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SUBCELLULAR LOCALISATION OF GUINEA PIG HEPATIC MOLYBDENUM HYDROXYLASES

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Molybdenum hydroxylase activity in guinea pig liver has been compared with that of marker enzymes in mitochondria(succinate dehydrogenase), microsomes(glucose-6-phosphatase) and cytosol (lactate dehydrogenase). Aldehyde oxidase activity was highest in the cytosol, with about 10-fold activity of xanthine oxidase. Significant molybdenum hydroxylase activity was found in mitochondria with minimal levels in microsomes. Mitochondrial and cytosolic aldehyde oxidase varied in substrate specificity and electrophoretic mobility with two major bands in each fraction, one of which was common to cytosol and mitochondria.

Aldehyde oxidase(Aldehyde-oxygen oxidoreductase EC 1.2.3.1) and xanthine oxidase(Xanthine-oxygen oxidoreductase EC 1.2.3.2) are molybdenum-containing enzymes which catalyse the oxidation of endogenous and exogenous N-heterocyclic compounds[1,2]. Unlike hepatic microsomal cytochrome P-450, most studies have indicated that the major part of molybdenum hydroxylase activity resides in the cytosol[3-7]. However Holmes and other workers, using electrophoretic techniques, have shown that there appears to be a small component of aldehyde oxidase activity in the microsomal pellet from horse, human and bovine liver [6,8,9].

In contrast Igo *et al*[10] reported significant levels of aldehyde oxidase in rat liver mitochondrial fractions. They included albumin in the fractionation buffer which was thought to prevent aldehyde oxidase leaching from the mitochondria during cell fractionation.

As the degree of integration of enzymes into an intracellular membrane or particle is highly variable, the absence/presence of a particular enzyme activity from a certain

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fraction may be misleading. The inherent uncertanties in subcellular localisation techniques are partially overcome by the selection of marker or indicator enzymes which are localised in a single intracellular site.

A systematic study was therefore undertaken which compared the subcellular localisation of guinea pig liver aldehyde oxidase and xanthine oxidase with that of characteristic marker enzymes to determine if microsomal or mitochondrial molybdenum hydroxylases are artifacts of the isolation procedure. Guinea pig was chosen in this investigation because of its similarity to man with respect to liver aldehyde oxidase[2]. These techniques have indicated the presence of a 'true' mitochondrial aldehyde oxidase isozyme with different kinetic properties to guinea pig liver cytosolic aldehyde oxidase.

MATERIALS AND METHODS

Phthalazine, p-iodonitrotetrazolium violet and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt were purchased from Aldrich Chemical Co. Ltd (Gillingham, UK) and menadione, sodium pyruvate, disodium succinate, nitro blue tetrazolium, phenazine methosulphate, xanthine, -NADH, Glucose oxidase Type II-S from Aspergillus niger, Peroxidase Type I from horseradish were obtained from Sigma Chemical Co. (Poole, UK). Protein assay dye reagent was from Pierce Ltd (USA) and carbazeran(6,7-dimethoxy-1-[4-(ethylcarbamoyloxy)piperidino]-phthalazine) was donated from the Compound Control Centre of Pfizer Central Research (Sandwich UK). 1-Phthalazinone, 2-methylphthalazinium iodide[2], 1-phenylquinolinium perchlorate and 1-methylquinolinium chloride[11] were synthesised according to published procedures.

Hepatic subcellular fractions were prepared from young, mature, male, Dunkin-Hartley guinea pigs, Sprague-Dawley rats or New Zealand White rabbits which were killed by cervical dislocation at approximately 0900 h. Freshly excised livers placed in ice-cold 0.05M Tris/0.25M sucrose/3mM $MgCl_2$ at pH 7.4 (8ml buffer/gm tissue) and all subsequent operations were carried out 4°C. The liver was homogenised using a Tri-R Stir-R Model s63C teflon homogeniser and the homogenates(10%, w/v) were centrifuged at 800 g for 10 min. The supernatant was centrifuged at 6000 g for 15 min and the mitochondrial pellet was washed twice by resuspension in buffer and centrifugation at 6000 g. The 6000 g supernatant was centrifuged at 100,000 g for 1 h and the microsomal pellet was washed twice by resuspension in buffer and centrifugation at 100,000 g.Each fraction (mitochondria, microsomes and cytosol) was reconstituted in the homogenisation buffer and stored as beads in liquid nitrogen. Partially purified aldehyde oxidase was prepared from these by heat denaturation and precipitation with ammonium sulphate[2].

