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Purification and properties of ATP–sulphate adenylyltransferase from liver

The sulphate-activating enzymes ATP-sulphurylase (ATP:sulphate adenylyltransferase, EC 2.7.7.4) and APS-kinase (ATP:adenylylsulphate 3'-phosphotransferase, EC 2.7.1.25) have been separated and purified from yeast by ROBBINS AND LIPMANN[1]. In recent years much attention has been focussed on these enzymes of mammalian tissue because of contradictory reports on the role of vitamin A in sulphate activation[2–5]. WOLF and his group[6,7] claim that ATP-sulphurylase activity is impaired in vitamin A deficiency and that a vitamin A metabolite can restore the lost activity. In the present investigation the enzyme has been partially purified and its properties have been studied. Evidence is also presented to indicate an intermediate complex formation as the first step in the ATP-sulphurylase reaction.

The enzyme activity was measured by estimating the disappearance of pyrophosphate in the presence of synthetic APS. APS was prepared according to the method of BADDILEY, BUCHANAN AND LETTERS[8] from pyridine sulphur trioxide and AMP, separated by paper chromatography and further purified using Bio-Gel P2. The incubation mixture consists of 20 μmoles Tris–HCl (pH 8.5), 0.6 μmole of sodium pyrophosphate, 0.2 μmole of APS, 0.2 μmole of $MgCl_2$ and enzyme in a total volume of 0.2 ml. It was incubated at 37° for 30 min and the reaction was stopped by keeping the tubes in boiling water for 90 sec after which they were chilled in ice. The system was subjected to the action of pyrophosphatase and the released phosphate was estimated according to the method of FISKE AND SUBBAROW[9]. A blank for each estimation was carried out omitting APS from the incubation mixture.

The enzyme was purified from fresh sheep liver by homogenization with isotonic potassium chloride in the cold and centrifugation for 1 h at 20 000 $\times$ g at 0°. The supernatant was subjected to ammonium sulphate fractionation and the precipitate obtained between 1.5 and 2.1 M concentration was dissolved in the minimum amount of 0.02 M Tris–HCl (pH 7.4) and passed through a Sephadex G-75 column. The enzyme obtained in the exclusion volume was subjected to ethanol fractionation at −5° and the precipitate obtained between 20–40% ethanol was dissolved in 0.02 M Tris–HCl (pH 7.4). It was further fractionated with ammonium sulphate, and the precipitate obtained between 1.5 and 2.1 M was dissolved in the minimum amount of 0.02 M Tris–HCl (pH 7.4). This solution was passed through a column of Sephadex G-200 and the fraction obtained immediately after the void volume contained the purified enzyme. This enzyme solution was added drop by drop to acetone at −10° (to a final volume ratio of 1:5); the mixture was centrifuged, the acetone removed by suction and the precipitate dissolved in the minimum amount of 0.02 M Tris–HCl (pH 7.4). This purified enzyme was able to catalyse the disappearance of 48 μmoles of pyrophosphate per mg protein at 37° within 30 min. By this procedure the enzyme was purified 200-fold with a recovery of 24%. The use of solvents like ethanol and acetone in the purification had not affected the enzyme activity, indicating the absence of an acetone- or alcohol-soluble cofactor for the enzyme. However, conclusive experiments were not carried out to study the role of vitamin A or its metabolites as a cofactor for the enzyme.

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

The enzyme is stable at −18° for four weeks at a protein concentration above 1 mg/ml but loses its activity on frequent freezing and thawing. $MgCl_2$ is essential for enzyme activity and the optimum proportions of pyrophosphate to $MgCl_2$ are 3:1. The optimum pH for activity is 8.5. The purified enzyme was found to be free from inorganic pyrophosphatase (EC 3.6.1.1), ATPase (EC 3.6.1.3) and APS-kinase. APS-kinase was measured by the formation of [^{35}S]PAPS[10]. The K_m values are $1.7 \cdot 10^{-3}$ M for pyrophosphate and $2 \cdot 10^{-3}$ M for APS, respectively. The enzyme is inhibited by ATP (80% at 10^{-4} M). When the enzyme was allowed to react in a way similar to yeast enzyme, to form APS from ATP and $^{35}SO_4^{2-}$, a high molecular weight radioactive compound was obtained which may be an enzyme–APS or a protein–APS complex, since this was obtained immediately after the void volume on Sephadex G-75 column chromatography (Table I). The formation of this complex was complete within 2 min, and experiments carried out at 0° and at 37° indicated a higher activity at 37°. BANDURSKI AND WILSON[11] have suggested that sulphate and yeast sulphurylase form a complex in the presence of $MgCl_2$. However, in the case of the present liver enzyme there is complex formation between APS and protein.

TABLE I

ENZYME-BOUND APS FORMATION

The complete system consists of 100 μmoles of Tris–HCl (pH 8.5), 10 μmoles of ATP, 10 μmoles of $MgCl_2$, 200 μC of carrier-free [^{35}S]sulphate and 0.5 ml (5 mg) of enzyme in a total volume of 1 ml. It was incubated at 37° for 15 min, cooled in ice and passed through a Sephadex G-75 column at 4° using 0.02 M Tris–HCl (pH 7.4). 2-ml fractions were collected immediately after the void volume and the radioactivity associated with protein was measured.

System	*Counts/min*
Complete	109 370
−ATP	2 240
$-MgCl_2$	2 250

The identity of the complex was established by three independent experiments. In the first experiment the complex was subjected to a reversal of the reaction in the presence of pyrophosphate and $MgCl_2$. It can be seen from Table II that 83% of the activity has been released, against 10% in the control. In the second experiment the

TABLE II

SULPHATE RELEASE FROM COMPLEX

The incubation mixture consists 100 μmoles of Tris–HCl (pH 8.5), 3 μmoles of pyrophosphate 1 μmole of $MgCl_2$ and complex (10 600 counts/min) in a total volume of 1 ml. It was incubated at 37° for 30 min, cooled in ice and passed through Sephadex G-75 at 4° using 0.02 M Tris–HCl (pH 7.4). 2-ml fractions were collected and the remaining radioactivity associated with protein was measured.

System	*Counts/min*	*% of release*
Complete	1790	83
$-Mg^{2+}$ and pyrophosphate	9340	10

complex was subjected to paper electrophoresis at 4° for 14 h at 4.5 V/cm using 0.02 M phosphate buffer (pH 8.5), along with authentic samples of APS and PAPS. PAPS moved 13 cm, APS 9 cm, and enzyme-bound complex 2 cm towards the anode; $^{35}SO_4^{2-}$ moved into the anodal buffer compartment. The radioactivity of the complex was shown to be associated with the protein portion by staining. The third experiment was the formation of [^{35}S]PAPS from the complex in the presence of ATP and $MgCl_2$ by the action of APS-kinase present in the Sephadex G-75 eluate. This preparation contains both ATP-sulphurylase and APS-kinase.

The release of $^{35}SO_4^{2-}$ in the reverse pyrophosphorylysis reaction, the association of radioactivity along with protein in electrophoresis and the formation of PAPS from the complex suggest that the initial step in sulphate activation may be the formation of a complex with enzyme and APS or a protein and APS. This complex is further acted upon by APS-kinase to form PAPS:

$$ATP + SO_4^{2-} + Enzyme \rightarrow Enzyme\text{–}APS + PP_i$$
$$Enzyme\text{–}APS + ATP \rightarrow PAPS + ADP + Enzyme$$

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