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ENZYME–SUBSTRATE COMPLEXES OF ATP-SULFURYLASE FROM MOUSE MASTOCYTOMA

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SUMMARY

1. A highly purified mouse mastocytoma ATP-sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4) preparation was observed to bind ATP, when incubated with the triphosphate. Upon addition of inorganic sulfate to the enzyme–ATP complex, the enzyme-bound ATP was converted to enzyme-bound adenylylsulfate.

2. When the enzyme–adenylylsulfate complex was incubated in the presence of adenylylsulfate kinase and ATP, a partial conversion of the bound adenylylsulfate to free 3′-phosphoadenylylsulfate was noted.

3. On the basis of these results a reaction sequence was suggested for the mechanism of sulfate activation, with ATP-sulfurylase–ATP and ATP-sulfurylase–adenylylsulfate complexes as intermediates.

INTRODUCTION

ROBBINS AND LIPMANN[1,2] and BANDURSKI and his group[3–5] established a two-step mechanism for the formation of 3′-phosphoadenylylsulfate (PAPS) involving adenylylsulfate (APS) as an intermediate.

$$ATP + SO_4^{2-} \rightleftharpoons APS + PP_i \quad (A)$$
$$APS + ATP \rightarrow PAPS + ADP \quad (B)$$

Reaction (A) is catalyzed by ATP-sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4), reaction (B) by APS kinase (ATP: adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25).

In the course of the studies presented below, high-molecular-weight radioactive compounds were observed after incubation of ATP-sulfurylase from mouse

Abbreviations: APS, adenylylsulfate; dAPS, deoxyadenylylsulfate; PAPS, 3′-phosphoadenylylsulfate.

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mastocytoma either with radioactive ATP alone, or with ATP and radioactive sulfate. These results were interpreted to indicate that enzyme–ATP and enzyme–APS complexes, respectively, were formed as intermediates in the sulfate activation process.

MATERIALS AND METHODS

Materials and analytical methods

Chemicals were C. P. or reagent grade, or purest preparations available. AMP, ATP and Tris were obtained from Sigma Chemical Company; [^{3}H]ATP (12 C/mmole) randomly labeled in the adenosine moiety, [8-^{14}C]ATP (18.5 mC/mmole), [8-^{14}C]dATP (20.5 mC/mmole), [8-^{14}C]ADP (20 mC/mmole) and carrier-free $Na_2{}^{35}SO_4$ from New England Nuclear Corporation; [^{32}P]ATP (21.7 C/mmole) labeled in the terminal phosphate group, APS and [^{35}S]PAPS were prepared as described earlier[6,7], and yeast APS kinase was purified by the method of ROBBINS[8].

Protein was determined according to the method of LOWRY *et al.*[9]. Radioactivity was measured using a liquid scintillation counter.

ATP-sulfurylase

ATP-sulfurylase from Furth mouse mastocytoma was purified according to a modification[10] of a procedure reported recently[6]. The specific activity of the highly purified ATP-sulfurylase was increased by a factor of 545. The purified enzyme catalyzed the formation of 556 μmoles ATP/mg protein per h (ATP-sulfurylase reaction in reverse), and it was free of ATPase, APS kinase, APS sulfohydrolase and inorganic pyrophosphatase; but it was not homogeneous when examined by acrylamide disc gel electrophoresis or ultracentrifugation, in part, probably, because the enzyme exists in more than one form[6]. As a matter of convenience, the highly purified ATP-sulfurylase fraction was designated enzyme.

Formation of enzyme–ATP complex

The reaction mixture, containing 30 μg enzyme protein, either 10 μC [^{3}H]ATP (randomly labeled) or 7.5 μC [γ-^{32}P]ATP, 0.4 μmole $MnCl_2$ and 20 μmoles Tris–HCl, pH 8.5, in a total volume of 0.2 ml, was incubated for 10 min at 37°. The solution was then chilled to 0°, and applied to a Sephadex G-75 column (32 cm × 1.4 cm) previously equilibrated with 0.02 M potassium phosphate buffer, pH 7.4. The mixture was eluted using the same buffer, with a flow rate of 8–9 ml/h; 2-ml fractions were collected, and aliquots were taken from each fraction for measuring radioactivity. The radioactive fractions containing the enzyme–ATP complex were pooled.

