Phytochemistry, Vol. 20, No. 4, pp. 601–604, 1981.
Printed in Great Britain.

0031–9422/81/040601–04 $02.00/0

A KINETIC INVESTIGATION OF THE APS-KINASE FROM *CHLAMYDOMONAS REINHARDII* CW 15

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(*Revised received* 6 *August* 1980)

Key Word Index—*Chlamydomonas reinhardii* CW 15; Chlorophyceae; ATP:APS phosphotransferase (EC 2.7.1.25); adenosine 3′-phosphate 5′-sulphatophosphate; reaction kinetic; HPLC of nucleotides.

Abstract—The reaction kinetics of APS-kinase from *Chlamydomonas reinhardii* showed that the enzyme formed PAPS from APS upon the addition of ATP. Evidence for a ^{35}S-labelled protein intermediate between APS and PAPS has been obtained. The APS-kinase activity could only be measured in the presence of low concentrations of APS ($20 \pm 10\,\mu M$) and of ATP (0.2 ± 0.05 mM) due to substrate inhibition. The inhibition was partially overcome by low concentrations of 3′,5′-PAP ($10\,\mu M$). The rates of PAPS formation obtained with cell extracts from the alga varied from 2 to 6 nM PAPS/mg protein/min ($33–100 \times 10^{-12}$ kat/mg).

INTRODUCTION

The activated sulphate PAPS has previously been identified as the product of enzymatic sulphate activation in green plants [1–3] and algae [4–6]. However, very little is known about the enzymatic process itself, which leads to the formation of this metabolite (see [7] for review) and evidence for the action of the enzyme APS-kinase in plants is at present indirect or presumptive. The ATP-dependent biosynthesis of PAPS from its precursor APS, so far, has only been demonstrated with the enzyme from bakers yeast [8]. Even in this investigation the true kinetic constants could not be determined because the enzyme was inhibited by the lowest concentrations of substrate employed.

The aim of the present work was to show that PAPS in plants is formed by the action of the APS-kinase:

$$APS^{2-} + ATP^{4-} \rightarrow PAPS^{4-} + ADP^{3-}$$

as was shown for the yeast sulphate activating system. The selectivity of HPLC and the sensitivity of continuous flow radio detection were found suitable to measure the disappearance of the substrate APS and simultaneously the appearance of the reaction product PAPS. The time course of the APS-kinase activity has been followed with partially purified extracts from the alga *Chlamydomonas reinhardii* CW 15.

RESULTS AND DISCUSSION

Under the conditions used (Fig. 1) [^{35}S]PAPS was formed in the presence of ATP from [^{35}S]APS by a partially purified protein extract from *C. reinhardii*. The reaction product was separated from its precursor by HPLC on a reverse-phase column as described in ref. [9]. The identification was obtained by co-chromatography with authentic [^{35}S]PAPS prepared according to Robbins [10] with the sulphate-activating enzymes from bakers yeast, using [^{35}S]sulphate and ATP as substrates. During incubation with the protein from *C. reinhardii*, PAPS was the only ^{35}S-labelled nucleotide formed from [^{35}S]APS. The occurrence of sulphate was due to non-specific hydrolysis of the [^{35}S]APS and [^{35}S]PAPS. The dependence of PAPS biosynthesis on ATP is depicted in Fig. 2A. The desalted protein extract formed the nucleotide from its precursor APS only after addition of ATP, indicating the action of a true ATP:APS phosphotransferase. The rate of PAPS biosynthesis in this sequential experiment was non-linear: after the addition of ATP a rapid initial phase was followed by a slower but more constant production of PAPS. The first initial formation of PAPS was suspected to be due to an enzyme-bound intermediate which may have released the reaction product when ATP became available. From previous investigations by Schiff and coworkers [11, 12] on the metabolism of APS in the alga *Chlorella*, a protein that specifically binds APS was known. Its further action was characterized as the transfer of the activated sulphate group from the APS onto an endogenous carrier forming an organic thiosulphate ('Bunte salt'). Since extracts from *C. reinhardii* also showed the capability of APS-binding, the resultant [^{35}S]APS-protein complex has been isolated by gel chromatography (see Experimental)—the complete assay mixture was investigated for sulphated nucleotides formed from [^{35}S]APS and for protein-bound intermediates. The kinetics of the fixation pattern (Fig. 2B) showed that immediately after the mixing of the protein extract with [^{35}S]APS and before the onset of [^{35}S]PAPS biosynthesis a ^{35}S-labelled protein occurred. The time course of its appearence is interpreted as the intermediate between APS and PAPS, since its decline coincided with the onset of PAPS biosynthesis. In experiments with very little PAPS formation (details not shown) the concentration of this compound remained practically unaltered.

