The Cryptic Nature of Hepatic Microsomal Sulphite Oxidase

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1. The activity of rat liver microsomal sulphite oxidase (EC 1.8.3.1) was increased several-fold on aging of microsomes, on delipidation by extraction with acetone or on solubilization with deoxycholate, suggesting its existence in a cryptic state. 2. In rat liver most of the sulphite oxidase was present in the nuclear fraction and only a small portion in the microsomes. 3. Microsomal sulphite oxidase activity was low in the developing embryo and increased rapidly after birth.

Sulphite oxidase (sulphite-oxygen oxidoreductase, EC 1.8.3.1), the enzyme catalysing the oxidation of sulphite, the final step in the catabolism of cysteine sulphur, is of wide occurrence in animals (Macleod, Farkas, Fridovich & Handler, 1961) and also in human tissues (Mudd, Irreverre & Laster, 1967). Although mitochondria seem to be able to oxidize sulphite (Hunter & Ford, 1954; Arrigoni, 1959), Macleod *et al.* (1961) found that ox liver sulphite oxidase was largely localized in the microsomal fraction. Also, they observed that 'direct assay of freshly prepared microsomes before acetone powdering indicated very slight activity', implying the masked state of the enzyme in the microsomal fraction but without giving it further recognition.

After establishing the constitutive nature of ubiquinone in rat liver microsomes (Jayaraman & Ramasarma, 1963), we investigated its possible participation in some oxidative systems such as NAD-cytochrome *c* reductase (NAD-cytochrome *b*₅ oxidoreductase, EC 1.6.2.2) (Jayaraman & Ramasarma, 1965), reactivation of reduced ribonuclease (Ramakrishna Kurup, Raman & Ramasarma, 1966) and sulphite oxidase. During these studies we found that microsomal sulphite oxidase activity increased six- to ten-fold on treatment with deoxycholate, on aging or on extraction of the lipids with acetone, suggesting that the native enzyme exists in a cryptic state.

EXPERIMENTAL

Materials. Cytochrome *c* (horse heart, type II), NADH and deoxycholic acid were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of microsomes. Adult albino rats from the stock colony were killed by decapitation and the livers were homogenized in 0.25 M-sucrose containing 5 mM-EDTA and 10 mM-tris-HCl buffer, pH 7.4 (10 ml. of medium/g. of tissue), in a Potter-Elvehjem-type glass homogenizer. The

nuclear and mitochondrial fractions were removed by centrifuging at 8800g for 10 min., and the microsomal fraction was obtained by centrifuging at 37000g for 60 min., washing the sediment once and resuspending it in 0.25 M-sucrose (1 ml./g. of liver). Portions (0.1–0.2 ml.) of this suspension were used for enzyme assay. The amount of sucrose remaining in the suspension did not exert any influence on the enzyme activity.

Preparation of cell fractions. The homogenate from 16 g. of liver (from two rats) obtained as described above was filtered through a double layer of cheesecloth and centrifuged at 800g for 10 min. The supernatant was decanted, and the sediment was washed once with the homogenizing medium and recentrifuged at 800g for 10 min. The combined supernatant was centrifuged at the following increasing speeds successively, the sediment being separated each time: 3000g for 10 min., 8700g for 10 min., 10000g for 10 min., 14500g for 10 min., 37000g for 60 min. and 105000g for 60 min. All the sediments were suspended in 0.25 M-sucrose (1 ml./g. of liver used). The final supernatant was used as such. All centrifugations were carried out at 0–5° in a refrigerated Sorvall RC2 centrifuge, and for the high speed in a Spinco model L ultracentrifuge.

Determinations. Ubiquinone from microsomal fractions was obtained after saponification and was measured by the decrease in *E*₂₇₅ on adding NaBH₄ to an ethanolic solution of the sample (Jayaraman & Ramasarma, 1963).

Protein in the cell fractions was determined by the biuret method (Gornall, Bardawill & David, 1949), deoxycholate (0.02%) being used for solubilization.

