Subcellular Localization of Chicken Kidney Aryl Acylamidase Activity

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The intracellular localization of aryl acylamidase (aryl-acylamide amidohydrolase, EC 3.5.1.13) in chicken kidney was investigated. By separation on density gradients of the silica sol Ludox AM, the enzyme was localized in the mitochondrial fraction. This mitochondrial fraction was shown to be substantially free of lysosomal contamination. Sub-fractionation of the purified mitochondria indicates that the enzyme is located on the outer membrane, can be solubilized, and may be a suitable marker enzyme for kidney mitochondria.

Aryl acylamidase (aryl-acylamide amidohydrolase, EC 3.5.1.13) was first described by Nimmo-Smith (1960) in chicken kidney preparations, but this enzyme has also been reported to occur in other mammalian tissues (Williams & Jacobson, 1966; Mahadevan & Tappel, 1967), in insects (Gleeson *et al.*, 1976), in micro-organisms (Lanzilotta & Pramer, 1970; Kearney, 1965) and in plants (Still & Kuzirian, 1967; Frear & Still, 1968).

In rice plants the enzyme is associated with the first event in the metabolic fate of the herbicide 3',4'-dichloropropionanilide (propanil) and was shown to be sedimentable in a relatively mild centrifugal field (Still & Kuzirian, 1967). Subsequent studies designed to determine the intracellular location of the enzyme in rice plants met with limited success and do not allow us to draw unequivocal conclusions; however, if the rice enzyme is similar to the aryl acylamidase described by Nimmo-Smith (1960), then we should have parallel evidence to support our interpretation of rice data. Unfortunately, the criteria of Nimmo-Smith (1960) are unacceptable today, and rigorous proofs are required before unambiguous conclusions of intracellular localization can be drawn. We have therefore undertaken the task of establishing the intracellular localization of the acylamidase in chicken kidney, and report here the results of our study.

Experimental

Mitochondrial isolation

Cross-bred, unsexed chickens were killed by cervical dislocation and the kidneys quickly removed (approx. 10 g of kidney/1500 g chicken) and placed

in ice-cold MESH medium {225 mM-mannitol, 0.1 mM-EDTA, 75 mM-sucrose, 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.0}. Extraneous material was carefully removed, the tissue rinsed twice and then minced into small pieces with scissors. The tissue was then homogenized by two passes through a Potter-Elvehjem homogenizer and the resulting brei (approx. 220 mg of protein/ml) centrifuged at 2000 rev./min for 10 min (IEC B-20 centrifuge; A-870 rotor) to sediment heavy cell debris and unbroken cells. The supernatant was filtered through a double layer of cheesecloth and centrifuged for 10 min at 7500 rev./min. The pellet was resuspended in 10 vol. of MESH medium and centrifuged for 15 min at 8000 rev./min. This final washing was repeated and the pellet was termed 'washed mitochondria'.

Preparation of gradients

The washed mitochondria were further purified by isopycnic centrifugation in self-forming gradients of the silica sol Ludox AM (E. I. DuPont de Nemours and Co., Industrial and Biochemical Department, Wilmington, DE 19898, U.S.A.). For standard gradients, 10 ml of MESH medium (5-fold concentrated) was added to 37.5 ml of purified Ludox AM (Price & Dowling, 1977), pH 6.8 ($\rho = 1.12$ g/ml). To this was added either 2 g of Dextran T-40 (Pharmacia Fine Chemicals, Piscataway, NJ 08854, U.S.A.) or 2 g of polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, MO 63178, U.S.A.) and deionized water up to 100 ml. These gradients were designated LD-MESH and LP-MESH respectively. On centrifugation, a gradient of colloidal silica is established, but the concentrations of Dextran T-40 (or polyvinylpyrrolidone) and MESH buffer are constant throughout the tube.

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Isopycnic centrifugation of the washed mitochondria was carried out in a SB-206 swinging-bucket rotor (Damon/IEC, Needham Heights, MA 02194, U.S.A.) at 20000 rev/min for 90 min ($J\omega^2 dt$ $2.45 \times 10^{10} \text{ rad}^2 \cdot \text{s}^{-1}$). The 12 ml analytical tubes were fractionated with an IEC no. 3851 gradient fractionator and 0.55 ml fractions collected. The tubes determined to contain the mitochondrial fractions were pooled and diluted 4-fold with MESH medium. The mitochondria were then washed twice by centrifugation at 8000 rev./min for 15 min (IEC B-20 centrifuge; A-870 rotor). The final pellet was termed 'purified mitochondria' and kept for further fractionation.

The density of these various fractions collected from the gradients was determined as described by Morgenthaler *et al.* (1975).