Formation of enzyme–APS complex

A reaction mixture containing 30 μg protein, 0.2 μmole ATP, 25 μC $Na_2{}^{35}SO_4$, 0.4 μmole $MnCl_2$ and 20 μmoles Tris–HCl in a total volume of 0.2 ml, was incubated, and the enzyme–APS complex was isolated as described for the enzyme–ATP complex.

Conversion of enzyme-bound ATP to enzyme-bound APS

Enzyme–[^{3}H]ATP complex (90 000 counts/min) was prepared as described

above and concentrated to a volume of 0.16 ml by ultrafiltration, using a Diaflo ultrafiltration apparatus with a PX 50 membrane (pressure, 60 lbs/inch^2). The enzyme–[^{3}H]ATP complex was incubated in the presence of 25 μC $Na_2{}^{35}SO_4$, 0.2 μmole $MnCl_2$ and 20 μmoles Tris–HCl, pH 8.5, and the doubly labeled enzyme–APS complex formed was isolated, as described for the formation of enzyme–ATP complex.

Paper electrophoresis of enzyme–ATP and enzyme–APS complexes

The enzyme–nucleotide complex was subjected to co-electrophoresis with ATP, APS, [^{35}S]PAPS and $Na_2{}^{35}SO_4$, on a Whatman No. 1 paper strip, 26 cm long, for 4.5 h, using 0.05 M potassium phosphate buffer, pH 8.5, at 8 V/cm. The sample was applied at room temperature, but the electrophoresis was carried out at 4°. The paper strip was then dried, and the radioactive spots were located using a strip scanner. For quantitative determination, the radioactive paper segments were cut out, and their radioactivities were measured using a liquid scintillation counter. Non-radioactive nucleotides were located on the paper strips using ultraviolet light and protein by staining with amido black[11].

Conversion of enzyme-bound APS to PAPS

ATP-sulfurylase–[^{35}S]APS complex was prepared as described above and incubated with 1 mM ATP, 2 mM $MgCl_2$ and 100 μg yeast APS-kinase in a final volume of 0.4 ml for 30 min at 37°. The reaction was stopped by addition of 0.14 ml of cold chloroform–isoamylalcohol (5:2, v/v). The denatured protein was removed by centrifugation, and an aliquot of the supernatant which contained the PAPS synthesized, was subjected to paper electrophoresis for separation of the [^{35}S]PAPS, under conditions described above.

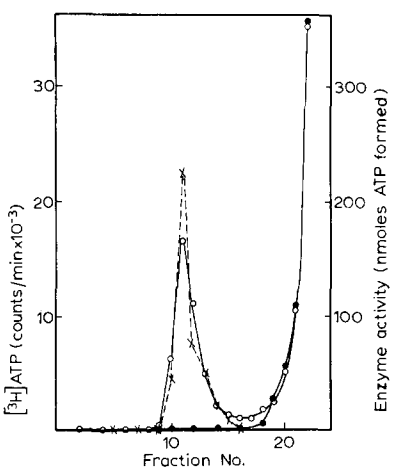

Fig. 1. Gel filtration on Sephadex G-75 of [^{3}H]ATP (labeled in adenosine group) incubated in presence and absence of ATP-sulfurylase (30 μg). Experimental conditions are described in text under *Formation of enzyme–ATP complex*. As the enzyme concentration was too low for activity determinations, results of gel filtration on Sephadex G-75 of a greater amount (2.5 mg) of ATP-sulfurylase alone are superimposed; enzyme activity determined as reported earlier[6]. ○—○, [^{3}H]ATP incubated in presence of enzyme; ●—●, [^{3}H]ATP incubated without enzyme or with boiled enzyme; ×—×, enzyme activity, ATP-sulfurylase alone.

