ACTA CHEMICA SCANDINAVICA 8 (1954) 1898—1906

On the Dephosphorylation and Deamination of Adenosine Triphosphate by Actomyosin Gel

ADAM DEUTSCH and ROY NILSSON

Institute of Chemistry, University of Lund, Sweden

Analysis by chromatographic methods shows rapid dephosphorylation of adenosine triphosphate to adenosine diphosphate by actomyosin gel, concurrent with the volume constriction of the gel. Dephosphorylation is followed by a slower process of deamination, yielding inosine diphosphate as the isolated end product of reaction. The deamination of adenosine diphosphate to inosine diphosphate represents a new type of deamination of adenine nucleotides by muscle preparations, in addition to the previously known route *via* adenosine monophosphate.

In previous investigations [1,2], the chemical aspects of the interaction of actomyosin and adenosine triphosphate (ATP) had been studied from the point of view of eventual chemical changes in the protein, accompanying the volume constriction of actomyosin threads. In the present work — short accounts[3,4] of which have already been published — the reaction between actomyosin gel and ATP has been investigated from the point of view of chemical changes in the nucleotide component of the reaction.

MATERIALS AND METHODS

Actomyosin gel. Rabbits were killed by stunning and packed in ice for 15 minutes. The muscles of the hind limbs and the back were excised and minced at 0° C, followed by extraction for 24 hours at 0° C with three times their volume of Weber's solution (0.6 mole potassium chloride, 0.01 mole sodium carbonate and 0.04 mole sodium hydrogen carbonate per litre). After dilution of the extract with the same volume of Weber's solution and filtering through madapolam, the actomyosin was precipitated by adding four volumes of distilled water to one volume of the extract. After standing for 12 hours at 0—3° C, the solution was decanted and the precipitate centrifuged for a few minutes at 8 000—10 000 g. The precipitate was brought into solution again by adding enough solid potassium chloride to make the resulting solution 0.5 molar. The viscous solution was centrifuged, thereby removing any insoluble protein and the clear solution reprecipitated by the addition of four volumes of distilled water to one volume of the solution. Three such reprecipitations were carried out. After the last reprecipitation, the precipitate was thoroughly washed with distilled water and centrifuged. Three such washings were carried out. Only glass-distilled water was used throughout the preparation.

The dry weight was determined for each preparation; it varied between 1 and 3 %. For the determination of the dry weight, duplicate samples were heated at 110° C to constant weight.

ATP was prepared as the barium salt from rabbit muscle by the method described by Needham[5] and purified by ion-exchange chromatography[6].

Adenosine diphosphate (ADP) was obtained from appropriate fractions of the effluent in the ion-exchange chromatography of ATP or prepared according to Bailey[7] as the barium salt and further purified by ion-exchange[6].

Adenosine-5'-monophosphate (AMP) and *inosine diphosphate (IDP)* were prepared and purified as described previously[6]. All the chemicals used were of analytical grade of purity.

Reaction between ATP and actomyosin gel. For the experiments, 10 g of actomyosin gel was placed in a centrifuge tube and after addition of 8 ml water or the buffer solution to be used and 2 ml of sodium ATP solution (20 mg/ml, calc. as the free acid) and thorough mixing with a glass rod, the tube was kept at 20° C for the desired reaction time. The reaction was interrupted by addition of 2 ml 50 % (w/v) trichloroacetic acid solution to the reaction mixture at 0° C. After centrifugation at 10 000 g, the ice-cold trichloroacetic solution was extracted with ether in order to remove the bulk of the acid, neutralized by addition of a drop of ammonia and freed from ether by aeration. The protein-free extract thus obtained was used for phosphorus analysis, paper chromatography and ion-exchange. Approximately 95 % of the total phosphorus in the reaction mixture were recovered in the protein-free extract.

For the purpose of direct spectroscopic examination of the protein-free extracts, perchloric acid was substituted for trichloroacetic acid in parallel samples in some experiments.

In each experiment, volume constriction of the actomyosin gel was measured in a duplicate tube containing the same concentrations of protein and ATP and compared with the gel volume in a third tube, in which ATP had been substituted by water or 0.04 *M* potassium chloride solution. The protein-free extract, prepared from the actomyosin gel without added ATP, gave on analysis nucleotide material not exceeding 2—3 % of that found in ATP-actomyosin mixtures.