Assay of sulphite oxidase and NAD-cytochrome *c* reductase activities. Sulphite oxidase activity was assayed by the reduction of cytochrome *c*. The reaction system consisted of tris-HCl buffer, pH 7.4 (250 μmoles), freshly prepared neutral KCN (0.6 μmole), cytochrome *c* (50 μmoles) and microsomes (0.5–1.0 mg. of protein) in a total volume of 2.4 ml. made up with water. The reaction was started by adding 0.1 ml. of freshly prepared 10 mM-Na₂SO₃ and the increase in *E*₅₅₀ was recorded in a Beckman DB recording spectrophotometer. The non-enzymic reduction was negligible under these conditions. Where mentioned, 0.1 ml. of neutral aq. 1% (w/v) deoxycholate was incorporated in the assay system.

For the assay of NAD-cytochrome *c* reductase activity

the same conditions were used with the following changes: cyanide and sulphite were replaced by antimycin A (1 μ g.) and NADH (50 μ moles) respectively, and added microsomal protein was about 0.05 mg.

The enzyme activities are expressed as milliunits, 1 unit being 1 μ mole of cytochrome *c* reduced/min.

RESULTS

Effect of aging of microsomes on sulphite oxidase activity. Freshly prepared microsomes from rat liver showed very low rates of reduction of cytochrome *c* with sulphite as the reducing agent (about 1–2 milliunits/mg. of protein) compared with a 200-fold higher rate with NADH. Samples of microsomes that were frozen and thawed a number of times showed the same low activity for 2 weeks, but the activity increased twofold after 3 weeks and nearly ninefold after 5 weeks (Table 1). At this late time NAD–cytochrome *c* reductase activity was completely lost.

Sulphite oxidase activity in acetone-extracted microsomes. In our attempts to test whether

ubiquinone participates in the sulphite oxidase system, the acetone-extracted microsomes were assayed for the enzyme activity. Under these conditions most of the ubiquinone and other lipids were removed (Jayaraman & Ramasarma, 1965) from microsomes, but sulphite oxidase activity was not lost, indicating non-involvement of ubiquinone

Table 1. *Effect of aging on microsomal sulphite oxidase activity*

Liver microsomal suspensions were stored in the frozen state for several weeks. The same sample was thawed to remove a portion at each stage for the assay of the enzyme activity and refrozen.

Storage time (weeks)	Sulphite oxidase activity (milliunits/mg. of protein)
Fresh	1.6
1	1.8
2	1.7
3	3.2
5	14.2

Table 2. *Sulphite oxidase activity in acetone-extracted microsomes*

Microsomal suspensions (5 ml., equivalent to 5 g. of liver) were added dropwise with stirring to cold (–30°) acetone (50 ml.). The mixture was centrifuged in a refrigerated centrifuge and the sediment was extracted twice with cold acetone (50 ml.) by stirring and centrifuged again. The residual acetone was removed *in vacuo* from the sediment, which was then suspended in 0.25 M-sucrose (5 ml.). The combined acetone extracts were evaporated, and after the traces of acetone were removed the extracted lipids formed a fine emulsion with the remaining water; this was made to volume (10 ml.) and used directly.

Fraction	Addition	Sulphite oxidase activity (milliunits/mg. of protein)
Microsomes	None	1.0
Microsomes	Acetone (0.1 ml.)	1.0
Acetone-extracted microsomes	None	6.5
Acetone-extracted microsomes	Acetone-extracted lipids (0.2 ml.)	6.4

Table 3. *Solubilization of microsomal sulphite oxidase by deoxycholate treatment*

Microsomal suspensions obtained from 5 g. of liver (5 ml. containing 138 mg. of protein) were mixed with 5 ml. of tris-HCl buffer (0.5 M), pH 8.0, and 2 ml. of neutral deoxycholate (1%), and the mixture was spun at 37000g for 90 min. in a refrigerated Sorvall centrifuge (extract I). The residue was re-extracted with 4 ml. of deoxycholate (1%) and 8 ml. of tris-HCl buffer (0.25 M), pH 8.0 (extract II). The two extracts and the residue, resuspended in water, were dialysed against water.

