Two New Reactions of the Activated Sulfates Adenylylsulfate and 3′-Phosphoadenylylsulfate with Ammonia

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Two substances, P1 and P2, have been isolated from the ammonium bicarbonate solutions of adenylylsulfate and 3′-phosphoadenylylsulfate, respectively. Using radiochemical, spectroscopic, chromatographic and enzymatic methods it could be shown that P1 was identical to adenosine 5′-monophosphoramidate and that P2 was a closely related phosphorylated derivative of the same substance, namely 3′-phosphoadenosine 5′-monophosphoramidate.

We reported earlier on the existence of an adenosine derivative occuring during the preparation of adenylylsulfate which was not identical to any of the expected products of decay [1]. In the meantime we have applied the same chromatographic methods to the isolation of 3′-phosphoadenylylsulfate. During the purification process we observed the quantitative conversion of the 3′-phosphoadenylylsulfate to an adenosine derivative which again was not identical to any of the adenylates normally encountered in work with the activated sulfates. In this paper we describe the isolation and structural identification of these two substances.

Formation and Isolation of P1 and P2

We synthesized 5 mmol of [^{35}S]PAPS* (spec. radioactivity = 0.1 mCi/mmol) using the enzymatic procedure of Robbins [2] and applied the chromatographic procedures described earlier for the isolation of APS [1] successfully to the isolation of the PAPS. A preliminary test showed that the lyophilisation of the purified PAPS fraction (10 mM in 0.9 M NH_4CO_3) led to about 30% decomposition of the sulfur nucleotide. Thus, we decided to use the ammonium bicarbonate solution of PAPS as is for our enzymatic studies. To our surprise we discovered after four months' storage at −18 °C that the solution contained only 5% of the PAPS originally present. The remainder had converted to inorganic sulfate and a UV-absorbing, non-radioactive substance (P2) whose chromatographic behavior was unlike that of any of the adenylates normally encountered in work with the activated sulfates (Table I). This prompted us to examine ammonium bicarbonate solutions of APS which had been stored for several months at −18 °C. Here, too, we observed the nearly quantitative conversion of the APS to the unknown derivative (P1) described earlier [1].

Table. I. Chromatographic data for P1, P2 and the reference substances used.

Substance	R_f-value (high performance thin layer chromatography)	Electrophoretic mobility (high voltage electrophoresis) [cm]
adenosine	0.80	0.0
AMP	0.42	4.8
ADP	0.23	8.0
ATP	0.15	10.3
PAP	0.25	9.4
APS	0.68	8.5
PAPS	0.52	12.2
P1	0.72	2.4
P2	0.36	9.4

P1 and P2 were isolated from these solutions using ion-exchange chromatography on Bio-Rex 5 resin as described by Cooper and Trüper [1]. The ammonium bicarbonate was removed by lyophilisation. P1 appeared as a sharp peak before sulfate, AMP and residual APS. P2 was eluted after sulfate and before PAP (slightly overlapping) and PAPS. The amorphous white salts were stored dry at −18 °C.

Structural Identification of P1

In two separate experiments 0.1 mmol each of [^{35}S]APS and [U-^{14}C]APS were prepared as de-

* Non-standard abbreviations: adenylylsulfate, APS; 3′-phosphoadenylylsulfate, PAPS; adenosine 3′,5′-bisphosphate, PAP.

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scribed earlier [1]. The lyophilized APS samples were redissolved in water and their components separated by renewed ion-exchange chromatography on Bio-Rex 5 resin. In the case of the [^{35}S]APS the P1 fraction contained no radioactivity. In the case of the [U-^{14}C]APS the ratio of radioactivity to UV-absorption in the P1 fraction was identical to that of the original APS. These results indicated that the AMP moiety of the APS molecule had been transferred to the P1 molecule while the sulfate group had been split off as inorganic sulfate.

The UV-spectrum of P1 was identical to that of AMP indicating that P1 was, indeed, an adenosine derivative. The ^{13}C-NMR-spectrum was very similar to that of ATP used as a reference. The fact that the signals at 65.1 and 84.9 ppm showed a coupling very similar to that of ATP indicated that the 5′-carbon atom was esterified with phosphate. The ^{31}P-NMR-spectrum confirmed the presence of one species of phosphor atom showing only one signal at 8.4 ppm.