Experiments on the reaction between ADP and actomyosin gel were performed analogously to those with ATP, ADP being used in the same molar concentration as ATP.

Paper chromatography[8]. One-dimensional chromatography in saturated ammonium sulphate solution-water-*iso*propanol (79 : 19 : 2) was used for the separation of the adenosine series of phosphates from the inosine series before and after hydrolysis to the corresponding purines for 1 hour in *N* hydrochloric acid at 100° C. Separation of the individual nucleotides was performed by two-dimensional chromatography, using *n*-propanol-ammonia (sp.gr. 0.880)-water (60 : 30 : 10) in the first direction and the ammonium sulphate-water-*iso*propanol solvent in the second.

50—100 μl of the protein-free extract containing 100—200 μg nucleotide material were applied to the paper in 5 μl portions, the spot being dried after each application in a current of cold air. The size of the starting spot did not exceed 1 cm in diameter. Descending chromatography on acid-washed Whatman No. 1 paper (46 × 53 cm) was used with developing times of 40—80 hours in the propanol-ammonia-water solvent and 6—8 hours — after previous equilibration overnight — in the ammonium sulphate-water-*iso*propanol system. The spots on the finished chromatogram were located by ultraviolet photography.

For the quantitative evaluation of the chromatograms the spots were cut out from the paper, cut into pieces of approx. 2 × 2 mm and extracted with 0.1 *N* hydrochloric acid for 4—8 hours. Optical densities were read against an extract from a paper blank of the same area, cut out from beside the spot; the same blank was used for phosphorus estimations. Prior to the application of the method to the analysis of protein-free extracts, adenosine and inosine phosphate preparations of known composition were subjected to analysis (Table 1).

Ion-exchange chromatography. The method of Cohn and Carter[9] in the modification previously described[6], was used.

Analytical methods. Phosphorus was determined by the method of Allen[10] using a Klett Summerson photoelectric colorimeter with a red filter and was differentiated in separate analyses into total phosphorus (P_T), inorganic phosphorus (P_0) and acid-labile

phosphorus (P_{10}) determined after 10 minutes hydrolysis in *N* hydrochloric acid at 100° C. In order to investigate whether the directly estimable phosphorus (P_0) in the protein-free extracts corresponded to inorganic orthophosphate or was partly liberated from labile organic phosphates during analysis, orthophosphate determinations were in addition carried out by the method of Lowry and Lopez[11].

Pentose was determined by the method of Mejbaum[12] as modified by Albaum and Umbreit[13]. A Klett Summerson photoelectric colorimeter was used with a red filter.

Optical densities were measured in 0.01 or 0.1 *N* hydrochloric acid solution in a Beckman Universal Spectrophotometer Model DU, the molecular extinction coefficients of 14 200 at 260 mμ for adenosine phosphates and 13 500 for adenine, 13 200 at 250 mμ for inosine phosphates and 11 500 for hypoxanthine being used.

RESULTS

Analysis of the deproteinised extract from the reaction between ATP and actomyosin gel shows that about one-third of the total phosphorus is present as orthophosphate, indicating that only the terminal phosphate group of ATP has been split off in the reaction. Orthophosphate values corresponding to 32—34 % of the total phosphorus are generally obtained after reaction for

Table 1. Analysis of adenosine and inosine phosphates by paper chromatography[8]. (For details see text).

Substance and chromatogr. method	μg Adenine		μg Hypoxanthine		μg Acid-labile P		μg Total P	
	found	calc.	found	calc.	found	calc.	found	calc.
ATP, 72 μg, one-dimensional	18.5 18.8	19.0			8.6 8.9	8.8	13.3 13.6	13.2
ATP, 56 μg one-dimensional after hydrolysis	14.4	14.8						
ATP, 119 μg two-dimensional	32.1	31.4			15.0	14.5	22.4	21.8
ATP, 160 μg two-dimensional					20.2	19.5	29.5	29.3
ADP, 70 μg + IDP, 104 μg one-dimensional	21.8 21.4	22.0	 32.5 31.3	 32.8	4.7 5.1 8.0 8.4	5.1 7.4	9.8 9.8 15.7 15.7	10.2 15.0
ADP, 54 μg + IDP, 104 μg one-dimensional after hydrolysis	17.0	17.0	32.5	32.8				
ADP, 157 μg + IDP, 104 μg, two-dimensional	47.6	49.3	 31.4	 32.8	10.5 8.1	11.4 7.5	22.1 15.6	22.8 15.0

