The photoaffinity probe 8-N_3[α-^{32}P]ATP labels the ATP-binding sites of rabbit neutrophil and skeletal muscle actin

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8-Azido-[α-^{32}P]ATP (8-N_3-ATP) was used as a photoaffinity label for ATP binding sites in the subcellular fractions of rabbit peritoneal neutrophils. The radioactive 8-N_3-ATP was specifically incorporated into one major protein of 43 kDa. The isoelectric point, molecular mass and subcellular distribution of this labeled protein closely resemble those of the actin. 8-N_3-[α-^{32}P]ATP was further tested as a photoaffinity label for the ATP binding site in the purified rabbit skeletal muscle G-actin. The radioactive 8-N_3-ATP was specifically incorporated into the actin band in SDS–polyacrylamide gel. The results indicate that 8-N_3-ATP can be used as a photoaffinity label for actin.

8-N_3-ATP *Actin* *Photoaffinity labeling*

1. INTRODUCTION

8-Azido-[α-^{32}P]ATP (8-N_3-ATP) has been used as a photoaffinity label for the ATP binding sites of the catalytic subunit of the cAMP-dependent protein kinase [1], the bovine heart mitochondrial ATPase [2], the bacterial ATPase [3], the glycoproteins of sarcoplasmic reticulum vesicles [4] and insulin receptor-associated protein kinase [5]. It competes specifically with ATP for the ATP binding sites in each case. Using 8-N_3-ATP as a photoaffinity label for ATP binding sites in rabbit neutrophils we have identified that it labels the cytoskeleton protein, actin.

2. MATERIALS AND METHODS

2.1. *Materials*

ATP, cAMP, ADP, AMP and rabbit skeletal muscle G-actin were purchased from Sigma; 8-N_3-[α-^{32}P]ATP from Schwarz-Mann (97 Ci/mmol); molecular mass standards for SDS-gel: thyroglobulin (330 000), ferritin (220 000), phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 000) from Pharmacia. Buffer A contained 50 mM Tris, 1 mM EGTA.

Abbreviations: 8-N_3-ATP, 8-azido-adenosine-5′-triphosphate; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetracetic acid

2.2. *Preparation of subcellular fractions*

Rabbit peritoneal neutrophils were collected 4–16 h after the intraperitoneal injection of 200–400 ml 0.1% glycogen in sterile saline. Subcellular fractions were prepared by a modification of the method in [6]. Neutrophils (2–10 $\times$ 10^9), kept at 4°C throughout the preparation except when noted, were washed once at 25°C with erythrocyte lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$ and 1 mM EDTA) and then in Hank's buffer (137 mM NaCl, 5 mM KCl, 0.7 mM KH_2PO_4, 10 mM *N*-2-hydroxyethyl piperazine *N′*-2-ethane sulfonic acid (HEPES), 17 mM $NaHCO_3$, pH 7.2.). The cells were suspended in ice-cold washing buffer (11.6% sucrose containing 10 mM HEPES, 1 mM EGTA, pH 7.2) and washed 4-times with the washing buffer. They were resuspended in the same buffer and homogenized