The kinetic experiments showed that under the conditions used, APS-kinase activity was only

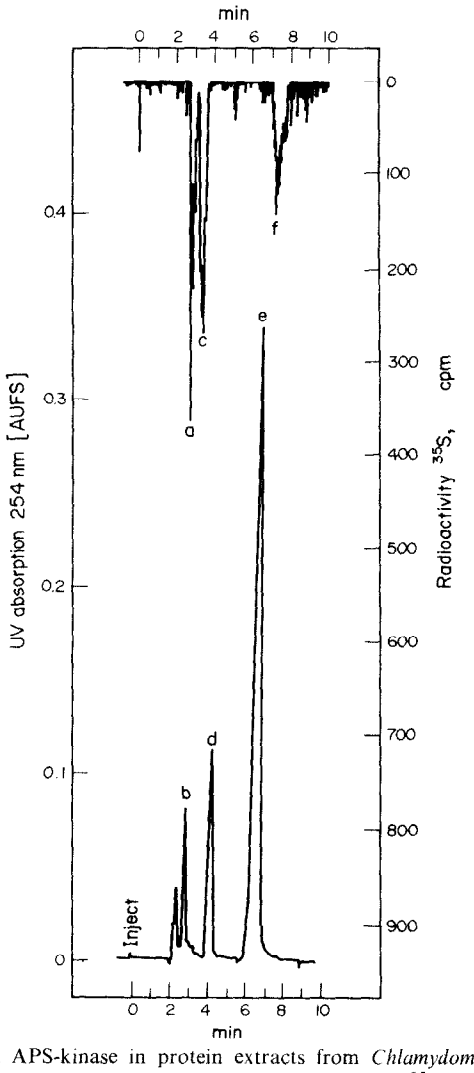

Fig. 1. APS-kinase in protein extracts from *Chlamydomonas reinhardii* CW 15. Compounds were formed from [^{35}S]APS in the presence of ATP. Assay conditions: triethanolamine buffer (pH 8.0) 25 mM, $MgCl_2$ 5 mM, ATP 1 mM, [^{35}S]APS 20 μM (sp. act. 280 Bq/nM) 32μg protein extract/100 μl volume, incubation for 30 sec under air at 30°. HPLC separation of nucleotides was extracted from the above assay on Li-Chrosorb RP18/10 μ, reverse phase, mobile phase 3 mM TBAH (pH 9.4) in 9.6% 2-PrOH, with other conditions as in ref. [9]. Simultaneous recording of radioactivity (upper trace) and UV absorbance (lower trace): (a) [^{35}S]SO_4, (b) AMP, (c) [^{35}S]APS, (d) ADP, (e) ATP, (f) [^{35}S]PAPS.

measurable for periods not longer than 4 min because the supply of [^{35}S]APS was rapidly exhausted. Further experiments, therefore, were designed to select the optimal concentrations for both the substrates, i.e. APS and ATP, of the APS-kinase. Undesired enzyme activities which hydrolyse ATP or APS have been reduced by fractionating the protein extract with $(NH_4)_2SO_4$ (30–60% saturation) in combination with gel and ion exchange chromatography. The purification procedure for APS-kinase from *C. reinhardii* will be published separately [13]. The partially purified enzyme showed a high affinity for APS (Fig. 3A) with an apparent K_m of 6 μM. Concentrations of APS exceeding 30–40 μM were inhibitory. A substrate inhibition by APS has already previously been observed with the APS-kinase from yeast [8] and from *Micrococcus denitrificans* [14]. ATP as the second substrate of this enzyme (Fig. 3B) was also metabolized at very low concentrations—the apparent K_m was 50–60 μM in the absence of an ATP regenerating system. Under these conditions ATP became inhibitory at concentrations exceeding 0.6 mM. When the APS-kinase assay was complemented with an ATP-regenerating system (2.0 mM creatine phosphate and 0.75 U creatine phosphokinase/assay) concentrations of 0.2 mM ATP were sufficient to cause severe inhibition. 3′,5′-PAP was found to alleviate this substrate inhibition when applied at low concentrations (10 μM). The nature of this effect is not yet known. It may either indicate a true regulatory site of the APS-kinase, or, as found for crude extracts from *Chlorella*, reflect an inhibition of the otherwise continued metabolic turnover (or degradation) of PAPS [4, 15].

EXPERIMENTAL

Growth and fractionation of the alga. Chlamydomonas reinhardii CW 15 was grown S-autotrophically as described in ref. [16]. The cells were harvested in the early phase of logarithmic growth, washed once in isotonic buffer and ruptured by osmotic shock in a homogenizing buffer containing 25 mM imidazole pH 6.8, 5 mM $Na_2S_2O_5$, 2.5 mM EDTA and 2% Polyclar AT. Protein extraction was initiated by sonication (6 × 15 sec with intervals of 15 sec) when the concn of cells had been brought to 2 mg chlorophyll/ml. The extract was clarified by centrifugation (45 000 *g*, 30 min) and the green supernatant was desalted by gel chromatography (Biogel P6, 2 × 20 cm, exclusion limit MW 4800, buffer: 25 mM Tris–Cl pH 8.0, 10 mM NaCl). The protein fractions were pooled and referred to as 'protein extract'.

