Biochimica et Biophysica Acta, 315 (1973) 394–401

BBA 66949

SEPARATION AND PROPERTIES OF THE SOLUBLE 3′-PHOSPHOADENOSINE 5′-PHOSPHOSULPHATE-DEGRADING ENZYMES OF BOVINE LIVER

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(Received February 23rd, 1973)

SUMMARY

3′-Phosphoadenosine 5′-phosphosulphate sulphohydrolase (PAPS sulphohydrolase), 3′-phosphoadenosine 5′-phosphosulphate 3′nucleotidase (PAPS 3′-nucleotidase) and adenosine 5′-phosphosulphate sulphohydrolase (APS sulphohydrolase) are enzymes present in the cytosol of the bovine liver cell which are capable of participating in the degradation of active sulphate (PAPS). A technique is described for the separation of the three enzymes so that they may be independently studied. In contrast to the general belief, the PAPS sulphohydrolase is either strongly inhibited or not affected by Co^{2+}, depending on the buffer used and it is concluded that the activation of the process of PAPS degradation by Co^{2+} reflects the marked activating effect of the metal on PAPS 3′-nucleotidase. Some general properties of PAPS sulphohydrolase and PAPS 3′-nucleotidase are described.

INTRODUCTION

The principal sulphate donor in biological systems is active sulphate (3′-phosphoadenosine 5′-phosphosulphate, PAPS). Apart from its participation in sulphate transfer reactions, PAPS is also subject to degradation. Thus, as a result of several studies of different tissues from different species, four distinct degrading enzyme activities have been reported: (a) PAPS sulphohydrolase[1–5], (b) PAPS 3′-nucleotidase[4,6] and/or PAPS phosphohydrolase[3,7], (c) adenosine 5′-phosphosulphate (APS) sulphohydrolase[8–13] and (d) 3′-phosphoadenosine 5′-phosphate (PAP) phosphohydrolase[4–6]. Two different pathways may therefore exist for the degradation of

Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulphate; APS, adenosine 5′-phosphosulphate; PAP, 3′-phosphoadenosine 5′-phosphate.

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PAPS, each one with identical end-products but with inorganic sulphate and phosphate being liberated in a different sequence of events (Fig. 1).

Many of the observations on PAPS degradation have been made with relatively crude enzyme systems over long incubation periods. Under such circumstances it is difficult to establish whether the production of inorganic sulphate has arisen by direct desulphation of PAPS by a PAPS sulphohydrolase (Route a, Fig. 1) or by the sequential activities of a PAPS 3′-nucleotidase (producing APS, Route b) and an APS sulphohydrolase (Route c). Similarly, Co^{2+} are known to stimulate PAPS degradation but this has been attributed by some workers to an effect on the PAPS sulphohydrolase and by others to an effect on the PAPS 3′-nucleotidase.

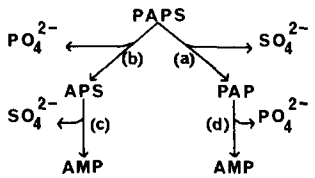

Fig. 1. Alternative routes for the enzymic degradation of PAPS. Enzymes on the pathways are (a) PAPS sulphohydrolase, (b) PAPS 3′-nucleotidase and/or PAPS phosphohydrolase, (c) APS sulphohydrolase, (d) PAP phosphohydrolase.

The present work describes the separation of PAPS sulphohydrolase, PAPS 3′-nucleotidase and APS sulphohydrolase activities from the cytosol of the bovine liver cell and clarifies the role of Co^{2+} for this particular source of the enzymes. A preliminary report on some of the work has already been made[14] and detailed accounts of the purification[13] and properties[15] of one of the enzymes (APS sulphohydrolase) have been given.

MATERIALS AND METHODS

Preparation of [^{35}S]PAPS and APS

[^{35}S]PAPS was prepared by an enzymic method based on experimental conditions established in these laboratories[16] and was purified according to the method of Banerjee and Roy[17] as modified by Bailey-Wood *et al.*[10]. The product was stored as an aqueous solution (0.3 mM, pH 7.5) at −5 °C. APS was prepared as previously described[13].

Other materials

All chemicals were Analar grade and all non-sulphated nucleotides were obtained from the Sigma Chemical Co. Ltd, St. Louis, Mo., U.S.A.