Fraction	Total protein (mg.)	Total ubiquinone (μ moles)	NAD–cytochrome <i>c</i> reductase activity		Sulphite oxidase activity	
			(milliunits/mg. of protein)	(total units)	(milliunits/mg. of protein)	(total units)
Microsomes	138	55	380	52.4	1.6	0.22
Microsomes + deoxycholate in assay medium	—	—	—	—	16.0	2.21
Extract I	66	13	110	7.26	20.8	1.37
Extract II	42	25	780	32.8	10.5	0.44
Residue	15	7	22	0.33	0.0	0.0

in this reaction. In fact, after delipidation, a suspension of acetone-extracted residue exhibited several-fold increased activity of the enzyme compared with the original microsomes (Table 2). This effect was not due to the mere presence of acetone, since added acetone in the reaction mixture had no effect on the enzyme activity. The activation seemed to be irreversible, since addition of equivalent amount of extracted lipids did not revert the activity to the initial value.

Solubilization of microsomal sulphite oxidase by deoxycholate treatment. Sulphite oxidase was recovered mostly in extract I (0.15mg. of deoxycholate/mg. of protein), whereas ubiquinone and NAD-cytochrome *c* reductase were largely found in extract II (0.3mg. of deoxycholate/mg. of protein). There was some sulphite oxidase in

extract II but none at all in the residue, which corresponds to the ribosomal fraction. Surprisingly, the total units of enzyme activity were eightfold higher than in the starting material. It was discovered that the presence of deoxycholate in the reaction mixture increased the enzyme activity nearly tenfold (Table 3). Addition of increasing concentrations of deoxycholate progressively increased the enzyme activity; maximum activity was attained at the concentration range 0.075–0.3ml. of aq. 1% (w/v) deoxycholate solution in the reaction mixture (Fig. 1). It appears that the enzyme was released at a critical concentration range, and excess was inhibitory. These characteristics are somewhat typical of a lysosomal enzyme, but treatment with Triton X-100 or incubation at pH 5.0 did not affect sulphite oxidase in microsomes. Further proof of its occurrence only in microsomes and not in lysosomes was obtained from a study of its intracellular distribution.

Distribution of sulphite oxidase activity in rat liver cell fractions. To establish whether the cryptic activity, exposed by treatment with deoxycholate or acetone, is localized in microsomes or not, the various cell fractions obtained by differential centrifugation of a sucrose homogenate of the liver were tested. The results in Table 4 show that fresh preparations of microsomes have low sulphite oxidase activity and that the activity increased on the addition of deoxycholate or extraction with acetone. This differential activity was primarily localized in heavy microsomes sedimenting at 37000g. The antimycin A-insensitive NAD-cytochrome *c* reductase activity, known to be localized in microsomes, and the cryptic sulphite oxidase activity (units/g. of liver) had parallel distributions in the various fractions (Fig. 2). Even

Fig. 1. Activation effect of deoxycholate on the microsomal sulphite oxidase. The enzyme activity was measured with 0.75mg. of microsomal protein with various amounts of added deoxycholate (1%) in the standard assay system.

Table 4. *Distribution of sulphite oxidase activity in rat liver cell fractions*

The preparation of the cell fractions by centrifuging sucrose homogenates of the liver at various speeds is described in the Experimental section.

Fraction no.	Centrifugal force (g)	Protein (mg./g. of liver)	Sulphite oxidase activity				Cryptic activity (milliunits/mg. of protein)
			Without added deoxycholate		With added deoxycholate		
			(milliunits/mg. of protein)	(% of total in fraction)	(milliunits/mg. of protein)	(% of total in fraction)	
1	800	57.6	45.0	75.0	46.5	67.0	1.5
2	3000	9.8	8.5	2.4	12.1	2.8	3.6
3	8700	7.9	8.0	1.8	8.9	1.8	0.9
4	10000	4.6	15.4	2.1	19.3	2.2	3.9
5	14500	3.7	3.1	0.3	18.2	1.6	15.1
6	37000	14.9	1.7	0.7	19.2	7.1	17.5
7	105000	5.6	2.8	0.5	8.4	1.1	5.6
8	Supernatant	40.8	14.7	17.2	16.3	16.4	1.6

when the full unmasked activity is taken into consideration the microsomal enzyme hardly accounted for 10% of the total in the rat liver, in contrast with nearly 80% found in ox liver (Macleod *et al.* 1961). In fact, the distribution of sulphite oxidase in the native state showed 75% in the nuclear fraction and about 17% in the soluble supernatant, with only 8% in the mitochondrial and microsomal fractions together (Table 4).

