

PROPERTIES AND SURFACE LOCATION OF A SULFHYDRYL OXIDIZING ENZYME IN FUNGUS SPORES

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Received for publication January 20, 1956

The sulfhydryl compounds, cysteine (CySH) and glutathione (GSH), have been proposed frequently as compounds of important metabolic activity because of their potential participation in cellular oxidation and reduction systems. These compounds can be oxidized through the mediation of a number of metal-containing enzymes such as the cytochrome system, peroxidase, ascorbic acid oxidase, and catalase. The oxidation here is indirect; only hematin compounds or organic salts of iron, copper, manganese, and sulfur have appeared capable of effecting direct oxidation (Lemberg and Legge, 1949; Fromageot, 1951). The present paper, however, describes a unique enzyme found in spores of the fungus *Myrothecium verrucaria*, which is apparently capable of bringing about the direct oxidation of CySH and GSH to the respective disulfides.

METHODS

Spores of *M. verrucaria* strain QM 460 were harvested from agar cultures containing filter paper as carbon source and were washed before use, as described previously (Mandels, 1951). Suspensions of these viable spores were either used directly, or were killed by shaking with toluene for 1 hr at 30 C to destroy respiratory and other metabolic systems which would interfere with measurements of substrate oxidation. Spores treated with toluene were washed with water, lyophilized, and stored at room temperature. Activity of the enzyme was not affected by this treatment. Weighed quantities of the dried spores were triturated with a few drops of distilled water before suspending in an appropriate volume of buffer or water as required. In some experiments, spores were killed by suspending in cold 95 per cent alcohol, washing, and suspending in buffer.

Spore extracts were prepared by grinding in a chilled Potter mill. They were freed of particulate matter by centrifuging at about 20,000 G.

Substrate oxidation was followed by conven-

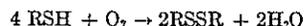
tional Warburg technique at 30 C. In general, the vessels contained 1.0 ml spores and 0.5 ml substrate in the sidearm. Controls to determine rates of autooxidation of substrates showed that in most instances the necessary corrections were hardly large enough to be significant except where homocysteine was the substrate.

RESULTS

Kinetics—stoichiometry. The courses of oxidation of CySH and GSH by spores treated with toluene are very similar (figures 1 and 2). They are not identical, however, since the oxidation of GSH is essentially linear for an appreciable period, whereas with CySH only the initial portion of the curve is linear. In neither case does the reaction follow first or second order kinetics. Similar curves for either substrate are obtained with untreated (viable) spores, except that respiratory activity is superposed on the oxidation curve.

The reaction rate is markedly affected by the concentrations of both RSH and O₂. The initial rate of oxidation of CySH is more concentration-dependent than is that of GSH (figures 1, 2, 5, and 6). The rate of oxidation of both substrates increases greatly up to at least 50 per cent O₂, as shown by sample data for GSH (figure 3). Over the range 10-100 per cent O₂, the rates increase linearly with log O₂ concentration. It should be noted that at 100 per cent O₂, the concentration in the liquid phase is about 1.1 mM, as compared with a substrate concentration of 24 mM.

Data indicate (table 1) that during the oxidation of CySH or GSH by treated spores, the substrates are converted quantitatively to the respective disulfides:



While oxygen uptakes found were only 85-90 per cent of theoretical, comparable relations were found employing ferric citrate as catalyst. This

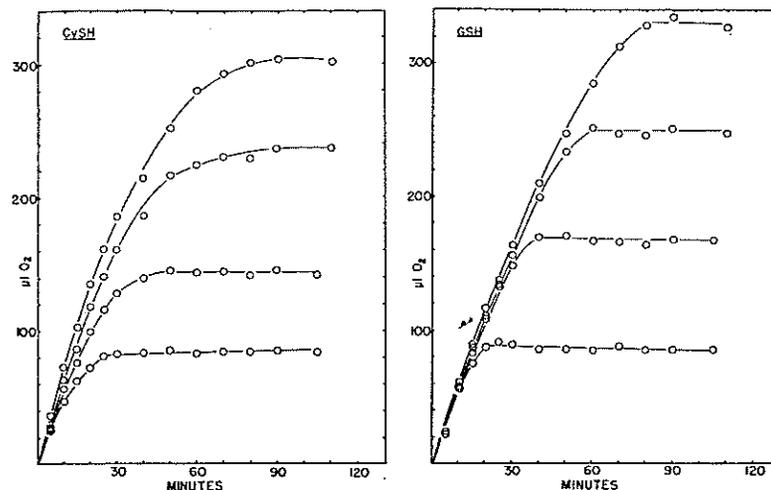


Figure 1 (left). Oxidation of cysteine (CySH) by toluene-treated spores. [CySH]—16, 32, 48, 64 μmoles/vessel; 6 mg spores/vessel; 0.2 M phosphate buffer pH 7.8; fluid volume 1.5 ml.