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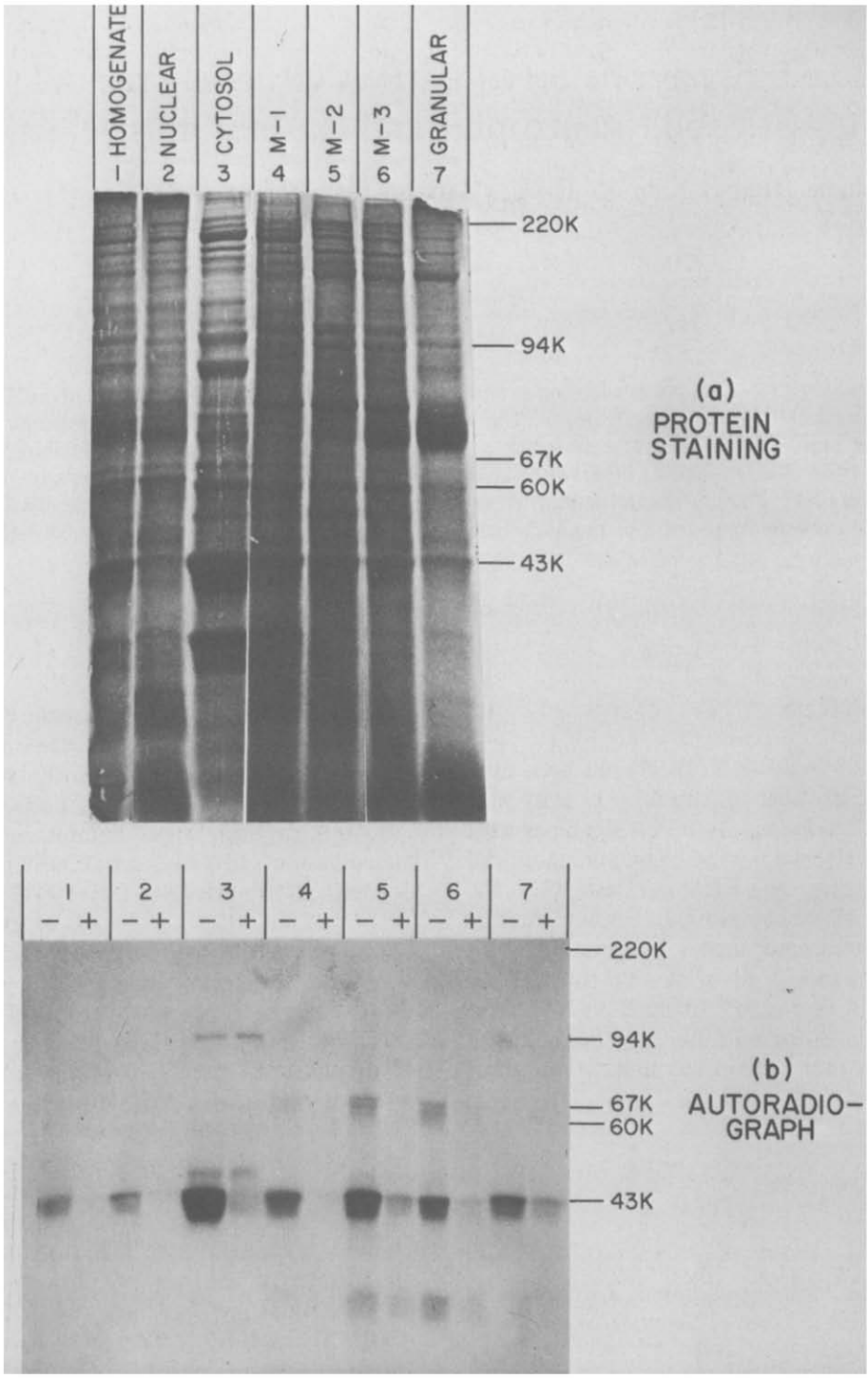

Fig. 1. 8-N_3-[α-^{32}P]ATP incorporation into the subcellular fractions of rabbit peritoneal neutrophils. Subcellular fractions (1 mg/ml) were pre-incubated at 4°C for 5 min in 100 μl of a solution of 50 mM Tris (pH 7.2), 0.125 μM 8-N_3-[α-^{32}P]ATP in the presence (+) or absence (−) of 500 μM ATP. Following irradiation for 5 min with UV light, the samples were analyzed by SDS–gel electrophoresis and autoradiography as described in the text: (a) Coomassie Blue-stained protein pattern; (b) autoradiograph.

for 3 min with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 × *g* for 10 min and the postnuclear supernatant layered onto a discontinuous sucrose gradient consisting of 10.8 ml of 30% sucrose, 8.4 ml of 40% sucrose and 10.8 ml of 50% sucrose in a nitrocellulose tube. All the sucrose solutions contained 10 mM HEPES and 1 mM EGTA at pH 7.2. After centrifugation in a Beckman SW 27 rotor at 120000 × *g* for 2 h, various subcellular fractions were obtained: cytosol (the supernatant fraction above 30% sucrose), membrane fraction M-1 (at the interface of supernatant and 30% sucrose), membrane fraction M-2 (at the interface of 30–40% sucrose), membrane fraction M-3 (at the interface of 40–50% sucrose) and the granular fraction (pellet). The membrane fractions were washed with buffer A and used immediately. The cytosol fraction was dialyzed against the same buffer for 3 h before use.