Tissue fractionation

In order to examine the overall cellular distribution of the acylamidase, the fractionation scheme of de Duve *et al.* (1955) was employed, with the exception that the isolation medium was MESH, pH 7.0, as described above. All steps were carried out at 4°C and the fractions produced were termed N (nuclear), M (mitochondrial), L (lysosomal), P (microsomal) and S (final supernatant). The final $J\omega^2 dt$ values necessary to produce these fractions were $4.4 \times 10^7 \text{ rad}^2 \cdot \text{s}^{-1}$ for the nuclear, $2.4 \times 10^8 \text{ rad}^2 \cdot \text{s}^{-1}$ for the mitochondrial, $1.8 \times 10^9 \text{ rad}^2 \cdot \text{s}^{-1}$ for the lysosomal and $2.2 \times 10^{10} \text{ rad}^2 \cdot \text{s}^{-1}$ for the microsomal fraction.

Subfractionation of mitochondria

Purified mitochondria were subfractionated into outer membrane, inner membrane and soluble fractions as described by Sottocasa *et al.* (1967). At variance from that procedure, sonication times were varied from 1 to 30 s and power was varied from 3 to 7 A with a Heat Systems Sonifier Cell Disruptor (model 185D; Heat System Ultra-Sonics Inc., Plain-

view, NY, U.S.A.) in order to optimize breakage. In addition, breakage was also attempted by using the swelling-contraction method of Sottocasa *et al.* (1967), followed by either hand homogenization in a Ten-Broeck homogenizer or by passage through a French pressure cell (American Instrument Co., Silver Springs, MD, U.S.A.) at 4000 lb/in² (27.6 MPa).

Enzyme assays

Aryl acylamidase activity was assayed as described by Ray & Still (1975), with 3',4'-dichloropropionanilide (propanil, Monsanto Co., St. Louis, MO 63178, U.S.A.) as the substrate. Reactions were carried out at 37°C and the amount of 3,4-dichloroaniline released was measured as described by Pease (1962). Succinate dehydrogenase activities were measured as described by Quigley (1966), modified from the method of Ells (1959). Monoamine oxidase was assayed as described by Tabor *et al.* (1954) with benzylamine as the substrate. Cathepsin D was assayed by the method of Anson (1938), as further modified by Canonico & Bird (1970), and glucose 6-phosphatase essentially by the procedure of de Duve *et al.* (1955). P_i was measured by the Taussky & Shorr (1953) method. Protein was assayed as described by Lowry *et al.* (1951) with crystalline bovine serum albumin (Fraction V; Sigma Chemical Co.) as standard.

Results

Tissue fractionation

On a gross level, the specific activity of aryl acylamidase was found to be highest in chicken kidney (see Table 1), although significant activities were also found in liver and brain.

In order to obtain a rough estimate of the intracellular distribution of aryl acylamidase in kidney tissue, the fractionation scheme of de Duve *et al.* (1955) was followed. The results of a representative

Table 1. Gross distribution of aryl acylamidase in various tissues

In this experiment whole organs were removed, rinsed with ice-cold MESH medium, minced with scissors into small pieces, resuspended in ice-cold MESH medium and then homogenized by 20 passes through a Ten-Broeck homogenizer. Samples of these homogenates were then assayed directly for aryl acylamidase by incubation in 0.183 mM-3',4'-dichloropropionanilide for 15 min at 37°C. Specific activity is expressed as μg of 3,4-dichloroaniline released/h per mg of protein.

Tissue	Activity (μg of 3,4-dichloroaniline released/h)	Protein (mg)	Specific activity	Activity (% of activity in kidney)
Kidney	183	11.3	16.2	100.0
Liver	70	19.0	3.7	22.8
Muscle*	5	31.6	0.16	1.0
Brain	11	5.1	2.2	13.6

* Breast muscle only.

Table 2. Intracellular distribution of enzymes

Fractionation scheme is based on that of de Duve *et al.* (1955). Key: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, final supernatant. Total protein used in this experiment was 220mg.

