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THE ORIGIN OF NATIVE G-ACTIN-BOUND ADENOSINE TRIPHOSPHATE

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SUMMARY

Acetone-dried rabbit-muscle powder, prepared according to classical procedures as a source of G-actin, contains only residual quantities of ATP. Water extraction of such powders, however, yields solutions of G-actin containing many times the initial level of ATP in association with the protein. The mechanism of synthesis of the newly formed ATP was investigated and found to be the result of residual ATP: AMP phosphotransferase (adenylate kinase, EC 2.7.4.3) action on the ADP retained in the powder.

INTRODUCTION

Acetone-dried, extracted minced muscle prepared according to the classical procedures of FEUER *et al.*[1] has continued to be almost universally employed in the preparation of so-called native, globular actin (G-actin). In spite of wide application of the preparative method, the basic processes leading to G-actin formation and extraction from such powders has remained obscure. In this respect, muscle powders although essentially free of ATP (ref. 2) yield on water extraction G-actin characteristically containing a mole of bound ATP per mole of protein[3,4]. The mechanism of ATP synthesis leading to G-actin formation and extraction from muscle powder has not thus far been determined.

Preliminary examination of the question of ATP formation accompanying actin extraction from muscle powder[4] revealed that synthesis was prevented in the presence of Dowex-1 (Cl^-) and further that addition of [^{32}P]P_i did not result in incorporation of the label into the newly formed ATP. The present report summarizes data which clearly demonstrate ATP synthesis to be the result of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3), fortuitously retained in muscle powder) action on the bound ADP present in the powder.

MATERIALS AND METHODS

Preparation and extraction of muscle powders

Acetone-dried, rabbit-muscle powders were prepared essentially as described by FEUER *et al.*[1]. The dried powders were further pulverized and sieved through a double layer of cheese cloth to provide uniform sampling and stored with dessicant at — 20°.

Weighed aliquots of the powder were extracted with 30–60 volumes of medium at near 0° in all instances. Extraction time and media varied with the experiment. Direct trichloroacetic acid extracts of the powder were prepared in certain instances to establish initial concentrations of nucleotide constituents. Analyses of such extracts followed the prior removal of acid with ether (3 extractions by shaking with 3 volumes ether). Water extracts of the powder were prepared, following appropriate extraction periods, by filtration under reduced pressure. The clear filtrates were treated with 1/20 volume Dowex-1 (acetate) when separation of free from actin-bound nucleotide[4,5] was required. Further deproteinization and release of bound nucleotide was accomplished with trichloroacetic acid and the acid removed in ether.

ADP and ATP analyses

ADP and ATP were determined on small aliquots (usually 0.1 ml) of the deproteinized extracts by enzymic coupling to pyridine nucleotide using fluorimetric analyses[6]. The sensitivity of the method allows ready analyses on less than a mμmole of either ADP or ATP and was based on the following sequence of reactions[7]:

$$\text{ATP} + \text{Glucose} \xrightarrow{\text{Hexokinase}} \text{Glc-6-}P + \text{ADP} \quad (1)$$

$$2\text{ADP} \xrightarrow{\text{Adenylate kinase}} \text{ATP} + \text{AMP} \quad (2)$$

$$\text{Glc-6-}P + \text{TPN} \xrightarrow{\text{Glc-6-}P\text{ dehydrogenase}} \text{Phosphogluconate} + \text{TPNH} \quad (3)$$

The assay procedure employed is described. To a final 2.5 ml volume were added: ATP and/or ADP containing sample, 50 μmoles glycylglycine buffer at pH 7.5, 0.5 μmole $MgCl_2$, 25 μmoles glucose, 10 μg crystalline hexokinase [EC 2.7.1.1] (Sigma Chemical Co.) and 1 μg crystalline Glc-6-*P* dehydrogenase [EC 1.1.1.49] (California Corporation for Biochemical Research). Following temperature equilibration (15° employed), the reaction was initiated with excess TPN (0.1 μmole in 10 μl) and maximum TPNH formation determined (within 5 min) as a measure of ATP. Crystalline adenylate kinase (California Corporation for Biochemical Research) was next added (5 μg in 10 μl) and the additional TPNH formation followed to completion (within 10 min) as a measure of ADP derived from ATP plus that initially present in the sample. All enzymes were dialyzed prior to their use. Fluorescence measurements were made with a Turner fluorometer equipped with a constant temperature sample chamber.

Chromatographic analyses

The nucleotide composition of certain extracts was quantitatively determined by application of paper chromatographic techniques. Measured aliquots of the deproteinized, trichloroacetic acid-free extracts were carefully flash evaporated to dryness and the total quantitatively transferred with water as a spot onto washed (0.1 N acetic acid) Whatman No. 3MM paper sheets. The papers were initially developed over a 24-h period, descending, using the following solvent mixture[8]: glacial acetic acid–isopropanol–water (3:6:1, v/v). Free bases, nucleosides and nucleoside monophosphates are readily moved in this solvent with the nucleoside polyphosphates remaining at or near the origin[9]. The papers were further developed overnight, descending, in the second dimension to complete the isolations[9] using the following solvent

mixture (modification of ref. 10): isobutyric acid–conc. NH_4OH–water (66:1.5:33, v/v). The isolated components were identified and quantitatively determined following their elution from paper according to previously described methods[9].