Further fractionation was achieved by stepwise pptn of the crude extract with solid $(NH_4)_2SO_4$. The APS-kinase was found in the fraction of 30–60% satn. This fraction was chromatographed on Sephacryl S-200 (2.4 × 37 cm, Tris buffer as above but NaCl omitted) and the most active samples were concd on Sephacel (1.5 × 7 cm). The column was developed with a Cl^- gradient in Tris buffer from 0 to 250 mM Cl^-. The active fractions were pooled and referred to as 'partially purified protein'.

APS-kinase. The assay is based on the ATP-dependent formation of [^{35}S]PAPS from [^{35}S]APS. The enzyme activity was routinely investigated in a final vol. of 0.5 ml triethanolamine/HCl, pH 8.0, 25 mM; $MgCl_2$ 5 mM; ATP 0.5 mM if not specified otherwise; creatine phosphate 3 mM; creatine prosphokinase (5 mg/ml) 125 mU; APS 20 μM (as [^{35}S]APS, 280 Bq/nM); 3′,5′-PAP 10 μM; and enzyme extract as indicated. The reaction was terminated by 2-PrOH (30% final concn). Nucleotides were extracted and analysed as described in ref. [9]. The measurement of labile sulphite from an aliquot of the APS-kinase mixture was carried out as described for the APS-sulfotransferase from plants [17] using scintillation vials and sealed mini-reaction vessels for the distillation process. Volatile sulphite was trapped directly into the trioctylamine. All ^{35}S-labelled compounds were counted in commercially available scintillant for aq. samples and processed into dpm applying appropriate quench corrections and internal standards ([^{14}C]toluene).

Preparation of [^{35}S]*APS.* The substrate for APS-kinase was prepared enzymatically from AMP and [^{35}S]sulphite with a

