HEXAMETHYLPHOSPHORIC TRIAMIDE AS A SOLUBILIZING AGENT

Purification and reactivation of diglyceride kinase

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1. Introduction

The tightly bound proteins of biological membranes are usually solubilized with the aid of detergents. Experimental problems arising are binding and inactivation by the detergent and difficulty of detergent removal in lipid/protein reconstitution.

An alternative way to purify membrane proteins is based on the observation that a small amount of membrane protein can usually be solubilized with organic solvents such as chloroform or butanol-1 [1,2]. Two hydrophobic bacterial membrane enzymes, isoprenoid alcohol kinase and diglyceride kinase, have been highly purified and reactivated using butanol-1 [3,4]. The aprotic organic solvents, HMPT and MP, have subsequently been found to be much more effective in solubilizing membrane proteins than chloroform or butanol-1 [5]. Precipitation with ethanol and gel permeation chromatography on Sephacryl were developed as methods for protein purification in HMPT. The following defined membrane proteins have so far been solubilized and purified in HMPT, the *Escherichia coli* phage lambda receptor protein [6], bacterioopsin [7], the pig kidney Na^+,K^+-ATPase subunits [S. Maier, H. S. unpublished] and certain erythrocyte and brain membrane proteins [R. Schmitt, S. Maier, H. S., unpublished].

The functional reconstitution from HMPT has remained a largely unsolved problem although certain soluble enzymes regained activity from HMPT-solution [5]. Reconstitution of lactose transport has been achieved in membrane vesicles prepared from a transport-negative strain of *E. coli* by addition of an HMPT-extract of transport-positive vesicles [8]. However, this reconstitution and that of the Na^+,K^+/ATPase subunits has so far not been reproducibly obtained in artificial proteoliposomes.

Since *E. coli* diglyceride kinase is easily purified and reactivated using butanol-1 [4] we have studied the behaviour of this enzyme in HMPT. Conditions allowing solubilization, purification and reactivation of diglyceride kinase in HMPT are reported.

Abbreviations: HMPT, hexamethylphosphoric triamide; MP,N-methylpyrrolidone; SDS, sodium dodecylsulphate

2. Experimental

A crude 'total' membrane fraction of *E. coli* K12 and inner membrane vesicles of *E. coli* ML 308-225 were prepared as in [4] and [8], respectively. Protein was determined in the presence of SDS [3]. SDS gel electrophoresis was performed on 13% (w/v) polyacrylamide slab gels [6], samples being dissolved in the presence of 2% (w/v) SDS (10 min, 100°C). *sn*-1,2-Dipalmitate and bovine heart cardiolipin were obtained from Fluka (Neu-Ulm) and Sigma (St Louis), respectively. [γ-^{32}P]ATP was purchased from Amersham-Buchler, (Braunschweig). A purified grade of HMPT was from Serva, (Heidelberg). A less pure grade (Merck, Darmstadt) was used for column chromatography.

Caution: HMPT has been classified as a potential carcinogen.

2.1. *Solubilization procedure*

A suspension of membrane vesicles (7.7 mg protein in 1 ml 0.1 M potassium phosphate (pH 6.6)) which had been stored in liquid nitrogen was thawed within ~5 min at 25°C and membranes were sedimented in the model 3200 Eppendorf centrifuge

(5 min, 25°C). The pellet was suspended in 1 ml 0.5 M Tris–sulphate (pH 7.5), 1 M LiCl, 3 mM dithiothreitol and re-pelleted. This procedure was repeated once more. The packed membrane pellet (1 vol. part) was then dispersed in 9 vol. parts of conc. HMPT. After shaking for 5 min at 25°C the dispersion was sonicated for 1 min in a Branson model 220 sonic bath. Undissolved material was removed by ultracentrifugation (Beckman rotor 65, 39 000 rev./min, 90 min, 15°C).

2.2. *Ethanol precipitation*

Ethanol (7 vol. parts) was added to the HMPT-solution (3 vol. parts), followed by occasional mixing on the Vortex mixer (5 min, 20°C). The protein pellet was isolated by low-speed centrifugation (model 3200 Eppendorf centrifuge, 2 × 2 min).