RESULTS

Preparations of dried muscle powder although containing only residual amounts of ATP, retain appreciable quantities of ADP (see Table I,A). It has been generally assumed that the powder contains actin in the polymerized form. Retained ADP therefore, presumably exists bound to the polymerized actin with ATP remaining as a residual contaminant. In support of this conclusion, extraction of the dried muscle powder in the presence of magnesium ion, which maintains actin in the polymerized state, results in the extraction of little nucleotide or actin (see Table I, B). Conventional water extraction of the dried muscle powder, on the other hand yields an extract containing ATP in large excess of that originally present in the powder (see Table I, C). The ATP is bound for the most part to actin monomers (G-actin), however, excess free ATP is also present.

TABLE I

ADP AND ATP COMPOSITION OF EXTRACTS OF ACETONE-DRIED MUSCLE POWDER

All extractions were for 30 min at 0° using 30 volumes of specified media per gram of dry powder. Free and bound nucleotide were separated using Dowex-1 as described in METHODS. Determination of ATP and ADP appearing in the extracts was by enzymic-fluorimetric analyses as described in METHODS.

	mμmoles/ml extract	
	ATP	*ADP*
(A) Trichloroacetic acid extract	3.4	35
(B) 0.005 M $MgCl_2$ extract:		
Total	4.5	0.0
Actin-bound	0.0	0.0
(C) CO_2-Free water extract:		
Total	17	0.0
Actin-bound	12	0.0

ATP, ADP changes accompanying water extraction

In view of the presence of appreciable quantities of ADP in muscle powder, it was reasonable to assume ATP formation in some manner from the ADP. An experiment was therefore carried out in which ATP and ADP levels were simultaneously examined during the course of water extraction of the dried powder. The results of one such experiment are summarized in Fig. 1. It can be seen from the results that ATP formation is accompanied by simultaneous decreases in the level of ADP. Examination of the stoichiometry indicates the disappearance of about two molecules of ADP for each ATP formed, with disappearance and formation proceeding at comparable rates. Termination of ATP synthesis and ADP loss occurred prior to total ADP disappearance and at approximately the same time, suggesting arrival at an equilibrium state. ATP synthesis is accompanied by corresponding increases in G-actin appearing in the extraction medium as evidenced by bound ATP analyses.

Synthesis of ATP from ADP

The results summarized in the preceding section leave little doubt that ATP formation occurs at the expense of ADP present in the powder. ADP phosphorylation could conceivably occur by (a) glycolysis (b) creatine phosphate and (c) ADP (adenylate kinase action). ADP phosphorylation by glycolysis was ruled out on the basis of the finding that [^{32}P]P_i (added during water extraction of muscle powder) was not incorporated into newly synthesized ATP[4]. Phosphorylation of ADP by creatine phosphate could also be ruled out on the basis of analyses indicating an absence of but trace quantities in the powder. Phosphorylation of ADP by ADP, a reaction mediated by adenylate kinase therefore, remained to be considered.

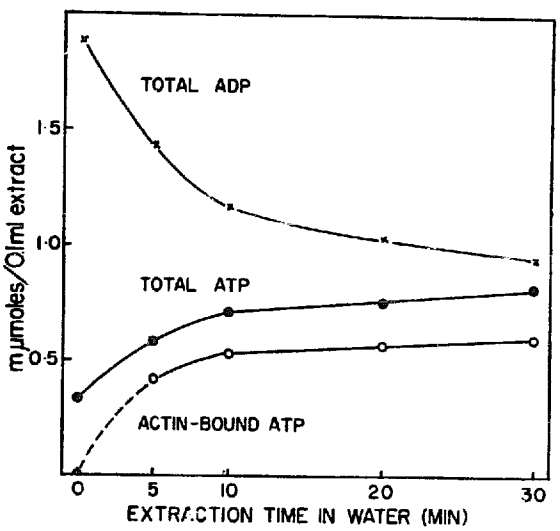

Fig. 1. Alterations in ADP and free and bound ATP accompanying water extraction of muscle powder. 1 g of finely pulverized muscle powder was added to 60 ml CO_2-free water and maintained at near 0° with gentle stirring. A 2.0-ml homogeneous aliquot was removed immediately after mixing and after each of the specified times and precipitated with 0.2 ml 30% trichloroacetic acid. Following removal of trichloroacetic acid with ether, 0.1-ml aliquots of the extracts were assayed directly for total ADP (×—×) and total ATP (●—●). Actin-bound ATP (○—○) was determined as follows. At the indicated times, 5-ml aliquots of the extracting mixture were removed, filtered, treated with 0.05 volume Dowex-1 (to remove free nucleotides), and treated finally with 0.1 volume 30% trichloroacetic acid. Aliquots of 0.1 ml of the resulting solutions following trichloroacetic acid removal, were tested directly for ATP present.