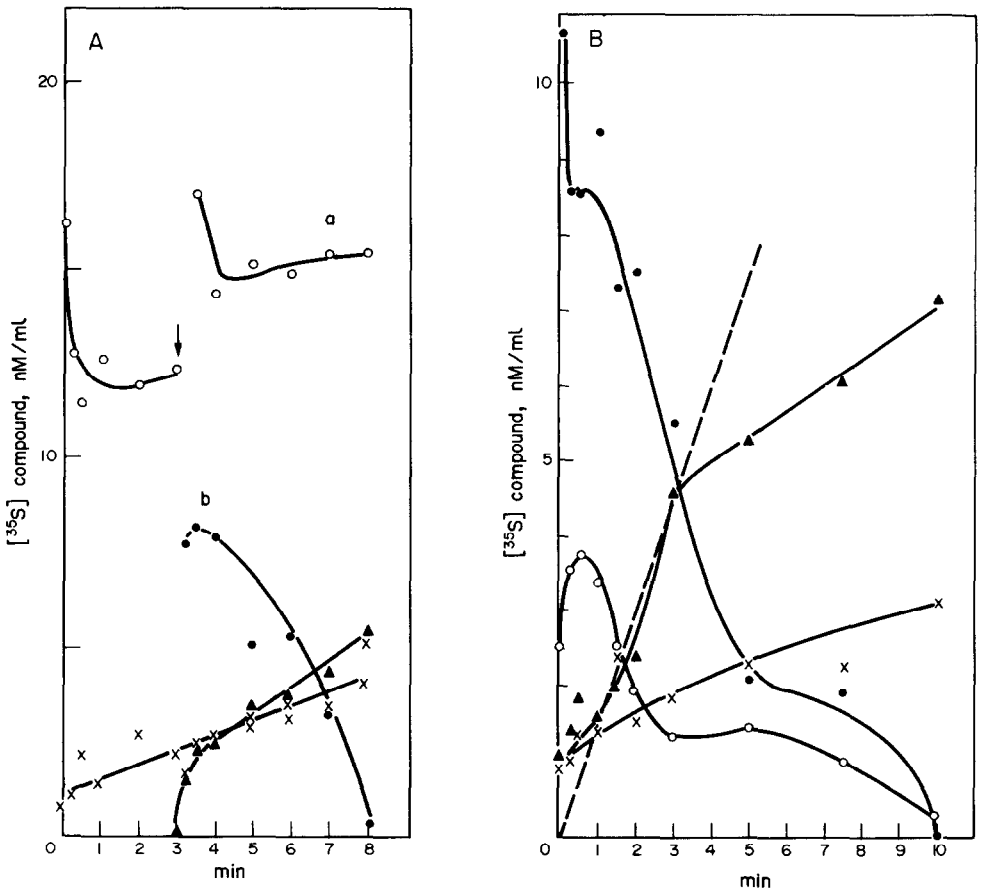

Fig. 2. Kinetics of PAPS-biosynthesis. (A) Initiation of PAPS-biosynthesis by ATP. The arrow indicates the addition of ATP to the enzyme pre-incubated with APS. The complete assay mixture has been separated into (a) reference without ATP and (b) sample with 0.5 mM ATP after 3 min. Total volume 10 ml, 18.2 mg protein extract, [^{35}S]APS 15 μM. (B) Initial rate of APS-kinase with intermediary formation of a protein-bound 'exchangeable sulphite'. The dashed curve represents the assumed initial slope of PAPS biosynthesis in the presence of ATP and APS. The rate limitation occurs when the protein-SO_3 has reached its apparent steady state (180 sec). Total volume 5.0 ml, 3.6 mg protein extract. [^{35}S]APS 20 μM, ATP 0.5 mM and creatine phosphate/creatine phosphokinase as ATP-regenerating system (see Experimental). Samples of 250 μl were withdrawn at intervals as indicated and analysed as in Fig. 1. 3′,5′-PAP was used as internal standard to eliminate errors caused by the extraction procedure of nucleotides. ○—○ APS in absence of ATP; ●—● APS + ATP; ▲—▲ PAPS; ○--○ protein-SO_3; ×——× SO_4^{2-}.

purified APS-reductase from *Thiobacillus denitrificans* strain RT according to ref. [18]. Further purification of the sulphated nucleotide was obtained by separation on DEAE-cellulose [19], but replacing the $(NH_4)_2CO_3$ gradient by Tris–Cl/NaCl as used for the purification of the APS-kinase on Sephacel. The samples containing [^{35}S]APS were concd and desalted by passage through a Biogel P2 column, equilibrated with 25 mM Tris–Cl buffer (pH 8.0). Radiochemical purity was higher than 98% for the samples used in the APS-kinase assay.

Protein was determined as TCA ppt. [20] or with Coomassie Brilliant Blue [21] and chlorophyll was measured according to ref. [22].

Acknowledgement—The financial support of the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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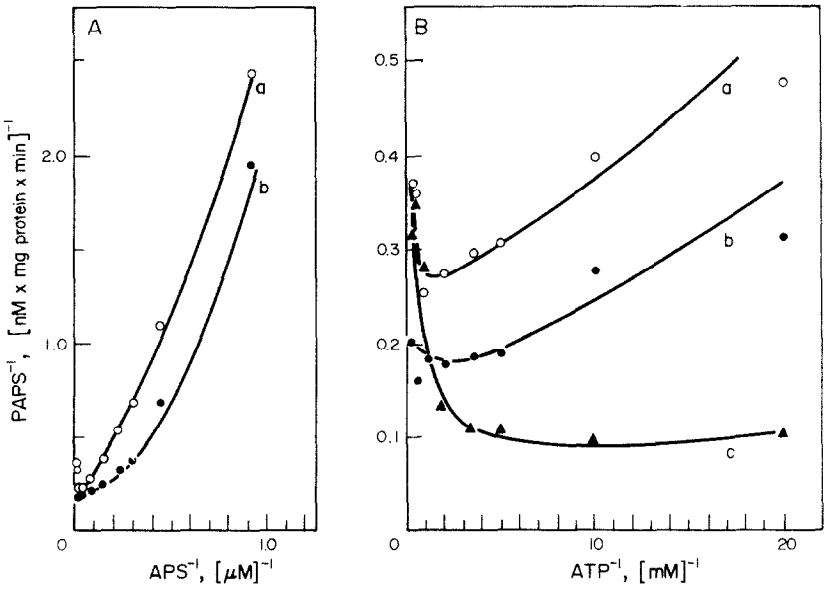

Fig. 3. Affinity of APS-kinase for APS and ATP. (A) Double reciprocal plot of the initial rate of PAPS formation vs the substrate APS. (a) without, (b) with 10 μM 3',5'-PAP. Partially purified protein 356 μg. ATP 1 mM. (B) Double reciprocal plot of the initial rate of PAPS formation vs the substrate ATP. (a) without, (b) with 10 μM 3',5'-PAP, and (c) as (b) but additional ATP-regenerating system. Partially purified protein (a) 340 μg, (b) 363 μg, (c) 304 μg, APS 20 μM. For other conditions see Fig. 1 and Experimental. Curves of both substrates indicate the substrate inhibition at higher concentrations.

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