On the basis of these results we developed a first working hypothesis suggesting that P1 be the symmetrical dimeric form of AMP: p^1,p^2-diadenosine 5′-pyrophosphate. Comparison of P1 with the authentic substance yielded significant differences in the R_f-values, electrophoretic mobilities, ^{13}C-NMR-, ^{31}P-NMR- and IR-spectra. Thus, we were forced to consider a possible reaction of the APS with the buffer solution. A reaction of the APS with the bicarbonate was unlikely due to the fact that no additional signal was detected in the ^{13}C-NMR-spectrum. We suggested, therefore, as a second working hypothesis that P1 be an amide of AMP. P1 could be converted to AMP by a phosphodiesterase from snake venom which specifically attacks nucleotides containing a free 3′-hydroxyl function yielding free 5′-nucleotide phosphates. In order to investigate our second working hypothesis we determined ammonia and AMP present in a sample of P1 (0.80 mg/ml) before and after treatment with phosphodiesterase from snake venom (Table II). The results presented above suggested that P1 was identical to the monoammonium salt of adenosine 5′-monophosphoramidate. This proposal was supported by the fact that comparison of P1 with an authentic sample showed identical chromatographic behavior and IR-spectra. We propose, therefore, that P1 be the product of the non-enzymatic reaction of adenylylsulfate with ammonia:

$$APS^{2-} + NH_3 \rightarrow SO_4^{2-} + \text{adenosine 5′-monophosphoramidate} + H^+.$$

Table II. Products of the enzymatic degredation of P1 (0.80 mg/ml) by phosphodiesterase from snake venom.

Substance	Before enzymatic treatment	After enzymatic treatment
NH_3 [mM]	2.07	4.05
AMP [mM]	0.0	2.0

Structural Identification of P2

P2 showed a UV-spectrum typical for adenosine derivatives. The ^{13}C-NMR-spectrum showed in addition to the adenine signals at 64.9, 75.0, 84.6 and 88.1 ppm. The fact that the signal at 75.0 ppm shows a double intensity with the same coupling constant suggests that it is composed of two coincidental signals. We deduced from the complex coupling pattern that both the 3′- and 5′-carbon atoms were esterified with phosphate. In view of our experience with P1 we proposed as an initial working hypothesis that P2 was the 3′-phosphorylated derivative of P1, namely 3′-phosphoadenosine 5′-monophosphoramidate.

In order to investigate this hypothesis we treated a sample (1.00 mg/ml) of P2 first with 3′-nucleotidase from rye grass which specifically removes the 3′-phosphate group of nucleotide 3′-phosphates [3]. We determined ammonia, phosphate and AMP separately before and after this enzymatic treatment and subjected samples to high performance thin layer chromatography and high voltage electrophoresis. Then the reaction mixture was treated with the phosphodiesterase from snake venom as described above. Again phosphate, ammonia and AMP were determined and samples were subjected to chromatographic analysis. The results of the enzymatic degradation of P2 are shown in Table III. The sample contained after treatment with 3′-nucleotidase one major UV-absorbing component whose chromatographic behavior was identical to that of authentic adenosine 5′-monophosphoramidate. A small amount of AMP was released from PAP which was present as a minor impurity in the P2 sample. Treatment of the sample with the phosphodiesterase from snake venom yielded equimolar amounts of

Table III. Products of the enzymatic degredation of P2 (1.0 mg/ml) by 3′-nucleotidase and phosphodiesterase from snake venom.

Substance	Control	+3′-Nucleotidase	+Phosphodiesterase
NH_3 [mM]	6.4	6.4	8.5
P_i [mM]	0.1	2.2	2.2
AMP [mM]	0.0	0.1	2.2

ammonia and AMP. These data confirm the proposed structure of P2 as 3′-phosphoadenosine 5′-monophosphoramidate which can spontaneously form from PAPS and ammonia in aqueous solution:

$$PAPS^{4-} + NH_3 \rightarrow \text{3′-phosphoadenosine 5′-monophosphoramidate} + SO_4^{2-} + H^+.$$

Experimental

UV-spectra: Perkin-Elmer double beam spectrophotometer 124. – IR-spectra: Pye Unicam SP 1025 (intensities: s = strong, m = moderate, w = weak). – NMR-spectra: Bruker WH 90. High performance thin layer chromatography: Fertigplatten für die HPTLC Kieselgel 60 F_{254} (Merck, Darmstadt), solvent system A: iso-propanol/NH_3 (33%)/H_2O (6:3:1 vol.). High voltage electrophoresis was carried out as described earlier [1].