ATP formation from ADP by adenylate kinase action is consistent with the findings previously presented in Fig. 1 with the observed stoichiometry between ADP loss and ATP formation being especially significant. That the stoichiometry obtained in these experiments was not simply fortuitous and did in fact reflect the result of adenylate kinase action was further examined. That more than sufficient adenylate kinase is retained in muscle powder to account for the findings is evident from the experimental results summarized in Table II. Maximum ATP formation occurred in the extraction mixtures containing added ADP even in the absence of crystalline adenylate kinase supplementation. It could be concluded from these findings that adenylate kinase retention in the powder was well in excess of that required for its postulated role.

ATP formation by adenylate kinase action on residual ADP

Confirmation of the postulated role of adenylate kinase in the conversion of residual ADP to ATP in the course of water extraction of muscle powder was sought in a more direct experiment which has been summarized in Table III. Since ATP synthesis from adenylate kinase action on ADP should also result in an equivalent amount of AMP formation, confirmation of the reaction was sought on this basis.

In view of the initial contaminating presence of residual ATP, AMP, and IMP in the powders, it was necessary to first establish the level of their presence from a

TABLE II

ADENYLATE KINASE RETENTION IN DRIED MUSCLE POWDER

All extractions were for 30 min at near 0° using 30 volumes of specified media per gram of dried powder (uniformally sieved): Extraction media contained 1 μg crystalline adenylate kinase and 60 mμmoles of ADP/ml of solution where indicated. Free ATP was separated on Dowex-1 (see METHODS) from actin-bound ATP. ATP and ADP determinations were by enzymic-fluorimetric analyses (see METHODS).

Extraction media		*mμmoles/ml extract*	
		ATP	*ADP*
H_2O only	(a) Total	16	—
	(b) Bound	11	—
H_2O + adenylate kinase	(a) Total	17	—
	(b) Bound	11	—
H_2O + ADP	(a) Total	43	13
	(b) Bound	13	—
H_2O + ADP + adenylate kinase	(a) Total	40	13
	(b) Bound	12	—

TABLE III

STOICHIOMETRIC EVIDENCE IN SUPPORT OF ATP SYNTHESIS BY ADENYLATE KINASE ACTION ON ADP DURING WATER EXTRACTION OF MUSCLE POWDER

Duplicate 2-g homogeneous aliquots of dried muscle powder were extracted for 30 min at 0° with (a) 60 ml 3% trichloroacetic acid solution and (b) 60 ml of CO_2-free water. Nucleotides were quantitatively isolated from the resulting extracts by paper chromatographic technique (for detailed procedures, see METHODS).

Nucleotide	*(A) Trichloroacetic acid extract (μmoles/30 ml)*	*(B) Water extract (μmoles/30 ml)*	*Changes in composition accompanying water extraction (μmoles)*
IMP	0.16	0.88	+ 0.72
AMP	0.06	0.09	+ 0.03
(IMP + AMP)	0.22	0.97	+ 0.75
ATP	0.15	0.81	+ 0.66
ADP	1.52	0.28	— 1.24

trichloroacetic acid extract of the powder. Additional ATP, AMP and IMP subsequently formed during water extraction of the powders could then be properly assessed. As apparent from the summarized results, ATP formation is accompanied by a nearly stoichiometric accumulation of AMP plus IMP (the latter resulting from a rapid deamination of AMP). The finding of slightly greater amounts of AMP plus IMP over ATP is presumably the result of degradative enzyme action on ADP or ATP accompanying the extraction. The over-all stoichiometry of the nucleotide changes accompanying water extraction of muscle powder would appear, therefore, to leave little doubt that ATP synthesis results from the action of residual adenylate kinase on the ADP present.

DISCUSSION

Elucidation of the mechanism of ATP formation during water extraction of muscle powder reveals a fortuitous retention of adenylate kinase as a contaminant of powders

prepared according to classical procedures[1]. In the absence of adenylate kinase contamination, polymerizable actin, (*i.e.*, G-actin containing bound ATP) could not normally be prepared from such powders by simple water extraction.

On the basis of the various evidence which has been obtained, formation of polymerizable actin during water extraction of muscle powder has been considered to proceed in the following manner. In a low ionic medium, polymerized actin in the powder undergoes depolymerization and the bound ADP dissociation. ADP on dissociation becomes available to adenylate kinase action and the newly synthesized ATP binds with the actin monomer to form G-actin appearing in the extracts. Since only a single ATP is formed from 2 ADP molecules by this reaction it can be speculated that only one-half of the actin in the powder can be expected to be maximally converted to G-actin. On the other hand, water extracts of the powder contain free ATP in excess of that bound with the G-actin (Table I,C), suggesting that G-actin formation is not limited by the available ATP. In this respect, exogenous ADP addition to water–powder mixtures results in the formation of a large excess of ATP with little additional G-actin appearing in the extract. Whether ATP synthesized from exogenous ADP is not available for G-actin formation due to steric considerations was not further examined.

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