TABLE I

FORMATION OF ENZYME–[^{3}H]ATP COMPLEX

ATP labeled with ^{3}H in the adenosine group was incubated with enzyme, and the enzyme–ATP complex formed was isolated as described under *Formation of enzyme–ATP complex*, except for the modifications of the incubation medium indicated.

Incubation mixture, additions or deletions	*Enzyme-bound ^{3}H found (counts/min)*
Complete mixture	20 900
– Enzyme	16
+ 1 mM PP_i	20 300
+ 1 mM Na_2SO_4 (non-radioactive)	28 700
– $MnCl_2$	20 500
– $MnCl_2$ + 1 mM EDTA	603
– Tris buffer + phosphate buffer, pH 8.5	26 700
– Enzyme, or – enzyme + boiled enzyme	0

RESULTS AND DISCUSSION

Formation of enzyme–ATP complex

After incubation of adenosine-labeled [^{3}H]ATP with enzyme, a significant portion of the label was eluted from Sephadex G-75 with the void volume, *i.e.* at the location where enzyme protein and ATP-sulfurylase activity were eluted as demonstrated in parallel experiments using more concentrated enzyme solutions (Fig. 1). Apparently, an enzyme–nucleotide complex was formed during the incubation which was not dissociated during gel filtration (Fig. 1, Table I). In the absence of enzyme, or in the presence of boiled enzyme (5 min at 100°) ,no radioactivity appeared at the location of the enzyme peak. Addition of PP_i to the incubation mixture did not influence the binding of label to the enzyme (Table I). When Tris buffer was replaced by phosphate buffer at pH 8.5, the binding of adenosine-labeled ATP was enhanced (Table I). The reaction was completed within 2 min. [8-^{14}C]dATP and [8-^{14}C]ADP also were found to form complexes when incubated with the enzyme under the conditions described, but to a smaller extent. The demonstration of an ATP-sulfurylase–dATP complex is in accord with our observation indicating that dAPS can serve as substrate in the reverse reaction catalyzed by the same enzyme, dAPS + $PP_i \rightleftharpoons$ dATP + SO_4^{2-} (see ref. 10).

When ATP labeled in the terminal phosphate group was incubated with ATP-

TABLE II

FORMATION OF ENZYME–[γ-^{32}P]ATP COMPLEX: INFLUENCE OF Na_2SO_4

ATP labeled with ^{32}P in the terminal phosphate group was incubated with enzyme and the enzyme–ATP complex formed was isolated as described under *Formation of enzyme–ATP complex*, except for the addition of non-radioactive Na_2SO_4, where indicated.

Incubation mixture	*Enzyme-bound ^{32}P found (counts/min)*
Complete	43 600
+ 1 mM Na_2SO_4 (non-radioactive)	4 760

sulfurylase, a significant portion of the label was again eluted with the enyzme peak (Table II). Since both adenosine- and γ-phosphate-labeled ATP were found to form radioactive complexes with ATP-sulfurylase, the results were interpreted to indicate that the entire ATP molecule was attached to the enzyme in the absence of inorganic sulfate, the second substrate of the ATP-sulfurylase reaction.

Formation of enzyme–APS complex

When ATP labeled in the adenosine group with ^3H was incubated with enzyme, the amount of enzyme-bound radioactivity was increased in the presence of Na_2SO_4 (Table I). However, when ATP labeled in the terminal phosphate group with ^{32}P was incubated under the same conditions, the addition of Na_2SO_4 to the incubation mixture reduced the quantity of enzyme-bound ^{32}P by about 90% (Table II). Apparently, the addition of sulfate resulted in the release of the γ-phosphate group of ATP from the complex, while the adenosine moiety remained bound to the enzyme.

When non-radioactive ATP and enzyme were incubated in the presence of $Na_2{}^{35}SO_4$, a significant portion of the radioactive sulfate was observed to be bound by ATP-sulfurylase. In the absence of ATP, sulfate was not bound by the enzyme (Table III).