Enzyme	Distribution (%)					Recovery (%)
	N	M	L	P	S	
Protein	14.7	20.2	7.4	22.5	35.2	95.0
Succinate dehydrogenase	10.1	70.2	16.8	2.8	0.1	92.1
Cathepsin D	5.9	31.0	49.8	9.7	3.6	95.6
Glucose 6-phosphatase	7.5	4.0	8.1	73.6	6.8	103.3
Aryl acylamidase	9.6	63.5	22.4	2.8	1.7	92.7

Fig. 1. Distribution pattern of enzymes

Fractions are represented in the order of their isolation, i.e. (from left to right) N, M, L, P and S (see Table 2). Each fraction is represented separately in the ordinate scale by its own relative specific activity (percentage of total recovered activity/percentage of total protein recovered). In the abscissa scale, each fraction is represented (cumulatively from left to right) by its protein content, expressed as a percentage of the total protein.

experiment are given in Table 2. With respect to the marker enzymes used, all three showed expected distribution patterns, i.e. succinate dehydrogenase was highest in total activity in the mitochondrial fraction, cathepsin D in the lysosomal fraction and glucose 6-phosphatase in the microsomal fraction. This can be seen even more clearly when expressed as relative specific activity (percentage of total activity/percentage of protein recovered) of the enzymes in their various fractions (Fig. 1).

Table 2 shows that approx. 85% of the amidase activity was recovered in the combined mitochondrial-lysosomal fractions. Although a greater total percentage of the enzyme was found in the mitochondrial fractions (63.5%) than in the lysosomal fraction (22.4%), the relative specific activities of the enzyme in these fractions are closely similar (3.1 for mitochondrial fraction; 3.0 for lysosomal fraction). Even so, the histogram of enzyme distribution for aryl acylamidase (Fig. 1d) more closely follows that of succinate dehydrogenase than that of cathepsin D.

Purification of washed mitochondria with Ludox AM

Since differential centrifugation was insufficient to localize the amidase, we turned to isopycnic separation in gradients of the colloidal silica Ludox AM. A unique feature of this silica sol is its ability to generate a density gradient when subjected to a centrifugal force. When washed mitochondria were centrifuged in Ludox containing either 2% Dextran T-40 or 2% polyvinylpyrrolidone, it was possible to resolve mitochondria and lysosomes by the use of marker enzymes. When washed mitochondria were sedimented in Ludox/Dextran gradients, mitochondria (succinate dehydrogenase activity) banded at a mean density of $1.095 \text{ g}\cdot\text{cm}^{-3}$, and lysosomes (cathepsin D activity) banded at a mean density of

Fig. 2. Sedimentation profile of washed mitochondria in Ludox/Dextran T-40 gradient

●, Density; ○, succinate dehydrogenase activity; △, cathepsin D activity; ■, aryl acylamidase activity. All activity is expressed as relative enzymic activity. For full details see the text.

Fig. 3. Sedimentation profile of washed mitochondria in Ludox/polyvinylpyrrolidone gradient

●, Density; ○, succinate dehydrogenase activity; △, cathepsin D activity; ■, aryl acylamidase activity. All activity is expressed as relative enzymic activity. For full details see the text.

$1.062 \text{ g}\cdot\text{cm}^{-3}$ (Fig. 2). The washed mitochondrial fraction behaved similarly in Ludox/polyvinylpyrrolidone gradients, except that mitochondria banded at $1.070 \text{ g}\cdot\text{cm}^{-3}$ and lysosomes were found at a density of $1.054 \text{ g}\cdot\text{cm}^{-3}$ (Fig. 3). In both cases the resolution of the mitochondrial and lysosomal fractions was good and showed little evidence of cross-contamination. In contrast with this, we have found very poor resolution between these two fractions when either Dextran or polyvinylpyrrolidone is omitted from the Ludox gradients (results not shown).

In all experiments where good resolution was obtained between the mitochondrial and lysosomal fractions, the amidase activity closely followed that of succinate dehydrogenase, indicating that this enzyme is associated with the mitochondrial fraction (Figs. 2 and 3). The pooled mitochondrial fractions from the Ludox gradients were washed and resuspended in MESH medium and served as the source of purified mitochondria for later fractionation.

Subfractionation of purified mitochondria

With the aryl acylamidase firmly established as a mitochondrial enzyme, we subjected purified mitochondria to several different fractionation schemes in an attempt at further localization. The methods used included swelling-contraction, homogenization by hand, sonication, and pressure disruption of the mitochondria. The results from several experiments are compiled in Table 3. In our experience, the amidase always behaves in a particulate fashion, sedimenting with a membrane fraction. In the subfractionation of purified mitochondria, however, we have found that sonication releases some of the enzyme into the soluble phase. As indicated in Table 3, sonication for 15 s at 3 A gave the highest specific activity of the enzyme ($54.1 \mu\text{g}$ of 3,4-dichloroaniline released/h per mg of protein) in the soluble fraction. This represented 39.7% of the total amidase activity. Increasing the sonication time to 30 s gave even higher percentages of total activity in the supernatant. When the sonication time was decreased to 5 s, the amount of total outer-membrane protein was decreased from 12.3 to 1.9%, but the specific activity of amidase in the soluble fraction was low ($18.9 \mu\text{g}$ of 3,4-dichloroaniline released/h per mg of protein), representing only 4.7% of the total activity.