Assay of PAPS-degrading activity

Initial tissue fractionation studies required a measure of the total PAPS-degrading activity. [^{35}S]PAPS (0.0375 mM), sodium acetate–acetic acid buffer (0.1 M, pH 5.6), $CoCl_2$ (5 mM) and enzyme, in a final volume of 400 μl, were incubated at 37 °C (usually for 5–10 min) before stopping the reaction by immersing reaction tubes in a boiling water bath for 30 s. Controls contained enzyme that had previously been

boiled for 1 min. The amount of [^{35}S]PAPS degraded was determined by the method of Denner and Stokes[18].

Assay of PAPS sulphohydrolase

This enzyme was assayed routinely by incubating [^{35}S]PAPS (0.0375 mM), Tris–HCl buffer (0.1 M) at pH 8.8 (unless otherwise stated) and enzyme in a final volume of 400 μl. After incubation at 37 °C (usually for 5–10 min) the reaction was stopped by immersing reaction tubes in a boiling water bath for 30 s. Controls contained enzyme that had previously been boiled for 1 min. Precipitated protein was removed by brief centrifugation and the amount of inorganic $^{35}SO_4^{2-}$ released was determined by the method of Denner and Stokes[18].

On some occasions a special buffer was employed in the assay procedure. The buffer was made by mixing 2.5 ml of a solution of 0.8 M glycine with an equal volume of 0.8 M glycylglycine and adjusting the pH with strong NaOH solution as required. The buffer was diluted to 10 ml and designated as 0.4 M glycine–glycylglycine–NaOH buffer.

Assay of PAPS 3'-nucleotidase

This was assayed routinely by incubating [^{35}S]PAPS (0.0375 mM), 0.1 M sodium acetate–acetic acid buffer, pH 5.6, $CoCl_2$ (5 mM) and enzyme in a final volume of 400 μl. After incubation for 5–10 min the reaction was stopped and precipitated protein removed as described in the preceding section. The [^{35}S]APS produced was determined as described by Denner and Stokes[18].

Cell fractionation, assay of marker enzymes and preparation of high-speed supernatant of bovine liver

The methods have been described previously[13].

Protein determination

This was determined by the method of Lowry *et al.*[19] employing solutions of bovine serum albumin as standards.

Ammonium sulphate fractionation

Fractionation was carried out at 4 °C using an amount of ammonium sulphate calculated according to the method of Dixon[20] without correction for temperature. In the routine preparation of the 0–40% saturation fraction of bovine liver high-speed supernatant the preparation was allowed to stand for 1 h after addition of ammonium sulphate before separating precipitated protein by centrifuging (*cf.* ref. 13). The protein was redissolved in the minimum volume of 0.02 M maleate–NaOH buffer, pH 5.6, and dialysed exhaustively against the same buffer.

ECTEOLA-cellulose chromatography

ECTEOLA-cellulose (Whatman ET11) was pre-cycled as recommended by the supplier and then washed extensively with equilibrating buffer. Eluates from equilibrated columns were checked to ensure that pH and conductivity values were identical with those of the applied buffer.

RESULTS

Sub-cellular distribution of PAPS-degrading activity in bovine liver

The distribution of PAPS-degrading activity in the subcellular fractions of bovine liver is shown in Fig. 2 and is related to the distribution of the marker enzymes, rhodanese, acid phosphatase and glucose-6-phosphatase. Relative specific activities have been plotted according to the procedure of De Duve *et al.*[21]. Some of the problems associated with the preparation of subcellular fractions of bovine liver have previously been indicated[13].

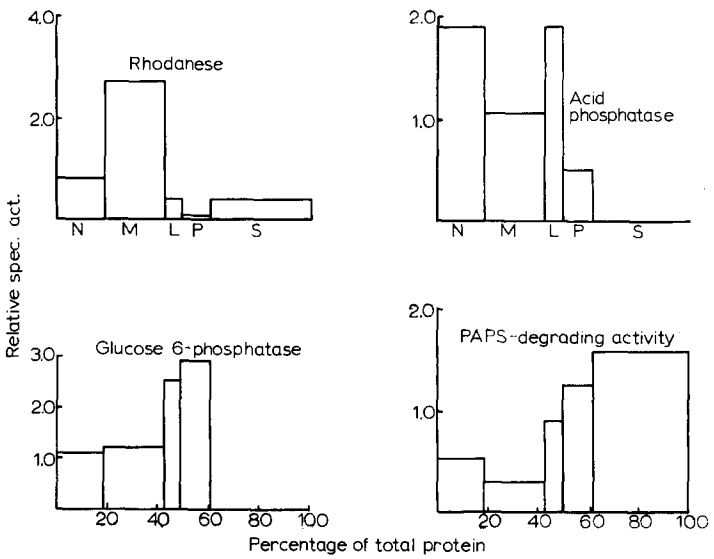

Fig. 2. Intracellular distribution of PAPS-degrading enzyme activity and various marker enzymes in the bovine liver cell. Fractionation procedure and determination of enzyme activities were as described in the text. N, M, L, P and S represent the nuclear, mitochondrial, lysosomal, microsomal and cytosol fractions, respectively. Results are the average of 5 experiments.