Table 2. Analysis of the eluted spots from two-dimensional paper chromatography of the protein-free extract from the reaction (30 min.) between ATP (4 μmoles/ml) and actomyosin gel (10 mg dry weight/ml). The extract contained 450 μg total purine, 104 μg inorganic phosphorus, 104 μg acid-labile phosphorus and 313 μg total phosphorus.

	μg Purine	μg Acid-labile P	μg Total P	Purine:acid-labile P: total P (calc. 1:1:2)
ADP spot	336	74	148	1:0.95:1.90
IDP spot	102	25	50	1:1.07:2.14

5 minutes as well as after 4 hours, suggesting that dephosphorylation is complete within the first minutes of reaction, no significant amounts of orthophosphate being formed thereafter.

Measurements on the volume constriction of the gel after 5 and 30 minutes reaction with ATP show that up to 90 % of the final value is already obtained after 5 minutes.

Spectroscopic examination of extracts prepared with perchloric instead of trichloroacetic acid reveals a progressive shift of the absorption maximum towards shorter wavelengths in the course of reaction, suggesting deamination of the original adenine nucleotide.

Paper chromatography of the protein-free extract confirms the results of direct spectroscopic examination and shows the presence of inosine nucleotide in amounts increasing with increasing times of reaction. A reaction time as short as 5 minutes gives only slight deamination, but after 4 hours most of the purine is transformed into hypoxanthine (Fig. 1). Similar results are obtained by paper chromatography of the extract before or after hydrolysis in *N* hydrochloric acid for 1 hour at 100° C.

Separation of the reaction products by two-dimensional paper chromatography results essentially in two ultraviolet-absorbing spots in positions corresponding to those of ADP and IDP, the latter spot increasing at the expense of the former, as the reaction proceeds (Fig. 2). Analyses on the eluted spots by measurement of ultraviolet absorption and phosphorus analysis give the

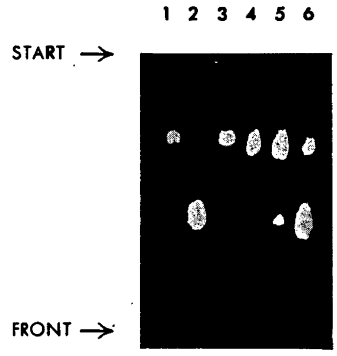

Fig. 1. Time course of deamination in the reaction between actomyosin gel (10 mg dry weight/ml) and ATP (4 μmoles/ml) as followed by paper chromatography of the protein-free extract, after hydrolysis for 1 hour at 100° C. Solvent: saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2). Time: 7 hours. (1) Adenine, 15 μg; (2) hypoxanthine, 30 μg; (3) hydrolysate of ATP employed, containing 20 μg adenine; (4), (5) and (6) hydrolysates of protein-free extract (containing 45 μg total purine) after 5 min., 30 min. and 4 hours reaction, showing spots for hypoxanthine corresponding to 0, 10 and 80 % of the total purine.