Marker enzyme activity, mitochondrial succinate dehydrogenase (EC 1.3.99.1), microsomal glucose-6-phosphatase (EC 3.1.3.9) and cytosolic lactate dehydrogenase (EC 1.1.1.27), was measured as described by Critchley[12]. Molybdenum hydroxylase activity was determined at 37°C in 0.067 M phosphate buffer containing 0.13

mM EDTA according to Beedham *et al*[2]. Protein was assayed using the method of Bradford[13] .

Preparation of the agarose plates, electrofocusing of enzyme samples and staining of the plates was carried out according to the method of Johnson *et al*[14]. The pH gradient of the plates and pI values for the enzyme bands were determined using an LKB 2117-111 Multiphor Electrode attached to a Philips pH meter. There was no statistical difference between pI values interpolated from a pH gradient profile and those obtained by direct measurement using a pH meter ($P > 0.5$).

RESULTS AND DISCUSSION

Marker enzyme levels in each subcellular fraction(Table 1) were similar to those previously reported for rat liver[15]. Aldehyde oxidase activity, measured under substrate saturating conditions, was found to be highest in the cytosol whereas the mitochondrial fraction contained maximal amounts of xanthine oxidase activity. Molybdenum hydroxylase activity was detected in double-washed microsomes but at similar levels to cytosolic cross-contamination. Therefore, it is probable that the activity observed previously in horse, human and bovine microsomes[6,8,9] was also due to cytosolic cross-contamination as unwashed

TABLE 1. Molybdenum hydroxylase and marker enzyme activity in guinea pig liver cell fractions (n = 4)

	ACTIVITY IN EACH ENZYME FRACTION[a]		
Enzym	Cytosol	Mitochondria	Microsomes
Lactate Dehydrogenase	1.00	0.04 ± 0.01	0.07 ± 0.01
Succinate Dehydrogenase	1.00	12.93 ± 3.52	1.23 ± 0.23
Glucose-6-phosphatase	1.00	2.25 ± 1.03	40.39 ± 27.81
Aldehyde oxidase	1.00	0.12 ± 0.03	0.05 ± 0.02
Xanthine oxidase	1.00	1.52 ± 0.18	0.03 ± 0.01

[a]Enzyme activity was calculated as a ratio with respect to the cytosolic activity of each enzyme.

Activity in cytosol of Lactate dehydrogenase, 10,820 ± 502 μmol/min/mg protein; Succinate dehydrogenase, 2028 ± 170 Arbitary units/mg protein; Glucose-6-phosphatase, 0.225 ± 0.015 μmol/min/mg protein; Aldehyde oxidase, 0.252 ± 0.012 μmol phthalazine transformed/min/mg protein; Xanthine oxidase, 0.032 ± 0.004 μmol xanthine transformed /min/mg.

microsomal pellets were used for these studies without marker enzymes to follow the fractionation procedure. Alternatively, guinea pig liver may differ from that of other species.

In contrast,the ratios of mitochondrial/cytosolic levels of aldehyde oxidase and xanthine oxidase are significantly higher ($P < 0.01$ and $P < 0.001$, respectively) than those of lactate dehydrogenase which is strongly indicative of genuine mitochondrial molybdenum hydroxylase activity.