Figure 2 (right). Oxidation of glutathione (GSH) by toluene-treated spores. [GSH]—18, 36, 54, 72 μmoles/vessel. Other conditions as in figure 1.

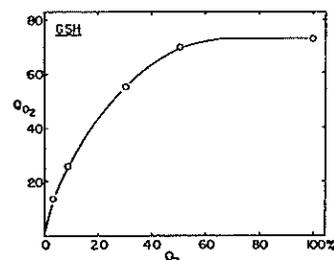


Figure 3. Effect of oxygen concentration on rate of oxidation of glutathione. 6 mg toluene-treated spores/vessel; 36 μmoles GSH; 0.2 M phosphate buffer pH 7.8; 1.5 ml fluid volume; Q₀₂ is initial rate of O₂ uptake in μL per mg dry wt spores per hr.

implicates some impurity or systemic error. No evidence was found indicating combination of substrate with some constituent of the spores. Evolution of H₂S, NH₃, or CO₂ could not be shown. Purity of the CySH was checked by titration with I₂ following the procedure of Lavine (1935). No significant impurities, such as cystine, were found.

Enzymatic nature and solubility. The enzymatic nature of the catalyst is indicated by its heat lability, either when associated with the

TABLE 1

Stoichiometry of CySH oxidation by toluene-treated spores and by Fe citrate (0.05 M phosphate, pH 7.8; 1.5 ml total volume)

System	CySH*	μL O ₂ †	Per Cent of Theor.
	mg/vessel		
4 mg spores...	2.83	78	87
4 mg spores...	5.65	154	86
4 mg spores...	11.3	324	90
8 mg spores...	5.65	169	88
8 mg spores...	11.3	305	85
Fe citrate‡...	11.3	313	87

* CySH·HCl·H₂O. H₂O content determined by drying *in vacuo* at 40 C.

† Final; about 60 min reaction time.

‡ 0.15 mg ferric citrate.

spores or when in spore extracts. Furthermore, the activity of spore extracts toward either CySH or GSH is not decreased by dialysis against phosphate buffer at pH 7.8. The enzyme is not associated with particulate matter in the extracts, since centrifugation at ca. 20,000 G does not effect sedimentation.

Effect of pH. The pH optimum for the oxidation of CySH is at about pH 7.5, with little or

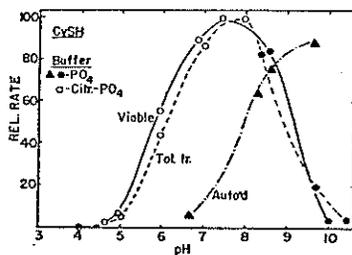


Figure 4. Effect of pH on oxidation of CySH by viable, toluene-treated, or autoclaved spores. On a spore weight basis, rate of oxidation by autoclaved spores is same at pH 9.5 as for toluene-treated nonautoclaved spores at pH 6.5. 0.2 M buffers; 32 μ moles CySH/vessel; 1.5 ml fluid volume.

no activity below 4.5 nor above pH 10 (figure 4). It should be noted that the pH-activity curve for viable spores is almost identical with that for alcohol (or toluene) treated spores. The effects of pH on the oxidation of GSH are essentially the same as for CySH. The activity curve for the "nonenzymatic catalyst" in autoclaved spores, as discussed below, is seen to be radically different from the other curves.

Substrate specificity. In addition to GSH and both D- and L-CySH, homocysteine and thiophenol are oxidized by the enzyme, whereas thiosalicylate, thioacetate, and thioglycolate are

TABLE 2

Oxidation of RSH compounds by toluene-treated spores (0.2 M citrate-phosphate buffer pH 8.0; substrate at $2.15 \times 10^{-2}M$)

Substrate	Source	Q_0 †
Glutathione	1*	63
D-Cysteine	2	57
L-Cysteine	3	72
DL-Homocysteine	1	28
Thiophenol	4	32
Thioglycolate	5	0
Thioacetate	4	0
Thiosalicylate	4	0
Isobutyl mercaptan	4	0

* Sources: 1—General Biochemicals, Inc.; 2—California Foundation for Biochemical Research; 3—Pfanstiehl Chemical Co.; 4—Eastman Kodak; 5—Fisher Scientific Co.

† μ L O_2 per mg dry wt toluene-treated spores per hr.

