Solubilization and purification of rat liver 5'-nucleotidase by use of a zwitterionic detergent and a monoclonal-antibody immunoabsorbent

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1. A variety of detergents were used to solubilize 5'-nucleotidase from rat liver plasma membranes. 2. The zwitterionic detergent Sulphobetaine 14 gave optimal solubilization by the criteria of release into a high-speed-centrifugation supernatant and the formation of the smallest and least polydisperse active enzyme observed on polyacrylamide-gel electrophoresis. 3. The Sulphobetaine 14-solubilized enzyme from rat liver was purified by using the conventional techniques of ion-exchange chromatography and gel filtration, or by an immunoaffinity step with a monoclonal-antibody immunoabsorbent. 4. 5'-Nucleotidase was purified at least 12000-fold relative to liver homogenate by the immunoaffinity purification scheme and had a specific activity in the range 285–340 $\mu\text{mol/min}$ per mg of protein. The yield was in the range 9–16%. 5. The purified enzyme shows a major polypeptide band of apparent M_r 70000 on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and a minor band of apparent M_r 38000. 6. A rational approach to the general problem of the purification of minor intrinsic membrane proteins is discussed, with the use of polyacrylamide-gel electrophoresis to determine the most appropriate detergent and monoclonal antibodies in subsequent immunoaffinity purification.

5'-Nucleotidase (EC 3.1.3.5) is an intrinsic glycoprotein of the plasma membrane that has been shown to be an ectoenzyme present in a variety of nucleate mammalian cells (Evans, 1978). It has an important physiological function in the production of adenosine from extracellular nucleotides (Newby, 1980; Pearson *et al.*, 1980; Newby & Holmquist, 1981).

The enzyme has been shown to circulate through the cell via intracellular membrane vesicles (Stanley *et al.*, 1980), though both the function of this circulation and its mechanism are unknown. Characterization of the enzyme and in particular of its relationships with other membrane proteins and cytoskeletal proteins has been hindered by lack of a suitable purified preparation. Previously, the enzyme has been partially purified from rat liver as a sphingomyelin-enzyme complex (Widnell, 1975), from which the phospholipid is difficult to remove (Merisko *et al.*, 1981), or solubilized in detergent with low yield and/or low specific activity (Evans & Gurd, 1973; Nakamura, 1976; Slavik *et al.*, 1977).

In the present work a systematic study led to the choice of a zwitterionic detergent to solubilize the active enzyme. Subsequent purification was achieved

either by classical chromatography methods or by use of an immunoaffinity step with an immunoabsorbent prepared from a monoclonal antibody to the enzyme (Siddle *et al.*, 1981).

Experimental

Materials

Sulphobetaine 14 [Zwittergent TM₃₋₁₄; 3-(*NN*-dimethyl-*N*-tetradecyl)ammonio)propane-1-sulphonate] was a gift from Calbiochem, C.P. Laboratories Ltd., Bishops Stortford, Herts. U.K. LKB Ampholines (pH 3.5–10) were obtained from LKB Instruments, South Croydon, Surrey, U.K. All other reagents were of the highest grade available.

Membranes

Rat liver plasma membranes were prepared as described by Pilkis *et al.* (1974), and suspended to 3 mg of protein/ml in 10 mM-Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonic acid)/NaOH buffer, pH 7.5.

Assays

5'-Nucleotidase activity was determined at 37°C

by the radioassay method of Avruch & Wallach (1971) or the spectrophotometric assay method of Ipata (1968). In each case 20 mM-sodium β -glycerophosphate was present to saturate non-specific phosphatases (Stanley *et al.*, 1980). In gels, 5'-nucleotidase activity was measured by a modification of the radioassay method or located by lead phosphate precipitation (Bailyes *et al.*, 1981).