2.3. *8-N_3-[α-^{32}P]ATP incorporation*

Photoactivated incorporation of 8-N_3-[α-^{32}P]ATP was performed as in [4]. The standard reaction mixture (100 μl) contained 50 mM Tris (pH 7.2.), 0.125 μM 8-N_3-[α-^{32}P]ATP (97 Ci/mmol) and 100 μg protein from subcellular fractions or 20 μg purified skeletal muscle G-actin. Controls were run in the presence of 500 μM ATP. After photolysis the samples were mixed with 50 μl 3× concentrated SDS–stop solution, heated in boiling water for 1 min and then subjected to SDS–polyacrylamide gel (10%) electrophoresis and autoradiography, as in [7]. Two-dimensional gel electrophoresis (isoelectric focusing followed by SDS–polyacrylamide gel electrophoresis) was performed as in [8].

3. RESULTS AND DISCUSSION

Fig. 1 shows that the major protein labeled by 8-N_3-[α-^{32}P]ATP in the subcellular fractions of rabbit peritoneal neutrophils is a protein with an apparent M_r of 43000 (43 kDa) in SDS-gel. The presence of ATP inhibits the labeling reaction. Another minor protein labeled by 8-N_3-[α-^{32}P]ATP with an M_r of about 67000, (67 kDa) is observed only in the membrane fractions (fig. 1. lane 4–6). The 67 kDa protein appears to be enriched in the membrane fraction M-2, the fraction which is also enriched in chemotactic receptors and plasma membrane Na^+, K^+-ATPase [9]. The 43 kDa protein is most enriched in the cytosol fraction and its distribution among subcellular fractions appears to match with that of the Coomassie Blue-stained 43 kDa protein.

Because it has the correct M_r and is one of the major cell proteins, the Coomassie Blue-stained 43 kDa protein is most likely actin [10]. To support this, the 8-N_3-[α-^{32}P]ATP labeled 43 kDa protein was further analyzed by two-dimensional gel electrophoresis (fig. 2a–c). The labeled 43 kDA protein matched closely with the Coomassie Blue-stained actin with an isoelectric point similar to what has been known for actin from platelets (pI 5.7) [11]. The labeled protein is slightly more acidic than the Coomassie Blue-stained actin. This could be due to the effect of the covalently attached 8-N_3-ATP.

Purified rabbit skeletal muscle G-actin was used to test the labeling of the ATP-binding site by 8-N_3-[α-^{32}P]ATP. Fig. 3 shows that 8-N_3-[α-^{32}P]ATP is incorporated into a protein band with the same M_r as G-actin. The presence of ATP or ADP during the incubation inhibits the labeling, while cAMP and AMP do not affect labeling. This is consistent with the property of the ATP binding site existing in the G-actin [12–15]. When the 8-N_3-[α-^{32}P]ATP-labeled G-actin was further analyzed by two-dimensional gel electrophoresis (Fig. 2d), the labeled protein closely matched with the Coomassie blue-stained G-actin. Also evident is that similar to the neutrophil, the bulk of the labeled actin is slightly more acidic than the Coomassie Blue-stained actin and its pI is also similar to the labeled protein from the neutrophil (fig. 2). Thus the results with skeletal muscle actin are essentially the same as those found with neutrophil actin. It is known that purified skeletal muscle G-actin contains 1 mol bound ATP with an association constant of $7.5 \times 10^9\ M^{-1}$ [12]. With the experimental procedure used here, about 25% of the available skeletal muscle actin ATP sites were labeled with 8-N_3[α-^{32}P]ATP. This low efficiency of labeling could be due to the low exchange rate between the bound ATP and 8-N_3-ATP. Higher efficiency of the labeling reaction may be obtained by using ATP-depleted actin prepared in 30–50% sucrose [16,17].