There was very little difference between the K_m values of phthalazine and carbazeran, determined with partially purified aldehyde oxidase from either mitochondria or cytosol(Table 2). In contrast markedly higher Michaelis-Menten constants were calculated for quaternary substrates with mitochondrial enzyme. Furthermore, the relative specific activities(V_{max} mitochondria /V_{max} cytosol x 100) for the uncharged heterocycles indicate that mitochondrial aldehyde oxidase levels are only 20-30% those of cytosolic enzyme yet similar or higher relative specific activities are obtained for cationic substrates. This indicates that there may be at least two forms of aldehyde oxidase, each specific for a particular subcellular site. This was further investigated by the use of analytical isoelectric focusing.

TABLE 2. Kinetic constants for heterocyclic substrates of cytosolic (n = 4) and mitochondrial (n = 2) guinea pig liver aldehyde oxidase

Substrate	Cytosol		Mitochondria		
	K_m (mM)	V_{max} µmol/min/mg protein	K_m (mM)	V_{max} µmol/min/mg protein	Ratio[a] x100
Phthalazine	0.06	0.263[b]	0.07	0.048	18.3
Carbazeran	0.04	0.019	0.06	0.004	21.1
2-Methyl-phthalazinium	0.10	0.110	2.20	0.268	243.6
1-Methyl-quinolinium	0.10	0.017	0.66	0.018	105.9
1-Phenyl-quinolinium	0.06	0.185	0.10	0.152	82.2

[a] Ratio V_{max} mitochondria/V_{max} cytosol.

[b] In each case oxidation of all substrates was completely inhibited by the addition of 0.1 mM menadione, a compound which strongly inhibits electron transfer between redox groups in aldehyde oxidase.

TABLE 3. Isoelectric values (pI) of guinea pig liver cytosolic and mitochondrial aldehyde oxidase

Enzyme fraction	pI (pH)		
	Band I	Band II	Band III
Cytosol (n = 5)	-	6.05 ± 0.08	6.29 ± 0.06
Mitochondria (n = 3)	5.3 ± 0.05	-	6.30 ± 0.03

The isoelectric points of the major bands in cytosolic and mitochondrial fractions are compared in Table 3. Two major bands of aldehyde oxidase activity were observed in each enzyme preparation, one of which appeared to be common to both fractions. However, the pI value for the second band in the mitochondrial fraction was significantly lower ($P < 0.001$) than that observed in the cytosol. Relative standard deviation(RSD) for pI values of the major cytosolic bands were 0.9%(n=6) and 1.2%(n=6) for the intra- and inter-guinea pig variation respectively. These low figures, which are close to the values quoted by Vesterberg and Svensson[16], indicate good reproducibility in the isoelectric focusing techinique .

Only one group of workers, Igo, Mackler and Duncan, have previously reported significant mitochondrial molybdenum hydroxylase activity[10]; these authors found that, in rat liver, the majority of aldehyde oxidase activity was sited in the mitochondria. Although other workers have reported variable cytosolic activity, they discarded the mitochondria and did not include albumin in their fractionation buffer[4]. We have also found low aldehyde oxidase activity in rat liver mitochondria. It would be interesting to repeat the fractionation of both rat and guinea pig liver in the presence of albumin to determine if higher mitochondrial levels of aldehyde oxidase are achieved.

Evidence for at least two isozymes of aldehyde oxidase in guinea pig liver comes from their varying substrate specificity and different pI values. However, it is not clear whether the same form of xanthine oxidase exists in each subcellular organelle. As there is little variation in substrate/inhibitor specificity between xanthine oxidase isolated from different

tissues or species[16,17] determination of the primary structure of the enzyme would be necessary to clarify the above point.

Guinea pig aldehyde oxidase shows a close resemblence to that in man[2,19], and thus a mitochondrial isozyme may also exist in human liver. Mitochondrial aldehyde oxidase could therefore be important in the metabolism of xenobiotics particularly cationic compounds. Perhaps more significant is the potential role of such an isozyme in the oxidation of aldehydes derived from endogenous amines in the mitochondria by monoamine oxidase. This is currently under investigation in our laboratories.

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