To determine K_m for AMP and K_i for ATP, initial velocities were determined by using the radioassay method in the presence of 1 mM-MgCl₂ throughout. The concentration range of AMP was 5–200 μ M and that of ATP 10–200 μ M. Apparent K_m values were obtained from Lineweaver–Burk plots (Dixon & Webb, 1964). The K_i for ATP was obtained from a plot of apparent K_m versus ATP concentration. The values of K_m and K_i and their statistical uncertainties were obtained from a linear regression by standard statistical methods (Armitage, 1971). Values reported are means $\pm$ S.D. (numbers of degrees of freedom).

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

Native polyacrylamide gels were prepared as previously described (Newby *et al.*, 1978), with a modified Ornstein–Davis buffer system (Davis, 1964) (System 2860; Chrambach *et al.*, 1976, p. 135); 40 mM-Tris/25.3 mM-phosphoric acid (final concentration) buffer, pH 6.89, was used in both stacking and resolving gels.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) was conducted on slab gels containing 10% (w/v) acrylamide with 0.27% (w/v) *NN'*-methylenebisacrylamide. Samples were reduced and alkylated (Lane, 1978) before application to the gels.

Gels were stained for protein by the method of Fairbanks *et al.* (1971) and scanned on a densitometer. When appropriate, tracks were cut into 2 mm slices for radioactivity counting.

Isoelectric focusing was conducted as described by McCormick *et al.* (1976), with 2% (v/v) (final concentration) Ampholines (pH 3.5–10). The voltage was maintained at 200 V for 4–24 h.

The pH gradient was determined with a pH electrode after elution of gel slices at 4°C overnight with degassed 25 mM-KCl.

Solubilization

Initial partial purification to produce a phospholipid–enzyme pellet was based on the method of Widnell (1975) (up to step 5), with the following modifications. Homogenization and detergent extraction of 16 rat livers (200–300 g) were as described in steps 1 and 2 of the method of Widnell (1975). In step 3 adjustment of the pH to 5.4 did not

reproducibly form a well-defined pellet, and so the centrifugation was omitted and the pH re-adjusted to pH 7.5. In step 4 the extract was maintained at 50°C for 90 s, and the protamine sulphate fractionation was omitted in step 5. The surface plug formed in 40%-saturated (NH₄)₂SO₄ (step 5) was resuspended in 900 ml of medium A, left overnight, and centrifuged at 88 000 g for 60 min at 4°C to produce a pellet, which was resuspended in 30 ml of 0.1 M-Tris/HCl buffer, pH 7.5, containing 1 mM-MgCl₂ and 2% (w/v) Sulphobetaine 14 and stirred for 30 min at 20°C. The solution was centrifuged (at 48 000 g for 30 min at 20°C), and the supernatant was warmed to 37°C for 10 min before DEAE-cellulose chromatography or immunoaffinity purification.

Ion-exchange chromatography and gel filtration

The first DEAE-cellulose column (40 ml of pre-swollen microgranular exchanger, Whatman DE-52) was equilibrated at room temperature (20–23°C) with 100 mM-Tris/70 mM-HCl buffer, pH 7.8, containing 1 mM-MgCl₂ and 0.1% Sulphobetaine 14. The second DEAE-cellulose column (10 ml bed volume) was equilibrated at 20–23°C with 10 mM-Tris/7 mM-HCl buffer, pH 7.8, containing 1 mM-MgCl₂ and 0.1% Sulphobetaine 14. Equilibration was continued until the eluate had the same pH and conductivity as the equilibrating buffers. The columns were prepared in 50 ml and 20 ml plastic syringes respectively and were loaded and eluted at 0.5 ml/min.

A column (2.5 cm $\times$ 90 cm) of Ultragel AcA-34 (exclusion limit 750 000) was equilibrated at 4°C with 0.1 M-Tris/HCl buffer, pH 7.5, containing 1 mM-MgCl₂ and 0.1% Sulphobetaine 14. Enzyme was applied and the column developed at 12–15 ml/h by gravity feed. The eluate fractions containing 5'-nucleotidase were concentrated by using an Amicon XM 50 membrane under N₂ pressure.

