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# SULPHATE ACTIVATION IN THE UNICELLULAR RED ALGA *RHODELLA*

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**Key Word Index**—*Rhodella maculata* Evans; Rhodophyceae; sulphate activation; APS; PAPS; ATP-sulphurylase; APS-kinase.

**Abstract**—ATP-sulphurylase (ATP:sulphate adenylyl-transferase, E.C. 2.7.74) from the unicellular red alga *Rhodella* has been purified 14-fold by $(NH_4)_2SO_4$ fractionation. It exhibits a temperature optimum of 31°, an activation energy of 10.8 kcal, has a pH optimum between 7.5 and 9.0 and forms unstable intermediates when incubated with ATP and group VI anions ($CrO_4^{2-}$, $MoO_4^{2-}$, $WO_4^{2-}$), resulting in the accumulation of pyrophosphate. Of the nucleotides tested, only ATP is acted upon by the enzyme. A divalent ion is required for activity and stimulation of the enzyme is 5 times higher with $Mg^{2+}$ than any other ion tested. The actual substrate for the reaction is a Mg–$ATP^{2-}$ complex. Free ATP inhibits the reaction. APS-[$^{35}$S] and traces of PAPS-[$^{35}$S] are formed when cell-free extract from *Rhodella* is incubated with ATP and sulphate-[$^{35}$S]. This indicates the existence of APS-kinase (ATP:adenylyl-sulphate 3$^1$-phosphotransferase, E.C. 2.7.1.25) as well as ATP-sulphurylase.

## INTRODUCTION

Marine algae which are major producers of sulphated polysaccharides provide an ideal system for investigating the enzymes involved in "sulphate activation". The unicellular red alga *Rhodella maculata* was chosen for the investigation. The cells are surrounded by a thick mucilaginous envelope which comprises 10% sulphate [1]. Sulphate-[$^{35}$S] is rapidly taken up from the medium in the light and incorporated into the mucilage which is continuously solubilized into the medium [1].

The sulphate activating system, ATP-sulphurylase and APS-kinase is widespread in nature. However, apart from the work of Goldberg and Delbruck [2] which deals very briefly with PAPS synthesis very little has been published on "sulphate activation" in marine algae. The present investigation was designed to find out whether ATP-sulphurylase and APS-kinase are present in *Rhodella* and, if so, to determine some of the properties of the ATP-sulphurylase, using the molybdolysis assay of Wilson and Bandurski [3].

## RESULTS

### *Purification and characterization of ATP-sulphurylase*

The purification steps for the enzyme are shown in Table 1. Maximum enzyme activity was found in fraction IV, i.e. between 40–60% $(NH_4)_2SO_4$ saturation. This fraction was used for all subsequent enzyme studies, unless otherwise stated.

ATP-sulphurylase activity was also measured in the reverse direction, and the amount of ATP produced, determined by the firefly luciferin–luciferase method. The sp. act., 0.72 nkat/mg protein, was of the same order as that determined by the molybdolysis assay. (The results are not directly comparable as the temperatures were not the same). Under standard assay conditions, ATP-sulphurylase activity was proportional to the amount of enzyme present, and activity was linear with time over a 60-min incubation period.

The ATP-sulphurylase from *Rhodella* shows a broad activity optimum between pH 7.5 and 9.0. Under standard assay conditions a pH of 8.4 was used.

Activity drops rapidly above 31°, and 29° was used for all assays. The activation energy was 10.8 kcal (45.5 kJ).

The effect of different group VI anions on ATP-sulphurylase activity was investigated (using standard molybdolysis assay conditions) and the following activities obtained, expressed as nkat/mg protein (in brackets): $SO_4^{2-}$ (0.17), $SeO_4^{2-}$ (0.25), $WO_4^{2-}$ (0.6), $CrO_4^{2-}$ (0.65), $MoO_4^{2-}$ (0.9). In the presence of $CrO_4^{2-}$, $MoO_4^{2-}$ or $WO_4^{2-}$ the enzyme forms unstable anhydrides, resulting in the accumulation of pyrophosphate. When $SO_4^{2-}$ or $SeO_4^{2-}$ are used as substrate, less phosphate is liberated.

The enzyme has an absolute requirement for ATP. GTP, UTP and CTP are not active in the reaction.