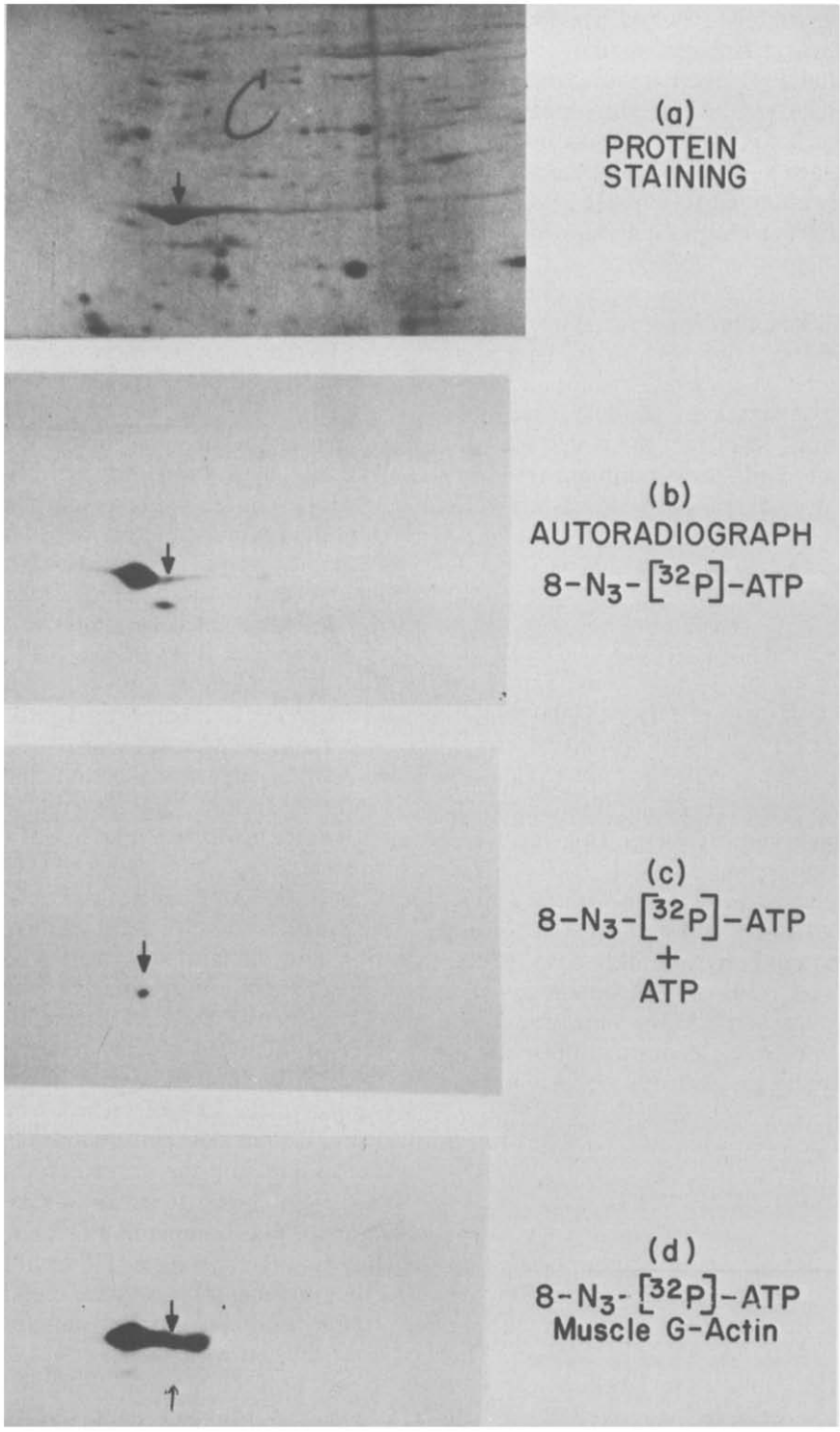

Fig. 2. Two-dimensional gel electrophoresis of 8-N_3-[α-^{32}P]ATP-labeled protein in the membrane fraction M-2 (a–c) and purified G-actin (d): (a) Coomassie blue-stained protein pattern; (b,c) autoradiograph of the labeled protein in the absence (b) and presence (c) of 500 μM ATP; (d) autoradiograph of the labeled G-actin. Arrows indicate the position of Coomassie Blue-stained actin.