Use of monoclonal-antibody immunoabsorbent

As an alternative to ion-exchange chromatography, immunoaffinity purification was performed as follows. A mouse monoclonal antibody capable of inhibiting rat liver 5'-nucleotidase was prepared, purified and coupled to diazo-cellulose as previously described (Siddle *et al.*, 1981). Portions (15 mg) of the consequent immunoabsorbent were washed with 3 $\times$ 10 ml of a buffer (used throughout immunoaffinity purification) containing 0.1 M-Tris/HCl, pH 7.5, 1 mM-MgCl₂ and 0.1% Sulphobetaine 14, then with 2 $\times$ 10 ml of 0.5 M-NaCl in the buffer, then with 2 $\times$ 10 ml of 50 mM-diethylamine, and finally with 2 $\times$ 10 ml of the buffer.

Samples [approx. 200 units (μ mol/min); 20 ml] of enzyme solubilized with Sulphobetaine 14 were incubated with 15 mg of washed immunoabsorbent for 90 min at 20°C. The immunoabsorbent was

separated by centrifugation (at 1000g for 2 min at 4°C) and then washed with 2 × 10 ml of buffer and then with 1 × 10 ml of 0.5 M-NaCl in buffer. Enzyme was eluted batchwise with 5 ml of freshly prepared cold (4°C) 50 mM-diethylamine for 8 min, followed by centrifugation (at 100g for 2 min at 4°C) and collection of the supernatant, to which was added 0.5 ml of 10-fold-concentrated buffer. The buffered eluate was immediately passed through a sterile Millipore GSWP filter (pore size 0.22 µm) before further purification by gel filtration as described above.

Preparation of iodinated 5'-nucleotidase

Purified enzyme was [¹²⁵I]iodinated by the iodogen method (Fraker & Speck, 1978) as previously described (Siddle *et al.*, 1981). The final specific radioactivity was 0.04–0.08 mCi/µg. Iodinated 5'-nucleotidase retained full enzyme activity.

Results

Solubilization

The release of 5'-nucleotidase from rat liver plasma membranes as demonstrated by its appearance in the supernatant after centrifugation is shown in Fig. 1. The zwitterionic detergent Sulphobetaine 14 was clearly the most effective of the detergents tested, in causing more than 95% release of 5'-nucleotidase with full retention of activity. Sodium dodecyl sulphate was the most effective detergent at solubilizing protein, but caused 60% inactivation of the enzyme at a concentration of 0.5% (w/v). In contrast, Sulphobetaine 14 concentration could be raised to 8% (w/v) without inactivation of the enzyme (results not shown).

Polyacrylamide-gel electrophoresis of the active solubilized 5'-nucleotidase required a system operating at pH 9.7 for adequate electrophoretic mobility. A high-speed supernatant from rat liver plasma membranes treated with a concentration of detergent producing maximal release of enzyme was electrophoresed into 7% (w/v) polyacrylamide gels (Fig. 2). Only Sulphobetaine 14, Lubrol 12A9 and Triton X-100 gave a sharp peak of 5'-nucleotidase activity in the resolving gel. Sulphobetaine 14 yielded 70% of the original membrane activity in a band of R_F 0.42, compared with 21% for Lubrol 12A9 and 20% for Triton X-100.

By using a range of gel concentrations from 4 to 11.2% (w/v) the R_F values of 5'-nucleotidase for the Sulphobetaine 14-solubilized enzyme were determined, and a Ferguson plot ($\log R_F$ versus gel concentration) was constructed. The slope of this plot (K_r , the retardation coefficient, a measure of molecular size; Ferguson, 1964; Rodbard & Chrambach, 1974) was determined as 0.094 ± 0.003 (45). This value of K_r was translated into a geometric