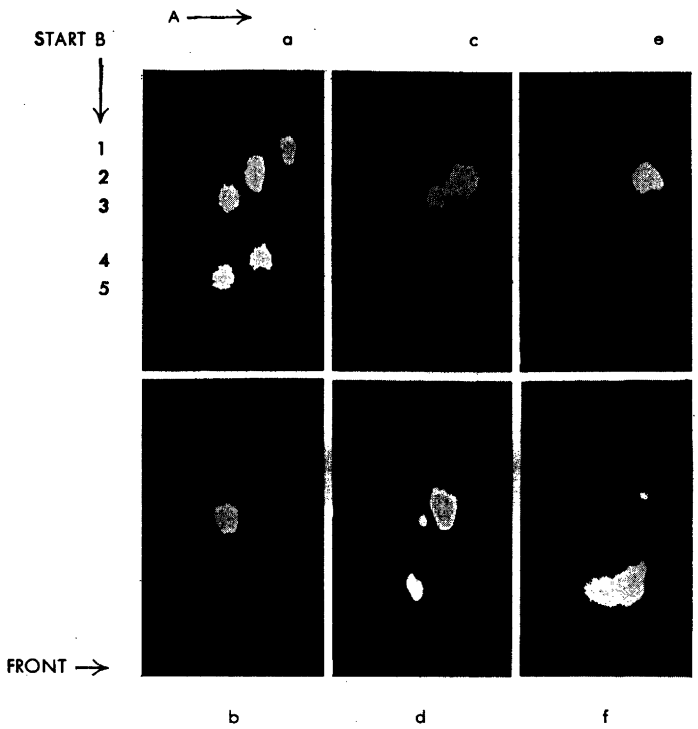

Fig. 2. The products of reaction between actomyosin gel (10 mg dry weight/ml) and ATP (4 μmoles/ml) as shown by two-dimensional paper chromatography of the protein-free extract. (a) first solvent: n-propanol-ammonia-water (60 : 30 : 10) 50 hours. (B) second solvent: saturated ammonium sulphate solution-water-isopropanol (79 : 19 : 2) 8 hours. (a) Mixture of (1) AMP, (2) ADP, (3) ATP, (4) IMP and (5) IDP, approx. 50 μg each; (b) ATP employed; (c) protein-free extract after 5 min. reaction, showing spots for ATP (38 μg), ADP (117 μg) and IDP (8 μg), corresponding to 80 % dephosphorylation and 5 % deamination; (d) protein-free extract after 30 min. reaction showing spots for ATP (8 μg), ADP (141 μg) and IDP (18 μg), corresponding to 95 % dephosphorylation and 11 % deamination; (e) and (f) extracts from another experiment, (e) after 8 min. reaction, showing a spot for ADP and a very faint spot for IDP, and (f) after 4 hours, with spots for ADP and IDP, corresponding to 94 % deamination.

values expected for ADP and IDP (Table 2). In some 5 and 30 min. experiments, a third spot in the position of ATP was observed (Fig. 2).

Ion-exchange chromatography gives three main fractions, corresponding in elution position and chemical analysis to orthophosphate, ADP and IDP (Table 3). In several experiments, however, ion-exchange chromatography of the protein-free extract from ATP-actomyosin reaction mixtures gave the IDP fraction in an elution position different from that of authentic IDP, the IDP fraction appearing first in the effluent by elution with the solvent of next highest anion concentration to the one which removes IDP from the column.

Isolation of the IDP fraction as the barium salt[14] from pooled fractions of the effluent from ion-exchange columns gave barium IDP, indistinguishable

Table 3. Analysis of the fractions obtained on ion-exchange chromatography of the protein-free extract from the the reaction (30 min.) between ATP (4 μmoles/ml) and actomyosin gel (10 mg dry weight/ml).

	μg Purine	μg Acid-labile P	μg Total P	Purine:acid-labile P:total P
Orthophosphate			1.26	
ADP	4.06	0.95	1.96	1:1.01:2.09
IDP	1.60	0.38	0.77	1:1.03:2.10
Calc. for conversion of ATP to diphosphate	5.52	1.27	3.80	1:1:2

from authentic IDP prepared by chemical deamination of ADP. The barium salt, isolated from IDP fractions with retarded elution position, was likewise identical with IDP, as judged by chemical analysis, paper chromatography and elution position on ion-exchange analysis performed on the isolated product, after removal of barium (Table 4).

Beside ADP and IDP, an adenine nucleotide fraction in the elution position of ATP was observed in the ion-exchange analysis in some 5 and 30 min. experiments, in agreement with the results of two-dimensional paper chromatography.

Dephosphorylation and deamination as measured by different methods of analysis, is seen from Table 5.

Essentially the same results were obtained by analysis of the reaction products from forty different actomyosin preparations after 30 minutes and 4 hours reaction with ATP. 20—30 % deamination was obtained after 30 minutes reaction, with the exception of three preparations which gave 50—65 %;

Table 4. Analysis of barium IDP ($C_{10}H_{11}O_{11}N_4P_2Ba_{1.5}$) isolated from the reaction between ATP and actomyosin gel, by precipitation with barium acetate and ethanol from the effluent in ion-exchange chromatography of the protein-free extract, followed by drying in vacuo over phosphoric oxide.