The effect of the following metal chlorides on ATP-sulphurylase activity was tested (at 10 mM) and the activities are given in nkat/mg protein (in brackets): Zn (0), Na (0.02), Ca (0.05), Sr (0.07), Mn (0.08), Co (0.1), Cu (0.1) and Mg (0.5). In the absence of metal ions activity was about 16–17 μkat/mg protein attributable to metal contamination. The enzyme has an absolute requirement for metal ions, $Mg^{2+}$ giving 5 times the activity of $Cu^{2+}$ and $Co^{2+}$.

Figure 1 shows that maximum ATP-sulphurylase activity is obtained when the concentrations of $Mg^{2+}$ and ATP are equal. This indicates that the substrate for the reaction is a 1:1 complex of $Mg^{2+}$ and ATP. When the concentration of ATP is increased above that of $Mg^{2+}$, the enzyme activity decreases. Free ATP is therefore an inhibitor of the reaction. An excess of $Mg^{2+}$ ions at a

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Table 1. Purification of ATP-sulphurylase. *Rhodella* cells (*ca* 20 g wet wt) were harvested by centrifugation, purified and enzyme activity determined by the molybdolysis assay (see Experimental)

| | Volume (ml) | Total activity (nkat ATP) | Sp. act. (nkat ATP/mg protein) | Purification factor | Recovery |
|---|---|---|---|---|---|
| 20000 *g* supernatant | 51 | 852 | 0.095 | 1 | 100 |
| 100000 *g* supernatant | 46 | 850 | 0.125 | 1.3 | 100 |
| 0–40% $(NH_4)_2SO_4$ ppt. | 13 | 110 | 0.022 | 0.2 | 13 |
| 40–60% $(NH_4)_2SO_4$ ppt. | 7 | 628 | 1.300 | 13.7 | 74 |

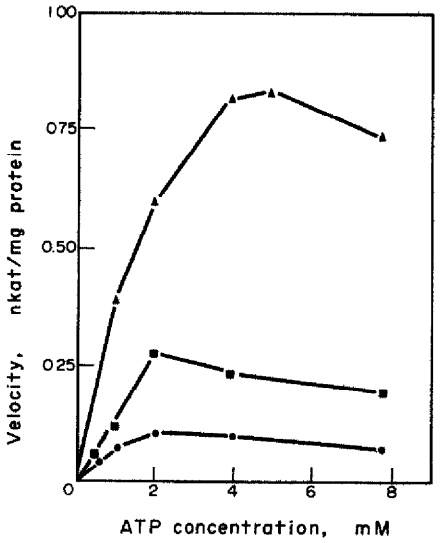


Fig. 1. Dependence of ATP-sulphurylase activity on ATP-concentration at constant concentrations of $MoO_4^{2-}$ and $Mg^{2+}$. Standard assay conditions were used as described in Experimental except that ATP- and $MoO_4^{2+}$-concentrations are varied as indicated. ●——● 2 mM $MoO_4^{2-}$, 2 mM $Mg^{2+}$; ■——■ 4 mM $MoO_7^{2-}$, 2 mM $Mg^{2+}$; ▲——▲ 8 mM $MoO_4^{2-}$, 5 mM $Mg^{2+}$.

constant concentration of ATP and $MoO_4^{2-}$ did not alter the reaction rate (unpublished results).

The ATP-sulphurylase activity of the 100000 *g* supernatant (fraction II) was stable for at least 2 months at −18°. Fraction IV was stable for 2 weeks at −18°. This fraction lost about 50% of its activity by freezing and thawing again, measured by method [2].

*Production of APS-[$^{35}$S] and PAPS-[$^{35}$S]*

On separation of the products resulting from incubation of fraction IV in 50 mM Tris–HCl buffer (pH 8.4) with $^{35}SO_4^{2-}$, ATP and $Mg^{2+}$ by HVE, APS-[$^{35}$S] and traces of PAPS-[$^{35}$S] (*ca* 20% of the activity of the former) are revealed.

