Histidine decarboxylase from rat mast cells

Enhanced recovery in cell-free extracts and isotopic labelling

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A procedure for obtaining rat mast-cell histidine decarboxylase in greater than 50 % yield in cell-free extracts was developed. The enzyme was found in the supernatant fractions from a 3500 *g* and a 105000 *g* centrifugation step and was demonstrated to be sensitive to inhibition by α -fluoromethylhistidine but not by phenylalanine. Although the enzyme shows a half-life of only 3 h in cell-free extract, the initial high recovery of activity allowed for active-site labelling of the enzyme by [^3H]histidine and NaBH_4 . Labelled protein migrated on non-denaturing polyacrylamide-gradient-gel electrophoresis as a 55000 Da species.

INTRODUCTION

The biogenic amine histamine has an important role in functions as diverse as inflammation, gastric acid secretion, tissue growth and repair, neurotransmission and the allergic response [1–3]. In this last-mentioned role, histamine is a key signal, causing, among other reactions, an increase in vascular permeability and vasodilation. The primary source of histamine for the allergic response is the mast cell, a widely distributed fixed tissue cell that is the principal non-neuronal site of histamine synthesis and storage [4–6]. The synthesis of histamine is catalysed in a single step from histidine by the enzyme histidine decarboxylase (EC 4.1.1.22). This enzyme has been purified to homogeneity from fetal rat liver [7] and from mouse kidney [8]; from both sources it appears to be a dimer composed of two subunits, each of approx. 54000 Da. Using chromatofocusing, Taguchi *et al.* [7] demonstrated that the fetal rat liver enzyme had an isoelectric point of 5.1, whereas the enzyme from mouse kidney was shown by isoelectric focusing to have three peaks of activity with pI values of 5.3, 5.5 and 5.7 [8]. Although mast cells show the highest level of histidine decarboxylase activity in whole-cell assays, attempts to isolate histidine decarboxylase from mast cells have been unsuccessful because more than 95 % of the enzyme activity is lost upon disruption of the cell [9,10]. As a result, most of what is known about histamine formation in mast cells has been derived from assays with whole cells. In the present paper we describe a cell-lysis procedure that allows for the recovery of over 50 % of the whole-cell activity. Although the enzyme is still quite labile in cell extracts, this initial high recovery of activity has allowed us to carry out subcellular localization of the enzyme. In addition, we have designed a procedure to affinity-label the enzyme to high specific radioactivity by using [^3H]histidine and NaBH_4 . This new labelling procedure should allow further purification and study of the enzyme.

MATERIALS AND METHODS

Purification of mast cells

Mast cells were obtained from the peritoneal and pleural cavities of male Sprague–Dawley rats (body wt.

250–300 g) as previously described [11]. Hepes/Locke buffer contained 20 mM-Hepes, 150 mM-NaCl, 5 mM-KCl and 1 mg of bovine serum albumin/ml at a final pH of 7.2. Cells were harvested by centrifugation at 400 *g* for 5 min, resuspended in Hepes/Locke buffer and purified to better than 95 % homogeneity by centrifugation through a 22.5 % (w/v) metrizamide step as described by Slutsky *et al.* [12]. Cell purity was judged by cell counts under phase-contrast microscopy with a haemocytometer.

Enzyme assay

Histidine decarboxylase activity was measured by the release of $^{14}\text{CO}_2$ from [*carboxy*- ^{14}C]histidine (New England Nuclear) as described by Beaven [13]. The standard reaction mixture contained 5×10^4 mast cells, 10 μM -pyridoxal 5'-phosphate, 20 nCi of [*carboxy*- ^{14}C]histidine, 145 μM total histidine in 20 mM-Hepes buffer, pH 7.2, containing Hanks balanced salts (GIBCO) and 1 mg of bovine serum albumin/ml in a total volume of 40 μl in a 1.5 ml microcentrifuge tube. The tube was placed in a 20 ml liquid-scintillation vial containing 50 μl of Protosol (New England Nuclear) and the vial was tightly capped. Samples were incubated for 60 min at 37 °C with shaking. The reaction was stopped by the addition of 20 μl of 2 M-HClO₄ and shaken for an additional 30 min. The reaction tube was then removed and 10 ml of Econofluor-2 (New England Nuclear) scintillation cocktail was added. One unit of enzyme activity is defined as that amount of enzyme that will liberate 1 pmol of CO_2 /h at 37 °C. Activity values are presented as means $\pm$ S.E.M. with the numbers of observations in parentheses. Intact cells were suspended in the Hepes/Hanks buffer described above for assay of histamine-synthetic capability.

