption by Nitro Blue Tetrazolium. Hence the reduction of O_2 appears to occur by a direct two-electron transfer process resulting in production of H_2O_2 . Finally, FAD, NAD^+ , $NADP^+$ and Nitro Blue Tetrazolium, examined under anaerobic conditions, were all inactive as electron acceptors for sulphydryl oxidase.

Discussion

Although the reaction catalysed by sulphydryl oxidase requires the binding of three substrate molecules, two of them are identical. Initial-rate patterns and substrate inhibition by thiol substrates suggest that these reactions follow a Ter Bi Ping Pong mechanism in which O_2 binds first. Since the oxidase catalyses a three-reactant two-product reaction, a ternary complex must be formed. Inhibition by thiols is consistent with the concept that formation of an initial $E \cdot RSH$ complex is non-productive, i.e. O_2 must bind first

for productive reaction. Moreover, 'substrate inhibition at low concentrations of the constant substrate [in this case, O_2] provides strong positive evidence for the substituted-enzyme mechanism' (Cornish-Bowden, 1979). By contrast, inhibition in the case of a quaternary-complex mechanism would be expected to occur only at O_2 concentrations considerably higher than the $K_m^{O_2}$ (Cornish-Bowden, 1979).

A Bi Uni Uni Ping Pong mechanism is consistent with these observations:

where ESH represents uncomplexed active enzyme bearing a thiol group. The initial-rate equation for this mechanism with GSH as substrate in the absence of products is given by eqn. (4), where $K_m^{GSH(1)}$ and $K_m^{GSH(2)}$ are the Michaelis constants for binding of the first and second molecules of GSH respectively:

$$v = \frac{V_{\max.} [O_2][GSH]^2}{K_1^{O_2} K_m^{GSH(1)} [GSH] + (K_m^{GSH(1)} + K_m^{GSH(2)}) [O_2][GSH] + K_m^{O_2} [GSH]^2 + [O_2][GSH]^2} \quad (4)$$

Defining $K_m^{GSH} \equiv K_m^{GSH(1)} + K_m^{GSH(2)}$, eqn. (4) reduces to eqn. (5):

$$v = \frac{V_{\max.} [O_2][GSH]^2}{K_1^{O_2} K_m^{GSH(1)} [GSH] + K_m^{GSH} [O_2][GSH] + K_m^{O_2} [GSH]^2 + [O_2][GSH]^2} \quad (5)$$

In this mechanism, a substituted-enzyme intermediate (ESSG) occurs that is a mixed-disulphide between the enzyme and the thiol. The existence of such an intermediate is substantiated by covalent immobilization of sulphydryl oxidase on cysteinyl-matrices (Sliwowski *et al.*, 1983). Covalent chromatography also suggests that the specificity of sulphydryl oxidase may reflect the binding of the first molecule of thiol, i.e. the enzyme binds to cysteinyl-matrices, but not to a mercaptopropyl-matrix nor to a 'sulphydrylcellulose' matrix (Schmelzer *et al.*, 1982). However, elution of the bound enzyme can be accomplished with substrates (GSH or cysteine) or with non-substrates (dithiothreitol) (Sliwowski *et al.*, 1983).

A relationship between $K_{m(app.)}$ and $V_{max(app.)}$ can be derived (with O_2 as the variable substrate):

$$K_{m(app.)}^{O_2} = \frac{K_1^{O_2} K_m^{GSH(1)}}{K_m^{GSH}} + \left[K_m^{O_2} - \frac{K_1^{O_2} K_m^{GSH(1)}}{K_m^{GSH}} \right] \frac{V_{max(app.)}}{V_{max.}} \quad (6)$$

The data shown in Figs. 1 and 2 require that $(K_1^{O_2} K_m^{GSH(1)} / K_m^{GSH}) \rightarrow 0$. Regarding $K_1^{O_2}$ as the limiting value of $K_m^{O_2}$ in the absence of thiol, or

roughly 0.1 mM, the value for $K_m^{GSH(1)}$ must be much less than that for $K_m^{GSH(2)}$, supporting the observed specificity for the first molecule of thiol.

The specificity of sulphydryl oxidase appears to be rather general for substrates containing cysteine or cysteine derivatives, including proteins (Janolino & Swaisgood, 1975; Janolino *et al.*, 1978; Clare *et al.*, 1981) and peptides (Sliwowski *et al.*, 1981). Perhaps of more interest are the thiols whose oxidations are not detectably catalysed, in contrast with the sulphydryl oxidase activity

associated with seminal-vesicle secretions, which was reported to exhibit greatest activity towards dithiothreitol (apparent $K_m < 1$ mM), 66% as much activity towards GSH ($K_m > 10$ mM) and 11% towards 2-mercaptoethanol (Ostrowski *et al.*, 1979). Skin sulphydryl oxidase activity was also greatest with respect to dithiothreitol and dithio-

erythritol, with 3.7% as much activity towards 2-mercaptoethanol, and only 0.7% towards GSH,

which was considered to be a 'poor substrate' (Takamori *et al.*, 1980).

Thiol oxidation as catalysed by sulphydryl oxidase is also mechanistically different from metal-ion-catalysed oxidation (Misra, 1974), since it appears to occur by a direct two-electron transfer without formation of free superoxide. This observation also differentiates sulphydryl oxidase from the well-characterized milk oxidase enzyme, xanthine oxidase (McCord & Fridovich, 1968). Although the $K_m^{O_2}$ (0.77 mM) is certainly above what might be considered a 'physiological' concentration, it is not unprecedented with respect to other O_2 -utilizing enzymes (see, e.g., Ogasawara *et al.*, 1966; Cohen & Fridovich, 1971; Flamm & Crandall, 1963).

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