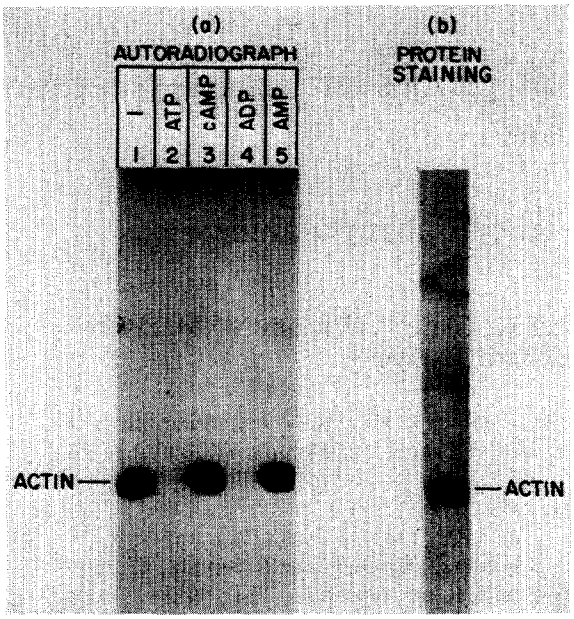

Fig. 3. 8-N_3-[α-^{32}P]ATP incorporation into the purified rabbit skeletal muscle G-actin. Purified G-actin (20 μg) was preincubated at 4°C for 5 min in 100 μl of a solution of 50 mM Tris (pH 7.2), 0.125 μM 8-N_3-[α^{32}P]ATP in the absence (lane 1) or presence of 500 μM of ATP (lane 2), cAMP (lane 3), ADP (lane 4) or AMP (lane 5). Following photolysis the samples were analyzed by SDS–gel electrophoresis and autoradiography as described in the text. (a) autoradiograph; (b) Coomassie Blue-stained G-actin.

Our results indicate that 8-N_3-[α-^{32}P]ATP can be used as a photoaffinity label for both muscle and non-muscle G-actin. Furthermore, proteins labeled by 8-N_3-[α-^{32}P]ATP are not necessarily protein kinase or ATPase. The primary amino acid sequence of mammalian skeletal muscle actin is known. 8-N_3-[α-^{32}P]ATP should be useful to identify the ATP binding site in the sequence as well as to study the role of ATP in actin polymerization [10,12].

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REFERENCES

[1] Hoppe, J. and Freist, W. (1979) Eur. J. Biochem. 93, 141–146.
[2] Wagenwood, R.J., Kemp, A. and Slater, G.C. (1980) Biochim. Biophys. Acta. 593, 204–211.
[3] Scheurich, P.L., Schaefer, H.J. and Dose, K. (1978) Eur. J. Biochem. 88, 253–257.
[4] Campbell, K.P. and MacLennan, P.H. (1983) J. Biol. Chem. 258, 1391–1394.
[5] Roth, R.A. and Cassell, P.J. (1983) Science 219, 299–301.
[6] Woodin, A.M. and Wienecke, A.A. (1966) Biochem. J. 99, 493–500.
[7] Ueda, T. and Greengard, P. (1977) J. Biol. Chem. 252, 5155–5163.
[8] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[9] Mackin, W.M., Huang, C.-K. and Becker, E.L. (1982) J. Immunol. 129, 1608–1611.
[10] White, J., Naccache, P.H. and Sha'afi, R. (1982) Biochem. Biophys. Res. Commun. 109, 1144–1149.
[11] Philips, D.R. and Jennings, L.K. (1980) J. Cell Biol. 86, 77–86.
[12] Korn, E.D. (1982) Pharmacol. Rev. 62, 672–737.
[13] Strohman, R.C. and Samorodin, A.J. (1962) J. Biol. Chem. 237, 363–370.
[14] Cooke, R. (1975) Biochemistry 14, 3250–3256.
[15] Martonosi, A., Gouvea, M.A. and Gergely, J. (1960) J. Biol. Chem. 235, 1700–1703.
[16] Kasai, M., Nakano, E. and Oosawa, F. (1965) Biochim. Biophys. Acta 94, 494–503.
[17] Nagy, B. and Strzelecka-Golaszewska, H. (1972) Arch. Biochem. Biophys. 150, 428–435.