Adenosine 5′-monophosphoramidate (sodium salt), p^1,p^2-adenosine 5′-pyrophosphate and 3′-nucleotidase from rye grass were obtained from Sigma, München. Bio-Rex 5 ion-exchange resin (200–400 mesh, Cl^- form) was purchased from Bio-Rad Laboratories, Richmond, California. Dowex ion-exchange resin was from Serva, Heidelberg. Phosphodiesterase from snake venom and all other enzymes and biochemicals were from Boehringer & Söhne, Mannheim. [^{35}S]sulfite (sodium salt, 5.5 mCi/mmol) and [U-^{14}C]AMP (sodium salt, 538 mCi/mmol) were from the Radiochemical Centre, Amersham, Radioactive samples (0.5 ml) were counted in 5 ml PCS (phase combining system, G. D. Searle, Heusenstamm) in a Beckmann LS 230 liquid scintillation counter.

AMP was determined enzymatically according to Jaworek, Gruber and Bergmeyer [4]. Phosphate was determined according to Taussky and Shorr [5]. Ammonia was determined using the Berthelot-reaction according to Fawcett and Scott [6]. The enzymatic conversions of P1 and P2 were carried out at 25 °C in 0.1 M sodium bicarbonate buffer, pH 8.5. Enzymes were used at a concentration of 10 units/ml. The progress of the reactions was followed by subjecting 0.1 µl samples to high performance thin layer chromatography using solvent system A. The plates were developed for 3 min.

P1

UV-spectrum (H_2O): $\lambda_{max} = 259$, 211 nm. – IR-spectrum (KBr): 3550–3140 (s), 1680–1650 (s), 1600 (s), 1568 (m), 1472 (m), 1414 (w), 1330 (w), 1300–1287 (w), 1213–1180 (s), 1108–1090 (s), 1082–1060 (s), 987 (w), 958 (w), 906–892 (w), 887–866 cm^{-1} (w). ^{13}C-NMR-spectrum (D_2O/CD_3OD): $\delta = 65.1$ (d, $J = 4.4$), 71.3, 75.3, 84.9 (d, $J = 8$), 88.2, 119.2, 141.0, 149.7, 152.5, 155.5. ^{31}P-NMR-spectrum (D_2O): $\delta = 8.4$.

p^1,p^2-diadenosine 5′-pyrophosphate

R_f-value (system A) = 0.68. Electrophoretic mobility (high voltage electrophoresis) = 3.4 cm. – UV-spectrum (H_2O): $\lambda_{max} = 259$, 211 nm. – IR-spectrum (KBr): 3620–3340 (s), 3310–3210 (s), 3020–2910 (w), 1658 (s), 1645 (s), 1608 (m), 1578 (w), 1480 (w), 1332 (w), 1255–1210 (s), 1134–1110 (s), 1088 (s), 978–938 cm^{-1} (w). – ^{13}C-NMR-spectrum (D_2O/CD_3OD): $\delta = 66.0$ (dd, $J_1 = 2$, $J_3 = 3$), 70.9, 75.7, 84.4 (dd, $J_1 = 4.3$, $J_2 = 5.2$), 88.3, 118.9, 140.2, 149.3, 153.5, 156.0. – ^{31}P-NMR-spectrum (D_2O): $\delta = 12.2$.

Adenosine 5′-monophosphoramidate

R_f (system A) = 0.72. – Electrophoretic mobility = 2.4 cm. UV-spectrum (H_2O): $\lambda_{max} = 259$, 211 nm. – IR-spectrum (KBr): 3550–3140 (s), 1680–1650 (s), 1600 (s), 1568 (m), 1472 (m), 1414 (w), 1330 (m), 1300–1287 (w), 1213–1180 (s), 1108–1090 (s), 1082–1060 (s), 987 (w), 958 (w), 906–892 (w), 887–866 cm^{-1} (w).

P2

UV-spectrum (H_2O): $\lambda_{max} = 259$, 211 nm. – ^{13}C-NMR-spectrum (D_2O/CD_3OD): $\delta = 64.2$ (d, $J = 4.5$), 75.0 (d, $J = 3.6$), 84.6 (dd, $J_1 = 3$, $J_2 = 8.7$), 88.1, 119.4, 141.4, 149.7, 151.9, 155.0.

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