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THE USE OF ARYL AZIDO ATP ANALOGS AS PHOTOAFFINITY LABELS FOR MYOSIN ATPASE

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The synthesis of ATP analogs containing a photoactive aryl azido grouping coupled to the 3′ hydroxyl of ATP is described. The potential effectiveness of these analogs in the investigation of nucleotide-binding regions is outlined and this effectiveness demonstrated by their photodependent inhibition of subfragment 1 ATPase. The use of ^{14}C-labeled azido ATP demonstrates an almost stoichiometric covalent binding of the analog.

Because of their potential application to other systems, a number of reactions describing the reactivity of the 3′ hydroxyl of the nucleotide ribose are outlined in an Appendix.

INTRODUCTION

The characteristic enzymatic activity of muscle myosin is the dephosphorylation of adenosine triphosphate to adenosine diphosphate and inorganic phosphate. This free energy-liberating reaction is intimately associated with muscle contraction (1) and a clarification of the interaction of adenine nucleotides with the myosin protein is one portion of the task to be accomplished in order to understand the molecular mechanism of muscle contraction. In point of fact, such ATPase activity is not restricted to the contractile protein alone, but is a property of almost all biological energy-requiring systems. Consequently, an understanding of the interaction of the adenine nucleotide with the myosin protein has relevance to a large number of energy-transducing systems.

The chemical modification of protein structure can often provide much information as to the structure of the active site or substrate binding region (2). As an example, the hydrolysis of ATP by myosin is inhibited by specific chemical modification of a tyrosine residue (3). These results are taken to indicate that the decomposition of ATP by myosin is accelerated by electron donation from the adenine base to a tyrosine situated at the nucleotide-binding site. A further indication of the interaciton between the adenine base of ATP and the myosin protein is the very tight Michaelis complex formed between ATP and myosin in which the ϵ-amino group of lysine has been trinitrophenylated. Presumably, there is conjugation between the adenine base of ATP and the trinitrophenyl grouping (4). Chemical modification of specific regions on an enzyme protein depends upon a presupposed chemical structure or reactivity within such regions. In the most general case, one cannot always differentiate on the basis of chemical modification between a direct attack on the active site and the modification of an enzymatic reaction by interaction at an adjacent site influencing reactivity only indirectly.

A valuable approach to the study of the mechanism of the myosin ATPase has been the utilization of a large number of structural analogs of ATP. The study of the interaction of such analogs with the myosin protein has resulted in the accumulation of rather specific information concerning those portions of the adenine nucleotide molecule which are important for proper orientation with the contractile protein. Such studies allow one to make as well an indirect assessment of the components present at the active site which might be responsible for nucleotide binding.

The ATP molecule contains three distinct structural groupings, i.e. the adenine base, the ribose sugar, and the triphosphate component. On the basis of the interaction of actomyosin with ATP analogs modified in these positions, a model of the enzyme-substrate complex has been postulated (5, 6). This model is based upon evidence suggesting that the amino grouping at the 6 position of the adenine base and the 3′ hydroxyl of the ribose ring are hydrogen bonded to the protein. The nucleotide binding is thought to involve an NH group at the trinitrobenzene sulfonate-interacting site (4), as well as conjugation of triphosphates with asparagine via metal interaction (7) and binding of the terminal pyrophosphate to a sulfhydryl and guanidinium group at the active site (8). An important aspect of actomyosin-nucleotide analog studies is the finding that the ribose ring can be drastically modified without affecting markedly the rate of myosin-catalyzed hydrolysis.

From such analog studies an appreciation of the forces responsible for the binding of substrate to protein must be gained by indirect analysis since destruction of the enzyme destroys those forces responsible for the specificity of substrate interaction. On the other hand, reagents which inhibit enzyme activity by virtue of forming covalent bonds with proteins do not normally possess sufficient specificity to assure that the reaction is taking place at the substrate binding site.

In our search for a general reagent which might be utilized in a comparative study of the nucleotide-binding region of a variety of energy-transducing systems we realized that what was required were assurances of "proper," i.e. active, site binding and that, subsequent to binding, the reagent be able covalently to insert itself at that region. Either the photochemical carbene probes first developed by Westheimer and his group (9–11) and extended to the mapping of the binding site of specific antibody proteins by Converse and Richards (12), or alternatively, the photoactive nitrene probes (13–20) appeared to be the most effective means of achieving such ends.

In view of the above facts it was decided to develop methods by which an active photosensitive adjunct could be attached to the ribose portion of the nucleoside triphosphate. With such analogs it was anticipated that coupled to substrate specificity would be the possibility of a controlled labeling of the active site by the photogeneration of an extremely active chemical species. An additional restraint which we imposed upon ourselves was that the photogenerated species be formed at wavelengths of light remote from regions of protein-damaging radiation. During the course of this work the interesting work on 8-azido adenosine triphosphate appeared in which the azido group on the adenine ring is converted to an active nitrene by UV irradiation (20).

This paper describes our attempts at the chemical coupling of diazo groupings to the ATP molecule, the successful coupling of aryl azido groupings to ATP in the synthesis of 3′-O-{3-[N-(4-azido-2-nitrophenyl)amino] propionyl} adenosine-5′-triphosphate (X), 3′-O-{4-[N-(4-azido-2-nitrophenyl)amino] butyryl} adenosine-5′-triphosphate (XI) and 3′-O-{6-[N-(4-azido-2-nitrophenyl)amino] caproyl }adenosine-5′-triphosphate (XII), and the photodependent inhibition of myosin subfragment 1 ATPase activity by these azido analogs. The synthesis of ^{14}C azido ATP analog, i.e. 3′-O-{3-[N-(4-azido-2-nitrophenyl)

amino] -1-^{14}C-propionyl} adenosine-5′-triphosphate and the use of the labeled compound in evaluating the covalent binding of the analog to myosin subfragment 1 are described. The photodependent inhibition and covalent labeling of the mitochondrial F_1 ATPase from ox heart has as well been accomplished and shall be detailed in another publication (21).