2.3. *Kinase assay*

Aliquots of organic solutions of 2 mM dipalmitate (50 μl) and 2.3 mM cardiolipin (100 μl) were pipetted into the test tube, followed by removal of organic solvents in vacuo. The HMPT-solution or membrane suspension to be tested (20 μl) and 75 μl 50 mM Tris–phosphate, 100 mM NaCl, 10 mM $MgSO_4$, 0.7% (w/v) Triton X-100, 1 mM 2-mercaptoethanol (pH 6.6) were then added. After 30 s in the model 220 Branson sonic bath the reaction was started by addition of 5 μl [γ-^{32}P]ATP (80 mM, ~100 000 cpm). Incubation was for 12 h at 40°C in a shaking water bath. The reaction was linear with time from 0.5–26 h incubation. Reactions were terminated by addition of 50 μl chloroform/methanol, 1:1 (v/v), followed by chromatographic analysis as in [14].

3. Results and discussion

3.1. *Solubilization*

Treatment of the 'total' membrane fraction of *E. coli* with 90% (v/v) HMPT or MP left all kinase activity in the insoluble pellet fraction. When membrane vesicles were employed, MP again left all kinase activity undissolved but HMPT gave complete solubilization. When butanol-1 solubilized enzyme was used after chromatography on DEAE-cellulose and drying in vacuo [4] HMPT and MP both gave complete solubilization of kinase activity along with 72% of the protein.

These results showed that solubilization with HMPT or MP depended to a high degree on the kind of starting material used. Previous studies have shown a drastic influence of divalent ions upon total solubilization and upon the nature of polypeptides dissolved by HMPT [5,6].

3.2. *Reactivation*

Due to the high boiling points of >200°C of HMPT and MP the previous assay procedure for diglyceride kinase activity [4] could not be used. HMPT- or MP-dissolved enzyme was therefore added directly to the test tube already containing the other required assay components [4] so that a final solvent concentration of 18% (v/v) of HMPT or MP was present. Addition of conc. HMPT or MP to the previous assay mixtures [4] gave ~35% inhibition at 18% (v/v) solvent concentration.

Lipid titration experiments with cardiolipin and with egg lecithin indicated that the optimal lipid cofactor concentrations were increased ~10-fold in the presence of aprotic solvent. This effect was taken into account in the reactivation procedure given under 2.3.

The kinase was furthermore observed to be unstable in HMPT when kept at 20°C or at 4°C ($t_{1/2}$ ~ 24 h at 4°C). The butanol-1 dissolved enzyme could be stored at 4°C for several months without loss of activity. When stored at −70°C, the HMPT or MP-kinase solutions were stable for several weeks. The reason for the loss of activity at 4°C and 20°C is unknown. A general proteolytic activity of the aprotic solvents was, however, excluded by showing that various soluble and membrane polypeptides remained undegraded upon storage in HMPT-solution at 20°C for several months (control by SDS gel electrophoresis).

3.3. *Purification*

The purification of diglyceride kinase from membrane vesicles is summarized in table 1. Solubilization with HMPT resulted in an apparent increase in total kinase activity. Gel permeation chromatography on a calibrated column of Sephacryl S-200 in HMPT gave a defined peak of kinase activity corresponding to M_r 16 000 (fig.1). M_r 15 400 had been determined in butanol-1 [4]. The column fractions were examined by SDS gel electrophoresis and this showed that polypeptides were eluted according to their monomeric M_r-values (fig.1B).

About 36% of the applied kinase activity was recovered after column chromatography. The loss

Table 1
Scheme for the purification of *E. coli* diglyceride kinase

	Protein (mg)[a]	Total activity (nmol product/ 12 h)	Spec. act. (nmol product . mg protein . 12 h^{-1})
Membrane vesicles	7.7	1220	158
HMPT-solubilizate	6.0	4130	688
Fractions 44–55 (of fig.1)	0.9	1520	1688
Fraction 48 (of fig.1)	0.06	325	5417

[a] The protein contents of fractions 44–55 and of fraction 48 were determined by integrating the corresponding areas of the absorption curve (280 nm) of fig.1. Control experiments showed that protein content determined in this way agreed within ± 3% with that determined according to [3]