The results show that, although some PAPS-degrading activity was recorded in all the subcellular fractions, the bulk of the activity was localized in the high-speed supernatant fraction. In contrast to the distribution of APS sulphohydrolase[13] a relatively small amount of PAPS-degrading activity was found in the lysosomal fraction. The high-speed supernatant fraction was used for all subsequent work.

Ammonium sulphate fractionation

The independent assay of PAPS sulphohydrolase (Fig. 1, a), PAPS 3'nucleotidase (Fig. 1, b) and APS sulphohydrolase (Fig. 1, c) when all three are present in the same fraction presents problems. When APS is employed as substrate APS sulphohydrolase can be assayed specifically[13]. However, the measurement of liberated $^{35}SO_4^{2-}$ from [^{35}S]PAPS would not distinguish between the action of the PAPS sulphohydrolase (a) and the rapid sequential action of the PAPS 3'-nucleotidase (b) and APS sulphohydrolase (c). It was therefore necessary to separate APS sulpho-

hydrolase before the other two enzymes could be satisfactorily studied. Following removal of APS sulphohydrolase any $^{35}SO_4^{2-}$ liberated from [^{35}S]PAPS would be a measure of PAPS sulphohydrolase, whilst any [^{35}S]APS produced would not be degraded further and would therefore constitute a measure of PAPS 3′-nucleotidase activity.

During ammonium sulphate fractionation of the high-speed supernatant, the fraction precipitating between 0–40% saturation degraded PAPS but contained only small amounts of APS sulphohydrolase. The bulk of the latter enzyme precipitated between 40 and 50% saturation (*cf.* ref. 13). When the time during which protein was allowed to precipitate was reduced from the 2-h period suggested by Bailey-Wood *et al.*[10] to 1 h no contaminating APS sulphohydrolase activity could be detected in the 0–40% fraction. Since this fraction still degraded PAPS it therefore contained both PAPS 3′-nucleotidase and PAPS sulphohydrolase activities.

Effects of Co^{2+} on PAPS degradation by the 0–40% ammonium sulphate fraction

[^{35}S]PAPS was incubated for 5 min at 37 °C with the 0-40% fraction at various pH values (0.1 M Tris–maleate buffer) in the presence and absence of $CoCl_2$ (5 mM). Fig. 3 shows the amounts of ^{35}S-labelled PAPS, APS and inorganic sulphate present at the end of the incubation expressed as a percentage of the total radioactivity. In the absence of Co^{2+} very little [^{35}S]APS was produced. The amount of $^{35}SO_4^{2-}$ produced increased with pH indicating that the PAPS sulphohydrolase has an optimum pH of 8.0 or higher. In the presence of Co^{2+} the amount of $^{35}SO_4^{2-}$ produced is very similar to that produced in the absence of Co^{2+}. However, considerable amounts of [^{35}S]APS are also produced at acid pH by the PAP 3′-nucleotidase, the optimum pH of which appears to be in the region of 6.0.

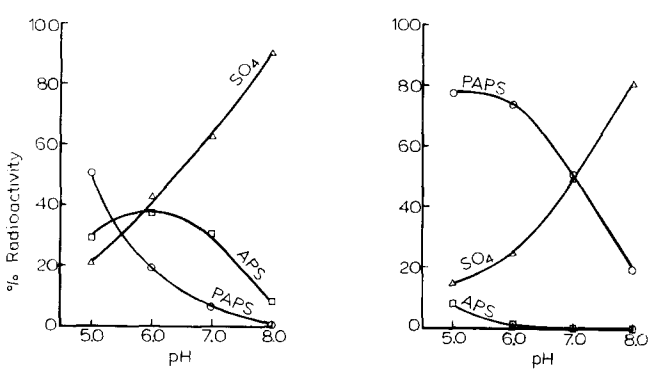

Fig. 3. The effects of 5 mM Co^{2+} on the degradation of PAPS (○), the production and degradation of APS (□) and the appearance of inorganic SO_4^{2-} (△). The control curves in the absence of Co^{2+} are on the right. Experimental details are described in the text.