Fig. 1. Sedimentation of 5'-nucleotidase and protein at high gravitational force

Rat liver plasma membranes were resuspended at 4°C to 3 mg of protein/ml in 10 mM-Tes/NaOH buffer, pH 7.5. Then 135 µl samples were each mixed with 15 µl of detergent solutions to give the final concentrations shown, in 300 µl conical tubes. Tubes were floated in groups of six to eight in water in 50 ml-capacity Sorvall tubes and centrifuged at 48 000g for 30 min at 4°C. Supernatants and uncentrifuged mixtures were then assayed for protein and 5'-nucleotidase activity. For full experimental details see the text. (a) Release of 5'-nucleotidase. (b) Release of protein. ■, Sulphobetaine 14; □, N-dodecylsarcosinate; ●, Lubrol; ▲, sodium dodecyl sulphate; △, Triton X-100.

mean radius of 3.6 nm for the Sulphobetaine-14-solubilized enzyme by linear interpolation from a standard curve as described by Rodbard & Chrambach (1974). Increasing the concentration of detergent in the sample up to 3.5% Sulphobetaine 14 with 2% Sulphobetaine 14 in the polyacrylamide gel, or adding ionic detergent in combination with Sulpho-

Fig. 2. *Electrophoresis of high-speed centrifugation supernatants in native polyacrylamide gels* Stacking gels (3.125% total monomers) and resolving gels (7% total monomers) were polymerized as described in the Experimental section. Rat liver plasma membranes were solubilized with 0.5% Sulphobetaine 14, 1.0% *N*-dodecylsarcosinate, 1% Lubrol 12A9, 2% Triton X-100 or 2% Triton X-100 + 1% sodium deoxycholate as described in the legend to Fig. 1. Ionic detergent or detergent mixture (0.1%) was present in the upper buffer. Non-ionic and zwitterionic detergents (0.1%) were present in the gels. Gel slicing and assay were as described in the Experimental section. The hatched line represents the position of the stacking/resolving gel boundary. The percentage of membrane activity in the peak is shown.

betaine 14, did not decrease the apparent size of the 5'-nucleotidase (results not shown). This suggested that Sulphobetaine 14 produced the smallest as well as the least polydisperse active enzyme, thus providing optimal solubilization (Newby & Chrambach, 1979).

Partial purification

The ability to solubilize 5'-nucleotidase reproducibly and effectively and to maintain it in solution with low detergent concentration with 0.1% Sulphobetaine 14 suggested that this detergent might be used in purification of the enzyme. Purification of 5'-nucleotidase from whole rat liver was conducted essentially as described by Widnell (1975) until and including the first 40%-saturated-(NH₄)₂SO₄ step (see the Experimental section). Addition of Sulphobetaine 14 at this stage resulted in more than 90% of the enzyme being in the supernatant after centrifugation at 48 000 *g* for 30 min, with 65–90% of the protein pelleted. Solubilization resulted in 3–8-fold

purification of the enzyme at this step alone. Subsequent steps were conducted at room temperature.

The supernatant, containing solubilized 5'-nucleotidase, was applied to the first DEAE-cellulose column, to which it did not bind.

The solution was then dialysed against 10 vol. of 1 mM-MgCl₂/0.1% Sulphobetaine 14 to lower the ionic strength and then applied to the second DEAE-cellulose column. All the enzyme activity bound to this column, and was eluted with 20 ml of column buffer containing 0.1 M-NaCl/1% Sulphobetaine 14. This eluate (approx. 5 ml) was further purified by gel-exclusion chromatography on Ultragel AcA-34.

A summary of the purification scheme with the use of ion-exchange and gel-exclusion chromatography is shown in Table 1(a). In three preparations the yield of enzyme was 8.3–9.5% (47–100 units), with a specific activity of 152–178 units/mg of protein.

Immunoaffinity purification

Purification of the enzyme with the use of the monoclonal-antibody immunoabsorbent was achieved by adding the immunoabsorbent to the Sulphobetaine-14-solubilized enzyme after the 40%-saturated-(NH₄)₂SO₄ step (Table 1b) and eluting with 50 mM-diethylamine batchwise at 4°C. This single immunoaffinity step achieved greater purification than did the ion-exchange chromatography and gel-exclusion chromatography steps described above.