In an effort to obtain better fractionation of mitochondria, homogenization by hand and the French pressure cell were utilized. After swelling and contraction, mitochondria were subjected to 40 passes through a Ten-Broeck homogenizer, which resulted in 91.6% of total protein in the inner membrane, 1.4% in the outer membrane and 7.0% in the soluble phase (see Table 3). The monoamine oxidase assay showed that all of the outer membrane was

Table 3. *Distribution of amidase activity on subfractionation of chicken kidney mitochondria by various procedures*

In each case, purified mitochondria were subjected to the swelling-contraction procedure as described by Sottocasa *et al.* (1967). Amidase activity is expressed as μg of 3,4-dichloroaniline released/h per mg of protein.

Fraction	Procedure ...			Hand homogenization			Sonication (15 s)			Sonication (5 s)			French press (4000lb/in ²)		
	Protein (%)	Amidase activity	Amidase recovery (%)	Protein (%)	Amidase activity	Amidase recovery (%)	Protein (%)	Amidase activity	Amidase recovery (%)	Protein (%)	Amidase activity	Amidase recovery (%)	Protein (%)	Amidase activity	Amidase recovery (%)
Intact mitochondria	100	15.1	—	100	13.2	—	100	94.2	—	100	94.2	—	100	94.2	—
Inner membranes	91.5	68.6	100	66.9	19.1	45.3	73.0	125.0	91.8	36.3	187.5	42.0	48.9	308.6	28.2
Outer membranes	1.4	0.0	0.0	12.3	34.6	15.1	1.9	180.0	3.4	14.8	308.6	28.2	48.9	98.8	29.8
Soluble proteins	7.0	0.0	0.0	20.7	54.1	39.6	25.1	18.9	4.8						

still associated with the inner membrane. Thus extensive homogenization by hand was unsuccessful in fractionating mitochondria. In addition, mitochondria were passed through a French pressure cell at 4000lb/in², which yielded 36.3% of total protein in the inner membrane, 14.8% in the outer membrane and 48.9% as soluble protein.

When a fractionation scheme was used that did not solubilize the enzyme, the highest specific activity of the amidase was found in the outer membrane of the mitochondria. When mitochondria were sonicated for 5 s or passed through a French press at 4000lb/in², the highest specific activities were located in the outer membrane fraction and were 180 and 308.6 μg of 3,4-dichloroaniline released/h per mg of protein respectively. In all cases the highest specific activity of monoamine oxidase activity was also located with the outer-membrane fraction.

The results of a typical experiment are presented in Table 4. Although the amidase activity was found in all fractions, the specific activity is highest in the outer membrane. Succinate dehydrogenase was found exclusively in the inner membrane, whereas monoamine oxidase activity was confined to the outer membrane. In this particular experiment the outer membrane represented only 1.9% of the total protein, but this value might be as high as 15% if more severe fractionation procedures are used.

Solubilization of aryl acylamidase

With the use of various detergents Nimmo-Smith (1960) was unable to solubilize the active enzyme. We have exposed purified mitochondria to various times of sonication (see Fig. 4). Exposure of the purified mitochondrial fraction to 20s of sonication is sufficient to remove approx. 80% of the total amidase activity, but the remaining 20% could not be appreciably solubilized even after 45 s of sonication. The solubilized activity could not be sedimented

Fig. 4. *Solubilization of aryl acylamidase by sonication* Portions of purified mitochondria were subjected to various times of sonication at 3A and the amount of activity released from the membranes was measured.

Table 4. *Distribution of enzyme activities in subfractions of chicken kidney mitochondria after sonication*

Purified mitochondria were sonicated for 5 s at 3A in a Branson sonicator. Amidase specific activity is expressed as μg of 3,4-dichloroaniline released/h per mg of protein. Succinate dehydrogenase specific activity is in units/min per mg of protein; one unit of activity is defined as that amount of enzyme causing a decrease of $0.001 A_{600}$ unit/min, corrected for endogenous activity. Monoamine oxidase specific activity is in units/min per mg of protein: one unit of activity is defined as that amount of enzyme causing an increase of $0.001 A_{250}$ unit/min.

Fraction	Protein (%)	Amidase		Succinate dehydrogenase		Monoamine oxidase	
		Activity	Recovery (%)	Activity	Recovery (%)	Activity	Recovery (%)
Intact mitochondria	100	94.2	—	534	—	65	—
Inner membrane	73.0	125.0	91.8	2382	99.9	0	0
Outer membrane	1.9	180.0	3.4	60	0.1	311	100
Soluble proteins	25.1	18.9	4.8	0	0	0	0

after centrifuging for 1 h at 60000 rev./min (IEC B-60 centrifuge; A-231 rotor). Increasing the amperage of sonication did hasten the removal of the 80%, but did not remove the residual activity (results not shown).