Cell lysis

Purified mast cells (1.4×10^6 cell/ml) were suspended at 4 °C in sonication buffer consisting of 20 mM-Hepes buffer, pH 7.2, containing 10 μM -pyridoxal 5'-phosphate, 100 μg of phenylmethanesulphonyl fluoride/ml, 0.1 mM-EGTA and 340 mM-sucrose. This cell suspension was exposed to ultrasonic oscillation from a Branson sonicator equipped with a microtip at an intensity of 90 W for 3 s, and was then centrifuged at 150 *g* for 5 min to

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Table 1. Histidine decarboxylase recovery in cell-free extracts prepared under different conditions of sonication

Purified mast cells were exposed to ultrasonic disruption as described in the Materials and methods section. The sonicated extract was centrifuged at 150 *g* for 5 min and then at 3500 *g* for 20 min, and assays were carried out on the final supernatant. The sucrose-free sonication buffer was either hypo-osmotic or made iso-osmotic by addition of 150 mM-NaCl+5 mM-KCl. The histamine released is measured in the 3500 *g* supernatant. Data are means $\pm$ S.E.M. and numbers of observations are given in parentheses.

Treatment	Histidine decarboxylase activity		Histamine released (% of that in whole cell)
	(units/10 ⁶ cells)	(% of that in intact cell)	
Whole cells	328 $\pm$ 6 (8)	100	100
Cell-free extract			
+ Sucrose	194 $\pm$ 3 (9)	59	24
- Sucrose (hypo-osmotic)	95 $\pm$ 5 (5)	29	77
- Sucrose (iso-osmotic)	90 $\pm$ 3 (4)	28	44

remove whole cells and large cellular debris. The resulting supernatant fraction (S1) was then centrifuged at 3500 *g* for 20 min to remove intact granules. The supernatant fraction from the 3500 *g* spin (S2) was used for assay of histidine decarboxylase activity. In previous work we have demonstrated that the pellet from the 3500 *g* centrifugation contains the membrane-bound secretory granules, as determined by their histamine content [14].

Radioactive labelling of histidine decarboxylase with [³H]histidine and NaBH₄

The S2 supernatant fraction prepared by ultrasonic disruption of 2×10^6 mast cells in 1 ml of sonication buffer was incubated with 50 μ Ci of [³H]histidine for 30 min at 4 °C. Freshly prepared NaBH₄ (1 M) was then added to a final concentration of 100 mM and incubation was continued for 20 min at 0 °C followed by 30 min at 25 °C. The incubation mixture was then adjusted to pH 7.2 by the addition of 0.1 M-HCl. This material was then analysed by electrophoresis on a 10–20 % polyacrylamide gradient gel in 30 mM-Tris/borate buffer, pH 8.3, for 8000 V·h. The sample lanes were then cut into 5 mm slices, placed in vials containing 10 ml of Econofluor-2/Protosol (97:3 v/v) and incubated for 15 h at 37 °C, and the radioactivity was determined by liquid-scintillation counting.

RESULTS

Histamine-synthetic capacity in whole mast cells

The histidine decarboxylase activity of purified, intact, rat peritoneal and pleural mast cells as measured by ¹⁴CO₂ release from [*carboxy*-¹⁴C]histidine was 345 ± 33 units/10⁶ cells (*n* = 42). In comparison, peritoneal and pleural cell washings obtained from the metrizamide-Locke buffer interface during purification, and deficient in mast cells, gave only 40 units/10⁶ cells. The relationship between CO₂ release and mast cell number was found to be linear up to 1.5×10^5 cells/assay (results not shown). To determine if this decarboxylation was due specifically to histidine decarboxylase, the specific histidine decarboxylase inhibitor α -fluoromethylhistidine was

utilized. Incubation of mast cells with 20 μ M- α -fluoromethylhistidine for 30 min before the addition of histidine resulted in an 88 % inhibition of ¹⁴CO₂ release.