A

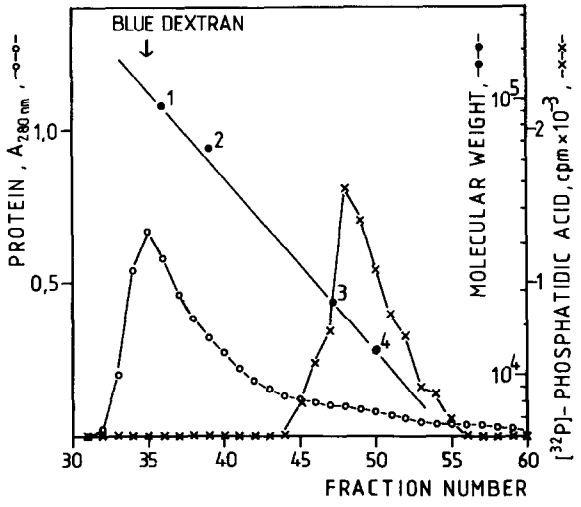
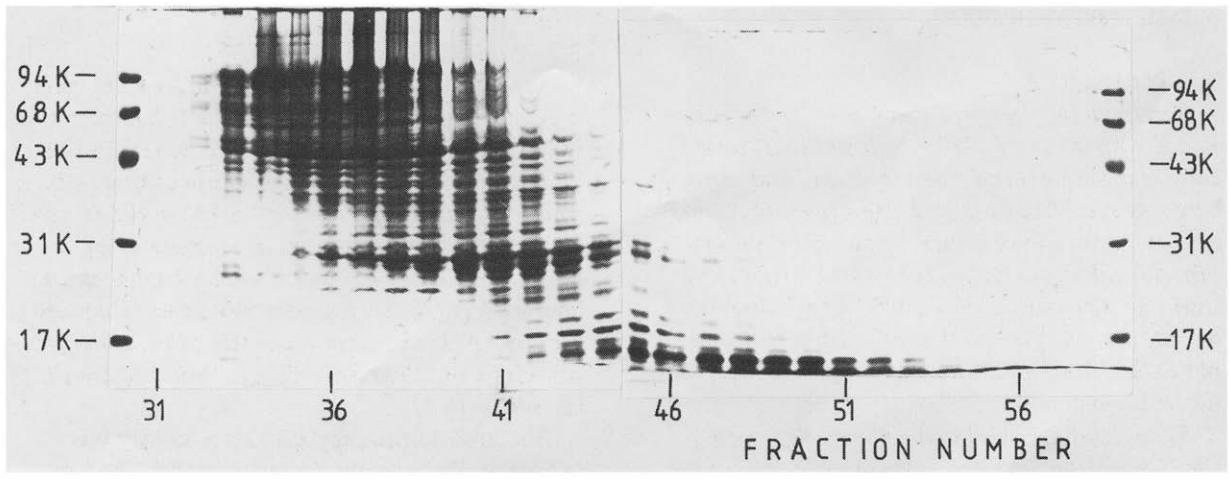


Fig.1. Chromatography on Sephacryl S-200. (A) A column (28 × 2 cm) was packed with Sephacryl S-200 (Pharmacia) in a buffer containing 90% (v/v) HMPT and 50 mM Tris–sulphate (pH 7.5), 0.1 M LiCl, 0.3 mM dithiothreitol. The solubilizate (~1.5 ml) was applied immediately after the ultracentrifugation step of section 2.1, followed by elution with the column buffer at 11 ml/h. Fractions (1 ml) were collected. The absorption at 280 nm was continuously registered ((○), arbitrary units). Aliquots (20 μl) of each fraction were assayed for kinase activity (section 2.3). The radioactivity (cpm) found associated with the phosphatidic acid product is plotted (X), as is the calibration curve (•) obtained in independent runs with the marker proteins (M_r): (1) phosphorylase *b* (94 000); (2) bovine serum albumin (68 000); (3) β-lactoglobulin (18 400); (4) cytochrome *c* (12 200). (B) SDS slab gels obtained with 400 μl aliquots of each column fraction after ethanol precipitation (section 2.3). Marker proteins were (M_r): phosphorylase *a* (94 000); bovine serum albumin (68 000); egg albumin (43 000); carboanhydrase (31 000); myoglobin (17 000).

B



of ~64% also occurred upon just storing the enzyme solution at room temperature for the time interval required for column chromatography (~5 h). The use of high-pressure liquid chromatography [9] should shorten the time required for protein fractionation and reduce the loss of enzyme activity.

The specific kinase activity of the peak fraction eluted (no. 48) was ~35-fold higher than that of the starting membrane and ~8-fold higher than that of the initial HMPT-extract. The SDS gel of fraction 48 showed that several major polypeptide bands were still present (fig.1).

The elution of phospholipid was determined by use of a [^{14}C]dipalmitoyllecithin marker. Radioactivity was only eluted near the inclusion volume (fraction 98).

The use of HMPT has led to a partially purified diglyceride kinase preparation which was free of lipid and detergent. Unexpected effects of HMPT were the 10-fold increase in the amount of lipid required for reactivation and the instability of kinase activity at 20°C and 4°C. The above results may provide an experimental basis for the isolation of other membrane proteins with retention of functional activity and without use of detergent.

Acknowledgements

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