These results indicate that this 0–40% ammonium sulphate fraction contains a PAPS 3′-nucleotidase which is stimulated by Co^{2+} and has an acid optimum pH and a PAPS sulphohydrolase which does not require Co^{2+} and which has an alkaline pH optimum.

Separation of PAPS sulphohydrolase and PAPS 3'-nucleotidase

PAPS 3'-nucleotidase. The 0–40% ammonium sulphate fraction was dialysed against 0.015 M maleate–NaOH buffer, pH 6.8, and a 2-ml portion (containing about 50 mg of protein) was applied to a column (18 cm × 1 cm) of ECTEOLA-cellulose that had previously been equilibrated with the same buffer. The column was eluted with the same buffer and the first 8 ml of eluate was discarded. The next 12 ml of eluate contained approximately 40% of the applied protein which had passed through the column unretarded. This fraction contained all the PAPS 3'-nucleotidase activity (within the limits of experimental error) but no PAPS sulphohydrolase activity.

PAPS sulphohydrolase. The 0–40% ammonium sulphate fraction was dialysed against 0.02 M maleate–NaOH buffer, pH 6.8, and a 2-ml portion was applied to a column (18 cm × 1 cm) of ECTEOLA-cellulose that had been equilibrated with the same buffer. The column was eluted with 30 ml of buffer and the eluate was discarded. Elution was then continued with 30 ml of the same buffer but containing 0.5 M NaCl. The eluate contained about 55% of the applied protein and PAPS sulphohydrolase activity.

Some difficulties were encountered in recovering PAPS sulphohydrolase from the column in good and reproducible yield. Recoveries varying from 20% to 70% were obtained on several different occasions. However, it became apparent that good recoveries were related to the age of the initial high-speed supernatant preparations. The best yields were obtained from high speed supernatant that had been stored in the frozen state for 7 days before ammonium sulphate fractionation. No obvious differences were apparent in the enzymological properties of batches of PAPS sulphohydrolase of different ages. This ageing process produced the opposite effect on the PAPS 3'-nucleotidase and reduced the yield by up to 80%.

Some properties of the separated enzymes

PAPS 3'-nucleotidase. In the presence of 5 mM $CoCl_2$ and 0.025 M maleate–NaOH buffer and over an incubation period of 10 min at 37 °C the optimum pH of the enzyme was 5.9 (Fig. 4). Activity of the enzyme in the absence of $CoCl_2$ at pH 5.9 was only 20% of that obtained in the presence of such ions.

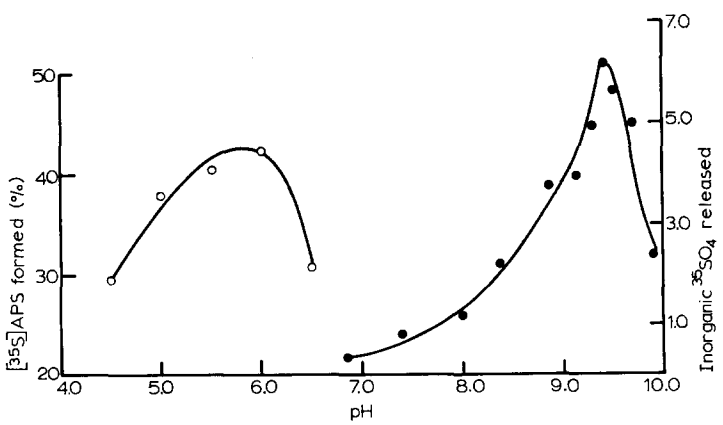

Fig. 4. pH–activity curves for PAPS 3'-nucleotidase (○) and PAPS sulphohydrolase (●). Experimental details are given in the text.