TABLE 3

Effect of enzyme inhibitors on the oxidation of CySH (ca. 9 mg alcohol-treated spores; 0.05 M citrate-phosphate buffer pH 8; inhibitors at $10^{-2}M$ incubated with spores about $\frac{1}{2}$ hr before addition of substrate; CySH at $2.15 \times 10^{-2}M$; fluid volume 1.5 ml)

Inhibitor	μ L O_2 at 10 min	Per Cent Inhibition
Control	99	—
KCN	96	3
Phenylthiourea*	100	0
Azide	100	0
Diethyldithiocarbamate	87	12
Iodoacetate	79	20
2,4-Dinitrophenol	69	30

* Not completely dissolved.

not (table 2). The relative rates of oxidation of CySH and GSH by spore extracts are the same as by intact spores or toluene-treated spores.

Effect of inhibitors. A number of typical enzyme inhibitors were tested and found to be ineffective, or only slightly inhibitory, at relatively high concentrations (table 3). The partial inhibition by iodoacetate is not due to combination with the CySH added, since it caused no diminution in total O_2 uptake. Apparently the iodoacetate reacted with spore constituents during incubation prior to addition of substrate.

Thioglycolate effectively inhibits the oxidation of both CySH and GSH (figures 5 and 6), apparently in a competitive manner. The extent of inhibition is a function of both substrate and inhibitor concentrations. Inhibition by thioglycolate is readily and completely reversed by washing the spores. No inhibition was encountered with any of the following compounds tested at the same molar concentration as the substrate (CySH): methionine, thiourea, thioacetic acid, isobutyl mercaptan, serine, phenol, and tyrosine.

Localization of the enzyme. It has been shown previously that treatment of spores of *M. verrucaria* with 0.1 N HCl or other acids does not kill the spores, yet inactivation of certain enzymes occurs (Mandels, 1953a, 1953c, 1954). It is presumed that such enzymes are located at the surface of the spores. Exposure to acid for as little as 5 seconds inactivates the RSH oxidizing enzyme of the spores (table 4). Both viable and toluene-treated spores behave similarly. Surface

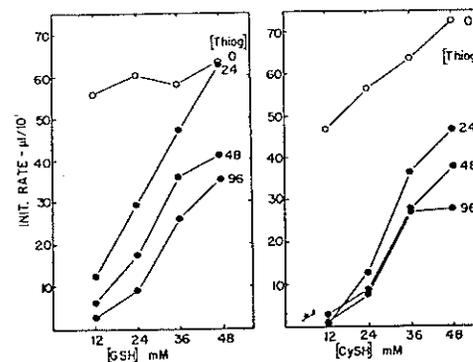


Figure 5 (left). Thioglycolate inhibition of GSH oxidation by toluene-treated spores. Figures at right of curves represent millimolar concentrations of thioglycolate. 6 mg spores/vessel; 0.2 M phosphate buffer pH 7.8; fluid volume 1.5 ml.

Figure 6 (right). Thioglycolate inhibition of CySH oxidation by toluene-treated spores. Conditions as in figure 5.

TABLE 4

Effect of acid treatment of spores on the oxidation of GSH

Spores	Treatment*	μ L O_2 at 10 min
Viable	0 sec acid	78
	5 sec acid	4
	30 sec acid	4
	Heated	0
Toluene treated	0 sec acid	47
	5 sec acid	0
	30 sec acid	0
	Heated	0

* 6 mg spores per vessel; 11 mg GSH per vessel; spores treated with 0.1 N HCl for indicated time at room temp., neutralized with 0.15 M K_2PO_4 . For 0 acid treatment, acid and buffer combined before adding to spores. Heated spores—placed in boiling water bath for 5 min.

localization of this enzyme is also indicated by the coincidence of the pH activity curves for viable and toluene-treated spores (figure 4).

Nonenzymatic catalyst in heated spores. Complete inactivation occurs by heating in phosphate at pH 7. However, if spores are autoclaved in citrate-phosphate at pH 8, CySH is oxidized at about half of the original rate. A number of experiments were carried out to establish the relation, if any, between the oxidation of CySH by non-heated and heated spores.

The pH characteristics alone (figure 4) separate the heat labile, enzymatic catalyst from that formed by heating spores. The high activity of autoclaved spores above pH 9.5, as contrasted with the low activity of nonheated spores at this pH, shows that a different catalyst is formed by heating spores and that it is not present in the unheated preparations. The specificity of this heat-formed catalyst is also different from that of the enzyme in that GSH is not oxidized. Homocysteine is oxidized at about one-half the rate of CySH. The catalyst is not inhibited by thioglycolate, but its activity is reduced about 50 per cent by cyanide or diethyldithiocarbamate at $3.3 \times 10^{-2}M$. It cannot be extracted from spores. Acid treatment does not destroy the activity of heated spores, nor does preliminary acid treatment prevent its formation.