Further purification after the immunoaffinity step was achieved by gel-exclusion chromatography as above, and a summary of purification via the immunoaffinity step is shown in Table 1(b). In three preparations the average final yield of enzyme purified via the immunoaffinity step was 9–16% of the initial homogenate enzyme, with a specific activity of 285–340 units/mg of protein.

Properties of the purified enzyme

5'-Nucleotidase was purified from rat liver at least 6000-fold via ion-exchange chromatography and at least 12000-fold via immunoaffinity purification (Table 1). The purified enzyme from each procedure showed a single major band of apparent *M_r* 70000 after fractionation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and staining with Coomassie Blue (results not shown). In addition, a clearly stained minor band of apparent *M_r* 38000 was observed in both preparations, and other lesser bands in the ion-exchange-chromatography-purified enzyme (Siddle *et al.*, 1981).

After iodination and fractionation by sodium dodecyl sulphate / polyacrylamide - gel electrophoresis the immunoaffinity-purified enzyme also

showed the same two labelled bands (Fig. 3). This iodinated enzyme could be cross-linked with dimethyl suberimidate or dimethyl 3,3'-dithiobispropionimidate used at 15 mM in 50 mM-sodium borate buffer, pH 8.5 (Peters & Richards, 1977), and after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed two new labelled bands of apparent *M_r* 134000 and 108000 (results not shown). No higher-molecular-mass aggregates were seen. The isoelectric point of the enzyme was 5.4–6.0.

The *K_m* for AMP of the immunoaffinity-purified

Fig. 3. Electrophoretic profile of iodinated immunoaffinity-purified enzyme on a sodium dodecyl sulphate/polyacrylamide gel

Immunoaffinity-purified 5'-nucleotidase was iodinated and prepared for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis as described in the Experimental section. Radioactivities (c.p.m.) per gel slice are expressed as percentages of total radioactivity (c.p.m.) on the gel. Specific activity of the enzyme was 340 units/mg of protein.

Table 1. Purification of 5'-nucleotidase

The first four steps listed are common to both ion-exchange chromatography and immunoaffinity purification. For full experimental details see the text.

Step	Specific activity (units/mg of protein)	Yield (%)
(a) Ion-exchange chromatography		
Homogenate (1180 units)	0.029	100
Resuspended plug after 40%-saturated (NH ₄) ₂ SO ₄	0.16	34
Pellet resuspended in Sulphobetaine 14 solution	0.9	23
Sulphobetaine 14 extraction	2.5	20
1st DEAE-cellulose column	9	13
2nd DEAE-cellulose column	19	10
Gel filtration	152	8.5
(b) Immunoabsorbent		
Homogenate (1364 units)	0.028	100
Resuspended plug after 40%-saturated (NH ₄) ₂ SO ₄	0.2	38
Pellet resuspended in Sulphobetaine 14 solution	0.5	27
Sulphobetaine 14 extraction	4	24.5
Eluate from immunoabsorbent	202	17.5
Gel filtration	285	16

enzyme was $8.1 \pm 0.7 \mu\text{M}$ (3). ATP was a competitive inhibitor, with a K_i of $75 \pm 11 \mu\text{M}$ (3). These values are in agreement with previous reports for rat liver 5'-nucleotidase (Widnell, 1975; Nakamura, 1976; Stanley & Luzio, 1978).

Discussion

The present paper describes a rational approach to the general problem of purification of minor intrinsic membrane proteins. Three increasingly stringent criteria of solubilization were used: non-sedimentation of enzyme activity after high-speed centrifugation, penetration of solubilized enzyme into a polyacrylamide gel and finally determination of the molecular size of detergent-enzyme complexes by Ferguson-plot analysis (Ferguson, 1964). These criteria lead to the definition of optimal solubilization as that which produces the 'smallest and least polydisperse active enzyme' (Newby & Chrambach, 1979).