## DISCUSSION

The enzymes ATP-sulphurylase and APS-kinase which together form the "sulphate activating system", have been studied in cell free extracts from yeast [3–5], from nitrifying microorganisms [6–8], from fresh water algae [9–11] and higher plants [12,13]. The present investigation indicates the existence of the "sulphate activating system" in the marine alga *Rhodella*. The recorded amount of PAPS-[$^{35}$S] formed was, however, very small and modifying conditions, e.g. giving a shorter incubation time, adding 1 μmol of carrier $SO_4^{2-}$ or adding half the amount of ATP did not increase the amount of PAPS-[$^{35}$S] produced. By using enzyme fraction II and standard radioactive assay conditions (see Experimental) the amount of APS-[$^{35}$S] increased compared with the PAPS-[$^{35}$S] produced. The presence of PAPS-degrading enzymes in the cell free extract may account for the small amount of PAPS-[$^{35}$S] produced. 3′-Nucleotidases 5′-nucleotidases [14] and PAPS-sulphatases [15] are all of general occurrence in animal and plant tissue.

The ATP-sulphurylase from *Rhodella* was only partly purified. The sp. act. of the crude extract (fraction I) was equal to that of a crude extract from yeast [16], i.e. 0.08 nkat ATP used/mg protein as determined by molybdolysis assay. The most highly purified fraction (fraction IV) had a sp. act. of 1.3 nkat ATP used/mg protein measured by the same method. The presence of polysaccharides in the enzyme extract made the solution very viscous, thus complicating further purification.

The ATP-sulphurylase had a pH optimum between 7.5 and 9.0. This is in agreement with work on enzymes from *Penicillium chrysogenum* [17], from yeast [5,16] and from soybean [18].

The optimum temperature for enzyme activity, 31°, is very low compared to sulphurylases from other sources. Akagi and Campbell [19] working with thermophilic bacteria, found that while the ATP-sulphurylase from *Clostridium nigrificans* (*Desulfatomaculum nitrificans*) was stable at 60–65°, the sulphurylase from *Desulfovibrio desulfuricans* was rapidly inactivated at the same temperatures. *D. desulfuricans* has a much lower growth temperature than *C. nigrificans* and this is consistent with the differences in temperature optima between the enzymes. *Rhodella* cells autolyze rapidly at temperatures above 30°, which may explain the rather low temperature optimum of the sulphurylase.

The specificity towards ATP and the utilization of different group VI anions is in agreement with the results obtained with the sulphurylase from yeast [3].

The ATP-sulphurylase is activated by $Mg^{2+}$ ions and the actual substrate is a $Mg\text{–}ATP^{2-}$ complex as shown for the enzyme from *P. chrysogenum* [20] and from yeast [16].

## EXPERIMENTAL

*Cultures* were maintained in the Leeds laboratory from the original made from a sample of sand as described in [21]. Cells were grown in SWM3 medium [22]. Cultures of 1.5 l in 3 l. flasks were kept in suspension by gentle shaking and illumination by 4000 lx from Northlight fluorescent tubes with a 16-hr photoperiod.

*Chemicals.* ATP, GTP, UTP, CTP, APS, yeast inorganic pyrophosphatase (485 units/mg), bovine serum albumin, firefly lantern extract, EDTA (Na salt) were obtained from Sigma Chemical Co. Carrier free sulphate-[$^{35}$S] was from The Radiochemical Centre, Amersham. PAPS-[$^{35}$S] was prepared from ATP and carrier free sulphate-[$^{35}$S] using a sulphate-activating enzyme complex from baker's yeast [5]. The method of Refs. [23] and [24] as modified in [6] was followed.

*Enzyme extraction and purification.* Cells from the logarithmic growth phase (*ca* 20 g wet wt) were harvested by centrifugation at 10000 *g* for 15 min. The cells were first washed in SWM3 medium, then in 0.1 M Tris–maleate–NaOH buffer, pH 8, containing 3 mM NaEDTA, and finally resuspended in this buffer (3 ml/g wet wt). Subsequent treatment was carried out at 0–4°. Cells were disrupted by passing the suspension (35 ml) 2× through a French pressure cell (100 kg/cm$^2$) at 4°. Microscopic examination showed that virtually all cells were broken by this treatment. The homogenate was centrifuged at 30000 *g* for 30 min, the pellet (which contained no ATP-sulphurylase activity) discarded and the supernatant (fraction I) centrifuged at 100000 *g* for 1 hr. Enzyme activity was entirely located in the subsequent supernatant (fraction II). This was fractionated by $(NH_4)_2SO_4$, sufficient being added to obtain 40% saturation. The crystals were added slowly to the supernatant while stirring to prevent local excess of electrolyte. The suspension was left to equilibrate for 30 min, the ppt. centrifuged at 30000 *g* for 30 min and the pellet (fraction III) resuspended in 0.1 M Tris–maleate–NaOH buffer, pH 8, containing 3 mM EDTA. The supernatant was then brought to 60% saturation with $(NH_4)_2SO_4$, the ppt. collected by centrifugation and resuspended in Tris buffer as above (fraction IV).