	Found %	Calc. %
Total phosphorus	9.76	9.96
Acid-labile phosphorus	4.88	4.95
Inorganic phosphorus	0	0
Pentose	25.2	23.8
Hypoxanthine	20.40	21.55
Total P:Acid-labile P:	2:1:	2:1:1:1
Pentose:Hypoxanthine	1.05:0.95	
Water (loss of weight at 110° C)	0	0

Table 5. Dephosphorylation and deamination of ATP (4 μmoles/ml) by actomyosin gel (10 mg dry weight/ml), as measured by different methods of analysis, on aliquots of the same protein-free extract after 30 min. reaction.

Method	% Conversion of ATP to the diphosphate	% Deamination
Direct analysis	96	
One-dimensional paper chromatography		22
One-dimensional paper chromatography after hydrolysis		18
Two-dimensional paper chromatography	94	18
Ion-exchange	94	22

4 hours reaction resulted in yields of 65—95 %. Only in two experiments was IMP detected among the reaction products, amounting to less than 5 % of the IDP fraction.

Reaction between ATP and actomyosin gel in buffer solutions between pH 6.0 and 8.0 results in dephosphorylation and deamination of ATP in all cases; considerably higher rates of deamination are found, however, at pH 6.0 than at higher pH values (Table 6), in analogy to the pH course of the deamination of AMP.

Substitution of ATP by ADP in the reaction with actomyosin gel results in deamination without dephosphorylation, and without any volume change

Table 6. Deamination in the reaction between ATP (4 μmoles/ml) and actomyosin gel (10 mg dry weight/ml) in buffer solutions, as measured by one-dimensional paper chromatography of the protein-free extract. Phosphorus analysis showed conversion of ATP to the diphosphate. The following buffer solutions were used: 0.1 M succinate at pH 6.0 and 6.6, 0.05 M acetate at pH 6.8, 0.1 M glycylglycine at pH 7.2 and 0.05 M Tris (hydroxymethyl)-aminomethane at pH 7.2 and 8.0.

Duration of the reaction	% Deamination at pH				
	6.0	6.6	6.8	7.2	8.0
60 min.	53		32	23	
4 hours	100	76		60	53

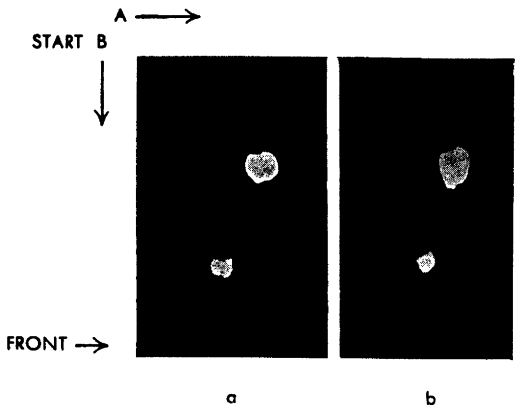

Fig. 3. Deamination in the reaction of actomyosin gel (10 mg dry weight/ml) with ADP (4 µmoles/ml) and ATP (4 µmoles/ml) as shown by two-dimensional paper chromatography of the protein-free extracts after 30 min. reaction. The solvents are the same as in Fig. 2. (a) Extract from the reaction with ADP (total nucleotide content 200 µg) and (b) extract from the reaction with ATP (total nucleotide content 150 µg), showing spots for ADP and IDP corresponding to (a) 37 % and (b) 27 % deamination.

in the protein gel. Similar results on deamination were obtained with ATP and ADP respectively, with somewhat higher deamination in the case of ADP in several 30 minutes experiments (Fig. 3).

DISCUSSION

The results of the present investigation establish two phases in the reaction between ATP and actomyosin gel, from the point of view of chemical changes in the nucleotide component of reaction.

In a first rapid phase, the terminal phosphate group of ATP is split off under formation of ADP. The dephosphorylation of ATP — concurrent with the volume constriction of the gel — is completed within the first minutes and even reaction times as short as a few seconds are reported[15] to result in complete dephosphorylation. In some of our experiments, however, only about 80 % dephosphorylation was obtained after 5 minutes reaction, as judged by chromatography.