PAPS sulphohydrolase. In the presence of 0.1 M glycine–glycylglycine–NaOH the enzyme exhibited an optimum pH of approximately 9.4 (Fig. 4). At this pH the K_m for the enzyme, calculated from a plot of $1/v$ against $1/S$ was $1.7 \cdot 10^{-5}$ M. AMP, ADP and ATP at concentrations of 0.1 mM inhibited to the extent of 55, 61 and 83%, respectively, and inhibited completely at 1.0 mM concentration. PAP inhibited slightly (12% at 0.2 mM concentration). Co^{2+} strongly inhibited enzyme activity (approximately 80% inhibition at a concentration of 0.2 mM). Other divalent ions were also inhibitory at the same concentration (Mn^{2+}, 71%; Hg^{2+}, 73%; Pb^{2+}, 66%; Mg^{2+}, 23%). In the presence of 0.1 M Tris–HCl buffer, pH 8.8 (optimum pH in this buffer), Co^{2+} (0.2 mM) had no significant effect on enzyme activity.

DISCUSSION

The aim of the present work was to separate the three enzymes PAPS sulphohydrolase, PAPS 3′-nucleotidase and APS sulphohydrolase from a single source and to resolve the problem of the requirements of the enzymes with regard to Co^{2+}. Balasubramanian and Bachhawat[1] were the first to report that PAPS degradation to PAP and inorganic sulphate was stimulated by Co^{2+} in a sheep brain preparation. Since this report it appears to have become the practice in PAPS degradation studies[3,5,7,11] to include Co^{2+} in the assay mixtures and, incidently, to incubate for long periods of up to 1 h. In addition some of the studies have been carried out using organs and species which were different from those on which the original claims were made. The appearance of inorganic sulphate has been regarded solely as a measure of PAPS sulphohydrolase activity in spite of the possibility that sequential action of PAPS 3′-nucleotidase and APS sulphohydrolase might well have been a contributory factor to inorganic sulphate production. In such cases any APS produced as an intermediate could well have remained undetected. For example, the use of long incubation periods could mean that any APS produced would be completely removed by APS sulphohydrolase. Certainly the routine procedure[1] for the assay of the PAPS sulphohydrolase involves removal of unreacted PAPS by absorption on charcoal, a procedure that also removes APS[10].

The present work has unequivocally established that the cytosol PAPS sulphohydrolase of bovine liver, in the complete absence of either PAPS 3′-nucleotidase or APS sulphohydrolase, is either inhibited or not affected by Co^{2+}, depending on the buffer used. The APS sulphohydrolase from the same source is not affected by Co^{2+} (ref. 15), whilst those from the cytosol and the lysosomes of rat liver cell[10] are slightly inhibited. These findings are in accord with the fact that no type of sulphohydrolase yet studied is activated by divalent cations such as Co^{2+}, Mn^{2+} or Cu^{2+}. The collective evidence from various studies from these laboratories[9,10,12–14] leads us to conclude that claims for the activation of PAPS sulphohydrolase by Co^{2+} in crude tissue preparations merely reflect the copresence of the Co^{2+}-activated PAPS 3′-nucleotidase enzyme and an APS sulphohydrolase.

The work of Bailey-Wood *et al.*[10] with rat liver supernatant preparations provided an early indication that PAPS sulphohydrolase has an unusually high optimum pH. This has now been clearly shown for the separated enzyme from bovine liver cytosol, the optimum pH being 9.4. No other sulphohydrolase has yet been described that has such a high pH optimum, the one that approaches the closest is

arylsulphohydrolase of *Alcaligenes metalcaligenes* with an optimum pH of 8.75 (ref. 22). It will be interesting to discover the implications of the high pH optimum for the mechanism of action of PAPS sulphohydrolase. Meanwhile, the pH optima for the PAPS 3′-nucleotidase and the APS sulphohydrolase[15] of bovine liver cytosol are 5.9 and 5.4, respectively, optima that are quite remote from that of the PAPS sulphohydrolase. The significance of this great difference in terms of the economy of the cell is indeed obscure. Like the APS sulphohydrolase, the corresponding PAPS sulphohydrolase is strongly inhibited by ATP. Presumably, therefore, both enzymes are effectively inhibited under normal conditions where biosynthesis of PAPS from ATP is proceeding. The possibility exists that the three enzymes concerned in the degradation of APS and PAPS are involved in the regeneration of ATP *via* AMP under circumstances where biological sulphation is no longer required. However, the situation is even more obscure when account is taken of the fact that the present work deals only with those enzymes present in soluble form in the cytosol. PAPS- and APS-degrading enzymes are present in other regions of the cell[3,5,9,11] but their properties, significance and relationship to the cytosol enzymes are still largely unknown.

ACKNOWLEDGEMENTS

The authors are grateful to the Wellcome Trust and the Science Research Council for financial support. A.M.S. was the holder of an S.R.C. Studentship.

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