TABLE III

FORMATION OF ENZYME–[^{35}S]APS COMPLEX

ATP and $Na_2{}^{35}SO_4$ were incubated with enzyme, and the enzyme–substrate complex formed was isolated as described under *Formation of enzyme–APS complex*, except for the modifications of the incubation medium indicated.

Incubation mixture, additions or deletions	*Enzyme-bound ^{35}S found (counts/min)*
Complete	11 700
+ 1 mM Na_2SO_4 (non-radioactive)	41
− ATP	145
− $MnCl_2$	4 630
− $MnCl_2$ + 1 mM EDTA	100
+ 1 mM PP_i	1 270
− Tris buffer + phosphate buffer, pH 8.5	15 300
− Enzyme, or − enzyme + boiled enzyme	0

These results were interpreted to indicate that the enzyme-bound ATP reacted with sulfate to form APS, with simultaneous release of PP_i, while the APS remained associated with the enzyme. The capacity of the enzyme to bind APS seemed to be greater than its ability to bind ATP, under the conditions of these epxeriments (Table I).

Formation of the enzyme–$^{35}SO_4{}^{2-}$ complex was reduced by omission of $MnCl_2$ from the reaction mixture (Table III). Apparently, an exogenous divalent cation such as Mn^{2+} was required for maximal conversion of enzyme-bound ATP to enzyme-bound APS, but not for binding of ATP by the enzyme (Table I). Both reactions were completely inhibited by EDTA (Tables I and III). Added PP_i decreased the binding of $^{35}SO_4{}^{2-}$ by the enzyme by about 90%. When Tris buffer was replaced by phosphate buffer, the complex formation was increased (Table III).

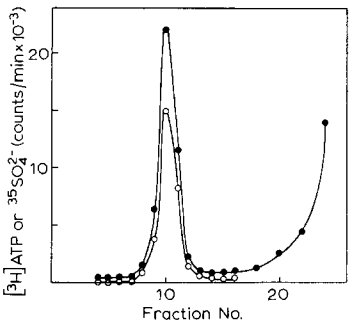

Fig. 2. Gel filtration on Sephadex G-75 of ATP-sulfurylase–[^{3}H]ATP complex (ATP labeled in adenosine group) incubated with $Na_2{}^{35}SO_4$. Experimental conditions described in text under *Conversion of enzyme-bound ATP to enzyme-bound APS*. ○—○, [^{3}H]ATP; ●—●, $^{35}SO_4{}^{2-}$.

The observations described in the preceding section were confirmed by experiments in which enzyme-bound ATP labeled in the adenosine group with ^{3}H was incubated with $Na_2{}^{35}SO_4$; ^{3}H and ^{35}S both were found to be eluted with the enzyme peak, indicating the formation of doubly labeled enzyme complex (Fig. 2). Apparently, enzyme-bound ATP was converted to APS, the latter remaining associated with the enzyme (Table IV). Added PP_i did not interfere with binding of ATP by the enzyme (Table I), but it inhibited again the conversion of ATP to APS almost completely (Table IV). The possibility is considered that PP_i reacted with APS to form ATP + $SO_4{}^{2-}$ (reaction catalyzed by ATP-sulfurylase in reverse which is favored energetically).

Characterization of enzyme–ATP and enzyme–APS complexes

When enzyme–[^{32}P]ATP and enzyme–[^{35}S]APS complexes were subjected to paper electrophoresis, both ^{32}P and ^{35}S, as well as enzyme protein, were found to remain at the point of application, while free ATP, APS, PAPS and inorganic sulfate moved 8.5, 8.0, 12.5 and 20.8 cm, respectively, towards the anode, under the same conditions.