*Enzyme assay for ATP-sulphurylase.* (1) Enzyme activity was determined by modification of the molybdolysis assay of [3] where molybdate replaces sulphate as substrate. The complete reaction mixture contained (total vol. 250 μl): Tris–HCl buffer, pH 8.4, 25 μmol; $MgCl_2$, 2.5 μmol; ATP, 2 μmol; $Na_2MoO_4$, 2 μmol, inorganic pyrophosphatase 1 unit; enzyme, 50 μl. The tubes were equilibrated at 29° before starting the reaction (by adding the enzyme) and then incubated with shaking for 45 min at 29°. The reaction was stopped by boiling for 90 sec. The tubes were then transferred to an ice-bath to minimize chemical breakdown of ATP. Phosphate[25] and protein [26] were then determined. Crude enzyme fractions were centrifuged at 5000 *g* for 5 min to remove precipitated protein and an aliquot of the supernatant used for Pi determination. To correct for ATP-ase activity, a control tube without $MoO_4^{2-}$ was included for each enzyme determination; control tubes without enzyme or with boiled enzyme were also included. Finally, to ensure pyrophosphatase was not rate-limiting a tube containing the following was used (total vol. 250 μl); buffer (as used in actual experiment), 25 μmol; $MgCl_2$, 2.5 μmol; $Na_4P_2O_7$, 1 μmol and inorganic pyrophosphatase 1 unit. In experiments where $MgCl_2$ was omitted from the reaction mixture, 2.5 μmol $MgCl_2$ and 1 unit of pyrophosphatase were added to each tube, after the reaction was stopped. Before Pi determinations were carried out, tubes were given a second incubation (15 min at 29°). The molybdolysis assay was used in all experiments unless otherwise stated. (2) Enzyme activity was also determined by the generation of ATP from APS and PPi. The incubation mixture contained (total vol. 0.5 ml): Tris–HCl buffer, pH 8.4, 10 μmol; $MgCl_2$, 0.25 μmol; PPi, 0.05 μmol; APS, 0.02 μmol and enzyme (fraction IV diluted 1:20) 50 μl. The reaction mixture was incubated for 5 min at 20°, the reaction stopped by boiling for 90 sec, the mixture cooled down in an ice-bath and 100 μl samples taken out for ATP determination by firefly assay [27]. The counting chamber was kept at 10°. The luciferin–luciferase extract, Sigma FFLE 250, was prepared according to [28].

*Enzyme assay of the sulphate activating system.* APS-kinase activity was determined by measuring the production of APS-[$^{35}$S] and PAPS-[$^{35}$S] from ATP and sulphate-[$^{35}$S]. The incubation mixture contained, unless otherwise stated (total vol. 200 μl): Tris–HCl buffer, pH 8.4, 10 μmol; $MgCl_2$, 2.5 μmol; pyrophosphatase, 1 unit; sulphate-[$^{35}$S] (carrier free), 15 μCi; enzyme (fraction II or IV, 100 μl). Boiled enzyme controls were included. Each reaction tube was flushed thoroughly with $N_2$ and sealed before the reaction was started. Incubation was at 25° for 1 hr, and the reaction was stopped by boiling for 1 min.

*Electrophoretic separation of APS and PAPS.* The labelled nucleotides were separated by HVE [6]. 50 μl aliquots from the reaction mixture were spotted onto Whatman 3 MM chromatography paper using 0.1 M Na citrate buffer pH 5 and separation carried out at 1000 V for 1.5 hr (30 V/cm). The paper was dried, cut into 3 cm wide strips and radioactivity detected by strip scanning. Nucleotides were also detected by UV light absorption. The mobilities of the radioactive products were the same as the mobilities of the standards.

*Abbreviations.* APS, adenosine-5′-phosphosulphate; PAPS, adenosine-3′-phosphate 5′-phosphosulphate.

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