DISCUSSION

The catalytic oxidation of certain sulfhydryl compounds by spores of *M. verrucaria* and extracts thereof is inferred to be enzymatic by virtue of its heat lability and resistance to dialysis. The participation of metal catalysts is further negated by the lack of inhibition by azide, cyanide, diethyldithiocarbamate or phenylthiourea. Resistance to these inhibitors also precludes involvement of RSH oxidation systems such as cytochrome, peroxidase, catalase, and ascorbic acid oxidase. Experiments involving dialysis also indicate that these indirect

systems, as well as the lipoxidase described by Mapson and Moustafa (1955), are not involved. Whereas the spores have relatively high catalase activity, this enzyme is completely inhibited by cyanide. Furthermore, the catalase of intact spores is resistant to acid treatment. Although the ascorbic acid oxidase of the spores is atypical in its resistance to copper and other heavy metal poisons, it is completely inhibited by isoascorbate (Mandels, 1953b). Tests not reported here show that isoascorbate does not inhibit the oxidation of CysSH or GSH. There is, therefore, no evidence identifying the enzyme with known indirect RSH oxidation systems. If some unknown system is involved, it or its components must be (a) nondialyzable, (b) surface localized and destroyed by acid, and (c) resistant to toluene or alcohol treatment of the spores.

Location of the enzyme at the spore surface is shown by coincidence of the pH activity curves for intact, as compared with toluene-treated, spores and by the rapid inactivation caused by brief exposures of intact spores to 0.1 N hydrochloric acid. If the enzyme were not surface localized, it should be relatively unaffected by variations in environmental pH with viable, as compared with toluene-treated, spores since the internal pH of viable cells is essentially constant. Similarly, since treatment of intact spores with acid does not kill the spores nor interfere with their respiratory metabolism (Mandels 1953c, 1954), it is presumed that the internal pH of the spores is not affected by the treatment. Enzymatic activities destroyed by acid treatment of viable spores are therefore inferred to be at the spore surface (see Mandels, 1953c, for further discussion). Myrbäck and Willstaedt (1955) have also employed this technique to localize yeast invertase at the cell surface.

ACKNOWLEDGMENTS

The author is indebted to Miss Anna B. Norton for capable technical assistance and to Drs. G. L. Miller and W. H. Stahl for their critical review of the manuscript.

SUMMARY

Spores of the fungus *Myrothecium verrucaria* contain a surface located enzyme which can oxidize both D- and L-cysteine, glutathione, homocysteine, and thiophenol to the respective disulfides. Thioacetate, thiosalicylate, and thioglycolate are not oxidized. As inferred from inhibitor studies, the enzyme does not require heavy metals for its activity. The enzyme in extracts of spores is not associated with particulate matter. Thioglycolate reversibly inhibits the enzyme, apparently in a competitive manner.

REFERENCES

- FROMAGEOT, C. 1951 Oxidation of organic sulfur. In *The enzymes*, Vol. 2, part 1, pp. 609-623. Ed. by J. B. Sumner and K. Myrbäck. Academic Press, New York.
- LAVINE, T. F. 1935 The iodometric determination of cysteine. *J. Biol. Chem.*, **109**, 141-145.
- LEMBERG, R. AND LEGGE, J. W. 1949 *Hematin compounds and bile pigments*. Interscience Publishers, Inc., New York.
- MANDELS, G. R. 1951 The invertase of *Myrothecium verrucaria* spores. *Am. J. Botany*, **38**, 213-221.
- MANDELS, G. R. 1953a The properties and surface location of an enzyme oxidizing ascorbic acid in fungus spores. *Arch. Biochem. and Biophys.*, **42**, 164-173.
- MANDELS, G. R. 1953b The atypical ascorbic acid oxidase in fungus spores—its inactivation by isoascorbate and its specificity. *Arch. Biochem. and Biophys.*, **44**, 362-377.
- MANDELS, G. R. 1953c Localization of carbohydrases at the surface of fungus spores by acid treatment. *Exptl. Cell Research*, **5**, 48-55.
- MANDELS, G. R. 1954 Metabolism of sucrose and related oligosaccharides by spores of the fungus *Myrothecium verrucaria*. *Plant Physiol.*, **29**, 18-26.
- MAPSON, L. W. AND MOUSTAFA, E. M. 1955 Oxidation of glutathione by a lipoxidase enzyme from pea seeds. *Biochem. J. (London)*, **60**, 71-80.
- MYRBÄCK, K. AND WILLSTAEDT, E. 1955 Studies on yeast invertase: localization of the enzyme in the cell and its liberation. *Arkiv Kemi*, **8**, 367-374.