Recovery of histidine decarboxylase activity from disrupted mast cells

As shown in Table 1, sonication of mast cells for 3 s in sonication buffer containing 340 mM-sucrose gave an S2 fraction that contained 59 % of the histidine decarboxylase activity found in non-disrupted cells. Under these conditions more than 95 % of the cells were broken, as indicated by visual inspection under the phase-contrast microscope ($\times 400$ magnification) and by the release of lactate dehydrogenase activity to the soluble fraction [14]. However, the S2 supernatant fraction contained at most 24 % of the total cell histamine (a range of 15–24 % was observed in different experiments); the remainder of the histamine was recovered in the granule pellet. Ultrasonic disruption in sonication buffer lacking sucrose gave only a 29 % recovery of the histidine decarboxylase activity found in whole cells. In this case the S2 supernatant contained 77 % of the total cell histamine, indicating extensive breakage of secretory granules. Sonication in an iso-osmotic Hepes/Locke buffer containing EGTA, pyridoxal 5'-phosphate and phenylmethanesulphonyl fluoride also resulted in greater breakage of secretory granules than in the sucrose-containing buffer and yielded only 28 % of the whole-cell histidine decarboxylase activity.

Compared with the results of previous workers, who recovered less than 5 % of the whole-cell histidine decarboxylase activity in cell-free extracts, these results are quite encouraging. It appears that the key to obtaining the recovery of activity is in ensuring minimal breakage of the secretory granules by using a combination of short sonication time, low power output and 340 mM-sucrose in the sonication buffer.

Intracellular distribution of histidine decarboxylase activity

Cell-free extracts were fractionated by differential centrifugation and the histidine decarboxylase activity was determined in each fraction. The granule fraction (3500 *g* pellet) contained only 4 % of the whole-cell

Fig. 1. Stability of histidine decarboxylase in cell-free extracts

Histidine decarboxylase assays were carried out at various times following cell lysis on the 3500 *g* supernatant stored at 4 °C. For experimental details see the text.

histidine decarboxylase activity while the supernatant fraction (S2) contained 49 % of the whole-cell histidine decarboxylase activity. Centrifugation of the S2 supernatant fraction at 105 000 *g* for 60 min resulted in the recovery of essentially all the histidine decarboxylase activity in the supernatant fraction, suggesting that this enzyme is not tightly associated with intact granules or membranes. Since the mast cell contains virtually all of its histamine within its secretory granules, the localization of the histidine decarboxylase to the cytoplasmic compartment suggests that the transport of histamine into the granule may not be a vectorial process during synthesis, but rather an ion-exchange uptake, perhaps due to the negatively charged heparin within the granule.

Inhibition by α -fluoromethylhistidine

To confirm that the activity that we have measured was indeed histidine decarboxylase and not a non-specific aromatic amino acid decarboxylase, the effects of 20 μ M- α -fluoromethylhistidine and 200 μ M-phenylalanine on activity in the S2 supernatant were compared. From a mast-cell preparation that demonstrated a histamine-synthetic capability of 370 units/10⁶ cells we obtained a 3500 *g* supernatant with histidine decarboxylase activity of 194 units/10⁶ cells. Preincubation of the 3500 *g* supernatant with 20 μ M- α -fluoromethylhistidine resulted in recovery of less than 5 % of the histidine decarboxylase activity, whereas preincubation with 200 μ M-phenylalanine yielded no loss of activity. These results indicate that the cell-free activity being measured is indeed histidine decarboxylase.