The zwitterionic detergent Sulphobetaine 14 was found to be the most effective of the detergents examined in solubilizing 5'-nucleotidase from rat liver plasma membranes, though the reason for its apparently superior solubilizing ability is not clear (Gonenne & Ernst, 1978; Bailyes *et al.*, 1981; Newby *et al.*, 1982). It has been used previously to solubilize and purify yeast plasma-membrane ATPase (Malpartida & Serrano, 1980), but denatures and inactivates some other membrane proteins such as cytochrome *P*-450 (Hjelmeland *et al.*, 1979). Ion-exchange chromatography and gel-exclusion chromatography of Sulphobetaine 14-solubilized 5'-nucleotidase resulted in a good yield (more than 8%) of enzyme purified at least 6000-fold from the initial liver homogenate. The specific activity of this enzyme (152–178 units/mg of protein) compared well with previous reports.

The immunoaffinity-purified 5'-nucleotidase had a specific activity of 285–340 units/mg, at least 12000-fold purified, and the yield was 9–16%. This specific activity is approx. 1.5 times the highest value previously reported for rat liver 5'-nucleotidase (Slavik *et al.*, 1977), with about 20 times more enzyme isolated in a single purification. The minimum apparent size of the detergent-solubilized active enzyme by polyacrylamide-gel electrophoresis was diameter 3.6 nm. This value may be converted into an apparent M_r of 155 000, assuming that the solubilized enzyme is a globular protein of partial specific volume 0.74 ml/g. A similar value of M_r is obtained by gel-exclusion chromatography (results not shown). It is noteworthy that Evans & Gurd (1973) have previously suggested that the mouse liver enzyme may be a dimer of two identical-size subunits (M_r 70 000–75 000) in the native state, and that both Naito & Lowenstein

(1981) and ourselves have obtained results with purified enzyme, by the use of cross-linking reagents, suggesting that the major native form is a dimer of subunits M_r 70 000. We have also detected a second dimer of subunits M_r 70 000 and 38 000.

A strategy involving the use of zwitterionic detergents and monoclonal antibodies may be of general application to the purification of minor membrane proteins. Cytochrome *P*-450 and the opiate receptor have been successfully solubilized by a sulphobetaine derivative of cholic acid {3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate (Hjelmeland, 1980; Simonds *et al.*, 1980)}, suggesting that different zwitterionic detergents will be useful for the purification of different membrane proteins. Similarly, the use of a monoclonal-antibody immunoaffinity step will be specific to the protein of interest. Our own experience suggests that a useful starting point for the raising of monoclonal antibodies to a minor membrane protein is a partially purified protein prepared by conventional techniques. It is also likely that successful elution from immunoabsorbent will vary, depending on the properties of the monoclonal antibody and the requirements for biological activity of the protein being purified. In the present experiments unsuccessful elution conditions included low pH, detergents at pH 7.5 and 11.5, and high concentrations of salt (0.5 M-NaCl) and chaotropic ions (2 M-KCl and 2 M-KSCN). These conditions have been widely used to disrupt other antigen-antibody links (Dandliker *et al.*, 1967; Miles & Hales, 1968; Crumpton & Parkhouse, 1972). Only freshly prepared 50 mM-diethylamine at 4°C could elute active enzyme (Parham, 1979). It was necessary to restrict elution time, since even at 4°C the enzyme was unstable in 50 mM-diethylamine, being inactivated by at least 40% at 60 min, but only by 10–15% at 10 min. Successful use of a monoclonal-antibody immunoaffinity step has been reported for purification of several membrane proteins (Parham, 1979; Lennon *et al.*, 1980; Momoi *et al.*, 1980; Brown *et al.*, 1981; Unkeless *et al.*, 1981).

It is possible to calculate from the present data that there are approx. 5×10^5 monomer molecules of 5'-nucleotidase per liver cell (assuming 169×10^6 cells/g wet wt. of liver; Hommes *et al.*, 1970), which is of the same order as the reported number of insulin receptors (1×10^5 – 2.5×10^5 per liver cell; Kahn, 1976). Thus the approach to purification reported may be of great use in studying other biologically important minor membrane components.

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