Sulphite oxidase in rat embryo microsomes. A comparison of microsomes prepared from rat foetuses at different stages of embryonic development showed that the sulphite oxidase activity was low before birth and appeared to increase rapidly after birth. The cryptic sulphite oxidase activity appearing on the addition of deoxycholate was very low and almost negligible in the 8-day embryo (Table 5). Therefore this feature of crypticity apparently developed only in the adult rat liver.

DISCUSSION

Three different types of treatment of microsomes, namely aging (which may be accompanied by some

Fig. 2. Intracellular distribution of NAD-cytochrome *c* reductase (□) and cryptic sulphite oxidase (■) activities in rat liver. The cryptic activity represents the increase over the native activity elicited by deoxycholate. The experimental conditions and the numbers for the fractions were the same as given in Table 4.

autodigestion), delipidation by acetone extraction and solubilization by deoxycholate involving rearrangement of lipid-protein organization, increased the activity of sulphite oxidase several-fold from the low native values. Therefore the enzyme is apparently present in a cryptic state when associated with the lipoprotein matrix of the membrane. One possibility is that some of the active sites in the native system are present in a latent state that may be unmasked on reorganization or removal of the lipids. The other alternative is that the molecular capacity of the active site(s) increased accompanying the conformational change on delipidation. In either case it is possible that this feature of crypticity might be utilized as a regulatory mechanism. Although this may be of value in ox liver, where most of the sulphite oxidase was found in microsomes (Macleod *et al.* 1961), it is of doubtful significance in the process of oxidation of sulphite in rat liver since here the activity in the whole homogenate increased only slightly from 25 to 28 milliunits/mg. of protein on the addition of deoxycholate, showing that most of the native enzyme was in the fully activated state. Also, most of the activity (67%) is present in the nuclear fraction (Table 4). This could not be ascribed to microsomal contamination since only a small fraction of the activity in the nuclear fraction was in the cryptic state. This poses the question whether the nucleus is in fact responsible for the oxidation of sulphite in the liver cell in this animal or whether the apparent activity was realized by sulphite reacting with some nuclear protein, e.g. an exposed disulphide group known to be reduced by sulphite. The specificity studies by Macleod *et al.* (1961) of the acceptors (oxygen, cytochrome *c*, ferricyanide and 2,6-dichlorophenol-indophenol) were carried out with the purified ox liver enzyme obtained from acetone-dried powders of whole tissue and the properties of the mixed enzyme may reflect a combination of the enzymes from different cell fractions.

It has been shown that the cryptic sulphite

Table 5. *Sulphite oxidase activity in microsomal preparations from developing rat embryo*

Microsomes from whole embryos were prepared in a manner similar to that used for rat liver. The age of the embryo was arbitrarily fixed depending on the day of mating and the size of the embryo.

Source of microsomal preparation	Sulphite oxidase activity (milliunits/mg. of protein)		
	Without added deoxycholate	With added deoxycholate	Cryptic activity
8-day embryo	0.3	0.4	0.1
18-day embryo	0.4	0.7	0.3
Newborn pup	0.8	1.5	0.7
Adult rat liver	1.6	20.8	19.2

oxidase activity in rat liver is localized in microsomes and is not of lysosomal origin. Another microsomal enzyme, glucose 6-phosphatase (EC 3.1.3.9), is known to be activated by deoxycholate (Beaufay & de Duve, 1954). It is also activated by digitonin and Triton X-100 (Segal & Washko, 1959) and by OH⁻ ions (Stetten & Burnett, 1966), unlike the activation of sulphite oxidase. Therefore the mechanisms of their activation are apparently different.

Sulphite oxidase assumed importance when an inborn error of absence of this enzyme was reported in a human infant who 'excreted in his urine abnormally large amounts of *S*-sulpho-L-cysteine, sulphite, and thiosulphate and virtually no inorganic sulphate' and died with 'neurological abnormalities, mental retardation, and dislocated ocular lenses' (Mudd *et al.* 1967). Despite the lack of knowledge on the nature of the enzyme in human tissues with regard to its intracellular distribution and contribution of the cryptic activity, it is evident that the amount of the enzyme was remarkably small in the defective infant. Our experiments with the rat embryo indicate that the enzyme was present in small amounts during development but increased after birth, presumably to meet the need of oxidative removal of sulphite. It may be conjectured that defective production

of the enzyme system during this stage may be responsible for the observed metabolic disorder.

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