The quick initial phase of dephosphorylation in the reaction between ATP and actomyosin gel is followed by a second slow phase, in which deamination of the diphosphate takes place. Deamination of adenine nucleotides by myosin and actomyosin has been repeatedly reported, whereby the reaction was assumed to take place *via* AMP giving IMP as the product of reaction. Deamination of ATP and ADP was interpreted as the result of the combined action of three enzymes, *viz.* 1) dephosphorylation of ATP to ADP by ATPase, 2) dismutation of ADP to AMP and ATP by adenylate kinase (myokinase) and 3) deamination of AMP to IMP by adenylate deaminase.

In our experiments, the absence of deamination *via* the AMP route to any noticeable degree is already indicated by the results of phosphorus analysis in the deproteinised reaction mixture, showing that only the terminal phosphate

group of ATP has been split off. Furthermore, the time course of deamination as contrasted with the rapid reaction found when AMP is added to our actomyosin preparations, points likewise towards a different type of deamination process. Finally, the fact, that IMP could not be detected in any quantity among the products of reaction, shows definitely that under the conditions of reaction the main route of deamination does not proceed through the AMP→IMP reaction, probably because our actomyosin preparations were practically free from adenylate kinase; in the presence of this enzyme, deamination *via* the AMP route would be the principal one at pH near 7.

Direct analysis of the protein-free reaction solution together with the results obtained by ion-exchange and paper chromatography show that deamination occurs at the diphosphate level; ADP formed by primary dephosphorylation of ATP is transformed by direct deamination into IDP. The direct proof of deamination at the diphosphate stage is furnished by isolation of IDP as the main product of reaction. In view of the diverging results as to the elution position of the IDP fraction at ion-exchange analysis in some of our experiments, the possibility of an IDP derivative not identical with, but easily transformed into IDP being the primary product of deamination, cannot be yet definitely excluded.

The demonstration of a new route of deamination, *viz.* direct deamination of ADP by actomyosin, as well as by washed myofibrils[16], in addition to the previously established route *via* AMP, necessitates further investigation of the specificity range of adenylate deaminase, and of the question of identity of the enzymes effecting the different types of deamination.

Investigations on these lines are now in progress in this laboratory; short reports [17,18] on some of the results have already been presented.

This work has been supported by grants from the *Swedish Natural Science Research Council* and the *Lilly Foundation.*

REFERENCES

1. Buchthal, F., Deutsch, A., Knappeis, G. G. and Munch-Petersen, A. *Acta Physiol. Scand.* **16** (1949) 326.
2. Buchthal, F., Deutsch, A., Knappeis, G. G. and Munch-Petersen, A. *Acta Physiol. Scand.* **24** (1951) 368.
3. Deutsch, A. and Nilsson, R. *Résumés des communications, IIe Congrès international de biochimie,* Paris 1952, p. 421.
4. Deutsch, A. and Nilsson, R. *Chemistry & Industry* **1953** 1149.
5. Needham, D. M. *Biochem. J. (London)* **36** (1942) 114.
6. Deutsch, A. and Nilsson, R. *Acta Chem. Scand.* **7** (1953) 1288.
7. Bailey, K. *Biochem. J. (London)* **36** (1942) 121.
8. Deutsch, A. and Nilsson, R. *Acta Chem. Scand.* **7** (1953) 858.
9. Cohn, W. E. and Carter, C. E. *J. Am. Chem. Soc.* **72** (1950) 4273.
10. Allen, R. J. L. *Biochem. J. (London)* **34** (1940) 858.
11. Lowry, O. H. and Lopez, J. A. *J. Biol. Chem.* **162** (1939) 421.
12. Mejbaum, W. *Hoppe-Seyler's Z. physiol. Chem.* **258** (1939) 117.
13. Albaum, H. G. and Umbreit, W. W. *J. Biol. Chem.* **167** (1947) 369.
14. Kleinzeller, A. *Biochem. J. (London)* **36** (1942) 729.
15. Kuschinsky, G., Lange, G. and Turba, F. *Biochem. Z.* **325** (1954) 321.
16. Webster, H. L. *Nature* **172** (1953) 453.
17. Deutsch, A. and Nilsson, R. *Acta Chem. Scand.* **8** (1954) 1106.
18. Deutsch, A. and Nilsson, R. *Acta Chem. Scand.* **8** (1954) 1106.

Received August 31, 1954.