Discussion

From the data presented we have confirmed the report of Nimmo-Smith (1960) that the aryl acylamidase of chicken kidney is associated with mitochondria, and more specifically with the outer membrane. We base these conclusions on the observation that the specific activity of the enzyme is highest in these fractions and that it is always associated with monoamine oxidase activity.

Previous attempts at localizing this enzymic activity by differential centrifugation proved inadequate (Nimmo-Smith, 1960; Still & Kuzirian, 1967) in that no resolution was obtained between the mitochondrial and other fractions. Most recent work by several investigators has demonstrated the utility of silica sols in subcellular fractionations [cf. reviews by Pertoft & Laurent (1968), Schmitt & Herrmann (1977) and Wolff (1975)]. Colloidal sols such as Ludox AM provide a nearly ideal medium for the isolation of cell particles, since they have low viscosity, are non-toxic to cells, and have only moderate osmotic effects. These sols have been successfully employed for the separation of lysosomes from HeLa cells (Wolff & Pertoft, 1972) and from rat liver parenchymal cells (Pertoft *et al.*, 1978), and the work reported here extends the general usefulness of this method. As noted by others (Morgenthaler *et al.*, 1975; Morgenthaler & Price, 1976), the addition of various polymers to the silica sols causes shifts in the banding densities of these particles. The present paper shows that the addition of either polyvinylpyrrolidone or Dextran T-40 to Ludox AM affords adequate resolution between mitochondrial and lysosomal fractions. However, we have only been able to obtain active mitochondria which

demonstrate respiratory control from the Ludox/Dextran T-40 gradients.

Another unique feature of these colloidal silica sols is their ability to form a density gradient in a centrifugal field (Pertoft, 1966). Owing to the heterogeneous nature of the silica particles, one obtains a sigmoidal density profile which can be alleviated somewhat by addition of polysaccharides. In this study we have used both self-forming and pre-formed gradients to separate mitochondria and lysosomes, and find no difference between the two. The ease of operation and the time saved in not pre-forming gradients should make the use of these self-forming silica gradients very attractive.

Since the osmotic pressure of Ludox AM is low (20 mosmol/kg of water; Pertoft, 1966), the banding densities obtained for mitochondria and lysosomes should be nearer their true values (Wolff, 1975). Indeed, the densities obtained in the present study agree well with those of other investigators who used silica sols, but, understandably, disagree strongly with those obtained in high-osmotic-pressure medium such as sucrose, which causes a 'wringing-out' effect on cells and membranous organelles. The reason for the density shifts of these organelles caused by polymer additions to the silica sol remains unclear. Morgenthaler & Price (1976) suggest that this may be due to varying degrees of hydration afforded by these polymers to the silica particles, a phenomenon that they have called the 'silica-exclusion' effect.

The observation that the aryl acylamidase can be selectively removed from the outer mitochondrial membrane may prove to be a useful technique for the further purification of this enzyme, although more data will be needed on the stability of this solubilized enzyme. It is noteworthy that not all of the enzymic activity can be removed from the membrane. We have shown that approx. 20% of this enzyme remains attached to the membrane even after extensive sonication.

Aryl acylamidase activity has been detected in a

wide variety of organisms (see the introduction) and shows remarkable similarities in both substrate specificity and sensitivity to inhibitors. Although the mammalian enzyme is found mostly in kidney (Nimmo-Smith, 1960; Mahadevan & Tappel, 1967), it has also been detected in brain tissue (Hsu *et al.*, 1977), and we have also shown it to be present in liver.

There is some evidence to suggest that this enzyme may have esterase activity. Frear & Still (1968) demonstrated the sensitivity of the rice aryl acylamidase to several esterase inhibitors, and several investigators have reported aryl acylamidase activity in acetylcholinesterase (Fujimoto, 1976; Oommen & Balasubramanian, 1978). The relationship between the esterase and the amidase activities is unclear, especially in light of the fact that the physiological substrate(s) for the latter is still unidentified.

The fact that this enzyme is located on the outer mitochondrial membrane is of interest, because of the relatively few enzymes associated with this fraction, and may eventually shed some light on its physiological significance. Owing to the simplicity of the assay and the stability of the enzyme, we suggest that aryl acylamidase might be a specific marker enzyme for the outer membrane of chicken kidney and other mitochondria.

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