Stability of histidine decarboxylase activity

Although we are able to obtain 10-fold higher levels of histidine decarboxylase activity in cell extracts than

Fig. 2. Non-denaturing polyacrylamide-gradient-gel electrophoresis of [³H]histidine-labelled protein

Cell-free extract was incubated with labelled histidine and then reduced with NaBH₄ before being loaded on a non-denaturing 10–20 % polyacrylamide gradient gel, which was run for 8000 V · h. Lanes were sliced into 5 mm bands and analysed for label by liquid-scintillation counting. In (b) and (c) the extract was preincubated for 30 min before addition of label with 20 μ M- α -fluoromethylhistidine (b) or with 200 μ M-phenylalanine (c). Arrows indicate the location of protein standards localized by Coomassie Brilliant Blue staining of an adjacent lane. Standards are: catalase (232 kDa), glyceraldehyde-3-phosphate dehydrogenase (134 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa).

previous workers, our preparation still shows a limited stability. No loss of activity at 0 °C was observed in the S2 supernatant over the initial 3 h following sonication, but a rapid loss then followed (Fig. 1). However, this initial recovery and stabilization has proven important, for it has allowed us to design a procedure for affinity-labelling the enzyme utilizing radiolabelled histidine of high specific radioactivity.

[³H]Histidine labelling of histidine decarboxylase

Previous workers have utilized the suicide inhibitor α -fluoromethylhistidine [15] as a means to label histidine

decarboxylase covalently [16,17]. Kubota *et al.* [16] examined the inactivation of the fetal rat enzyme and found that decarboxylation was necessary for activity loss, and Taguchi *et al.* [7] demonstrated that stable incorporation of α -fluoromethylhistidine required reduction of the inactive enzyme by NaBH_4 . A more detailed analysis of the mechanism of inactivation was carried out by Hayashi *et al.* [17], who examined the pyridoxal 5'-phosphate-dependent enzyme from *Morganella morganii* AM-15. However, in all these studies the low specific radioactivity of the α -fluoromethylhistidine available (12 mCi/mmol) has meant that the enzyme could not be highly labelled. We have found that it is possible to obtain a labelled protein with high specific radioactivity by the use of high-specific-radioactivity histidine (20 Ci/mmol) and NaBH_4 . The S2 supernatant fraction was incubated with [^3H]histidine followed by reduction with NaBH_4 to yield a stable secondary amine between the labelled histidine and the enzyme-bound pyridoxal 5'-phosphate. The labelled S2 supernatant fraction was then subjected to non-denaturing polyacrylamide-gradient-gel electrophoresis as described in the Materials and methods section. Fig. 2 shows that labelled material migrated as a rather broad band with a peak between 53000 and 60000 Da, the expected histidine decarboxylase subunit molecular mass. That this labelled peak was indeed histidine decarboxylase was indicated by the fact that preincubation with 20 μM unlabelled α -fluoromethylhistidine gave a 67% inhibition of [^3H]histidine incorporation whereas preincubation with 200 μM unlabelled phenylalanine gave no decrease in label incorporation (Figs. 2b and 2c).

This procedure should bind histidine (or its decarboxylation product) covalently to the pyridoxal 5'-phosphate via a stable secondary amine. Although the pyridoxal 5'-phosphate-histidine complex is not covalently bound to the enzyme, pyridoxal 5'-phosphate cofactors are generally very tightly bound to their associated proteins [18]. Indeed, conditions expected to denature the enzyme, such as acid precipitation or SDS/polyacrylamide-gel electrophoresis, resulted in a loss of the protein-associated label (results not shown). Migration of the label as a species of approx. 55000 Da suggests that under these conditions the enzyme is fully dissociating into its subunits without loss of any cofactor. It will be of interest to determine if the loss in histidine decarboxylase activity is associated with a dissociation of the enzyme into its subunits.

Even though mast-cell histidine decarboxylase is unstable in cell-free extracts, our ability to obtain an extract with high initial recoveries of activity has allowed us to label the enzyme to high specific radioactivity.

Furthermore, label incorporation is stable as long as non-denaturing conditions are maintained. This result suggests that it will be possible to design a purification scheme for histidine decarboxylase based on radiolabel localization rather than activity and that the purified enzyme could then be used to obtain information about the sequence and other physical properties of the enzyme.

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