Conversion of enzyme-bound APS to PAPS

When enzyme-bound [^{35}S]APS was incubated with yeast APS kinase in the presence of ATP and Mg^{2+}, under the conditions described, [^{35}S]PAPS was found to be

TABLE IV

CONVERSION OF ENZYME-BOUND ATP TO ENZYME-BOUND APS: INFLUENCE OF PP_i

Enzyme-bound [^{3}H]ATP was prepared and incubated with $Na_2{}^{35}SO_4$, and the enzyme–[^{3}H, ^{35}S]APS complex formed was isolated under conditions described under *Conversion of enzyme-bound ATP to enzyme-bound APS*

Incubation mixture	*Protein-bound radioactivity found*	
	^{3}H (counts/min)	*^{35}S (counts/min)*
Complete	31 100	46 000
+ mM PP_i	30 500	1 500

formed and released from the enzyme protein, with a yield of approximately 10%. Apparently, introduction of a phosphate group into the 3′ position of the nucleotide resulted in a dissociation of the latter from the enzyme. The limited extent of the reaction of yeast APS kinase with the APS bound to mammalian ATP-sulfurylase might result from the heterology of the sources of these two enzymes.

Mechanism of sulfate activation

On the basis of the results presented above, the following sequence is suggested for the process of sulfate activation:

$$\text{ATP-sulfurylase} + \text{ATP} \rightleftharpoons [\text{ATP-sulfurylase–ATP}]$$

$$[\text{ATP-sulfurylase–ATP}] + SO_4^{2-} \rightleftharpoons \text{ATP-sulfurylase}\begin{matrix}\text{ATP}\\ |\\ SO_4\end{matrix}$$

$$\text{ATP-sulfurylase}\begin{matrix}\text{ATP}\\ |\\ SO_4\end{matrix} \rightleftharpoons [\text{ATP-sulfurylase–APS}] + PP_i$$

$$[\text{ATP-sulfurylase–APS}] + \text{ATP} + \text{APS kinase} \rightarrow \text{PAPS} + \text{ATP-sulfurylase} + \text{APS kinase}$$

The ATP-sulfurylase complexes with ATP and APS, respectively, and the conversion of the former to the latter were demonstrated, as described above. The intermediate, however, was postulated without direct evidence, and the results on the formation of PAPS from enzyme-bound APS ought to be considered tentative, until a better yield of PAPS is obtained.

Our results are in agreement with findings of PANIKKAR AND BACHHAWAT[12], indicating that sheep liver ATP-sulfurylase catalyzed the formation of an enzyme–APS complex, but they fail to confirm suggestions by BANDURSKI AND WILSON[5], made on the basis of experiments using yeast ATP-sulfurylase, that an enzyme–Mg–sulfate complex was formed prior to the reaction with ATP. The mechanism proposed by us is also in conflict with conclusions reached by LEVI AND WOLF[13], who postulated an enzyme–AMP complex as an intermediate in the reaction catalyzed by rat liver ATP-sulfurylase.

If it is assumed that APS formed as a result of ATP-sulfurylase action remains attached to the enzyme, until it is either phosphorylated by ATP and APS kinase to form PAPS, or converted back to ATP and sulfate, in the presence of PP_i (reverse ATP-sulfurylase reaction), it is to be expected that no free APS would be found. In agreement with these considerations, PASTERNAK[14] reported the presence of endogenous PAPS in mouse mastocytoma (P-815), but he was unable to detect free APS. In our laboratory, very small and somewhat variable amounts of APS were found in some of the experiments concerned with the enzymatic preparation of PAPS, in the presence of a high-speed supernatant of mast cell tumor[7]. However, this APS originated most likely through hydrolysis of PAPS catalyzed by PAPS phosphohydrolase, an enzyme shown to be present in similar mastocytoma preparations (unpublished work from our laboratory).

In connection with our observations on the formation of an ATP sulfurylase–ATP complex, it is interesting that an enyzme–ATP complex was reported to be an intermediate in the activation of leucine by leucyl-RNA synthetase[15].

The ATP-sulfurylase used in the experiments described was purified 545-fold, and it was free of ATPase, APS kinase, APS sulfohydrolase and inorganic pyrophosphatase; but it was not homogeneous, when examined by acrylamide disc gel electrophoresis or ultracentrifugation, as mentioned. Although the inhomogeneity may be attributed, in part, to the fact that the enzyme exists in more than one form[6], it is realized that the results will be more meaningful when a pure enzyme is used for these studies. Efforts are in progress to obtain a homogeneous ATP-sulfurylase preparation.

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