The general utility of our procedure for the formation of nucleotide analogs by substitution at the ribose hydroxyl group is indicated by the synthesis of spin-labeled analogs of ATP (XV), ADP, AMP, and NADP having 3-carboxy-2,2,5,5-tetramethyl-pyrroline-1-oxyl (XIV) coupled to this position.

METHODS

Esterification of ATP with N-t-Butoxycarbonyl-α-Alanine

Carbodiimidazole (71 mg, 0.43 mmole) and N-t-BOC-α-alanine (70.8 mg, 0.40 mmole) dissolved in 0.2 ml dimethylformamide (dried over molecular sieves) were stirred for 10 min at room temperature (22). The solution was added to ATP (24.2 mg, 0.04 mmole in 1 ml water) and the reaction mixture was allowed to react at room temperature for 3.5 hr. Solvent was evaporated under vacuum and the resulting oil washed with ether to remove imidazole and excess N-t-BOC-α-alanine. The residue from ether extraction (87.8 mg) was dissolved in water and chromatographed on 20 × 20-cm Whatman No. 42 paper using n-butanol: water: acetic acid (5:3:2) as eluent. A resulting, well-defined, UV absorbing band at R_f 0.37, a diffused band at R_f 0.45, and a narrow band at R_f 0.69 were eluted from the paper with water and lyophilized, resulting in the collection of 7.3 mg, 1.0 mg, and 2.9 mg, respectively. The fractions with R_f values of 0.45 and 0.69 were the 2′ isomer and 2′, 3′-disubstituted compound. The fraction with R_f 0.37 was the 3′ isomer (XIX) (see Appendix).

Esterification of ATP at the 3′ Position with p-Azidobenzoic Acid

The 3′-p-azido benzoyl ATP (I) was prepared with p-azidobenzoic acid and ATP using carbodiimidazole as a catalyst as indicated above for the esterification of ATP with N-t-BOC-α-alanine. The reaction mixture was subjected to chromatographic resolution with n-butanol:water:acetic acid (5:3:2)as solvent. The reaction product had a UV absorption band with an R_f of 0.37. The material was eluted from the paper with water and found to have absorption peaks at 266 and 285 (sh)nm. Irradiation of the aqueous solution with UV light shifted the 266 nm absorption maximum to 257 nm. The starting p-azidobenzoic acid had a maximum absorption peak at 271 nm which was broadened by UV irradiation.

Preparation of 4-Fluoro-3-Nitrophenyl Azide[1] (II) (14)

4-Fluoro-3-nitroaniline (4.38 g, 0.028 mole) dissolved in 30 ml of concentrated HCl and 5 ml of water at 45°C was filtered and then chilled to −20°C in dry ice-acetone bath. Sodium nitrite (2.4 g, 0.034 mole) in 5 ml of water was added slowly to the well-stirred acid medium while the flask temperature was kept at −15° to −20°C. The reaction mixture following a 10-min stirring was filtered into a flask at −20°C. To this filtrate, sodium azide (2.2 g, 0.032 mole) dissolved in 8 ml of water was added dropwise while the reaction mixture was stirred and kept in the dark at −15° to −20°C. The light brown solid obtained

[1]Azido derivatives of this nature are known to be light sensitive. They are explosive in large quantities when dry and caution is urged in their preparation. We always utilized a ventilated hood and reactions were carried out in the dark.

by filtration was washed with ice water and dried in a vacuum dessicator. Recrystallization from petroleum ether yielded straw-colored needles. Yield: 3.2 g, 63%. MP: 52–52.5°C; MS: m/e 182; NMR ($CDCl_3$): 7.47 ppm (2 H), 7.78 ppm (1 H).

Preparation of N-4-Azido-2-Nitrophenyl-β-Alanine (IV) (14)

To 5.4 ml of an aqueous solution of β-alanine (534 mg, 6 mmoles) and sodium carbonate (1080 mg, 10 mmoles) was added 4-fluoro-3-nitrophenyl azide (900 mg, 4.9 mmoles). Ethanol (6.75 ml), water (5.4 ml) and another portion of ethanol (13.5 ml) were added subsequently to enhance the homogeneity of the reaction mixture. The reaction mixture suspension was stirred at 52°C overnight with an attached cooling condenser. The resulting dark red mixture was first concentrated under vacuum to about one-third of its volume and then diluted with 18 ml of water. Two extractions with 45 ml of ether removed all of the excess starting azide. The aqueous layer was acidified with 3 N HCl to pH 2 and extracted with three 90-ml portions of ether. The ether layer following three washings, each with 50 ml of saturated NaCl solution, was dried over sodium sulfate and then evaporated to dryness. The residue was recrystallized from hot ethanol (yield: 736 mg, 59%). MP: 142.5–145°C; MS: m/e 251; UV: $\lambda_{max}^{CH_3OH}$ 260 (molar extinction coefficient 27.2×10^3), 280 (sh) and 460 (molar extinction coefficient 5.9×10^3) nm; IR: λ_{max}^{KBr} 2.96, 3.43, 4.74, 4.80, 5.83, 6.13, and 6.37 μ.

Preparation of N-4-Azido-2-Nitrophenyl Amino Carboxylic Acid Derivatives

The N-4-azido-2-nitrophenyl derivatives of glycine (III), α-alanine (IVa), 4-aminobutyric acid (V), 5-aminovaleric acid (VI), 6-aminocaproic acid (VII), 11-amino-undecanoic acid (VIII), and 12-aminododecanoic acid (IX) were prepared following the procedure as outlined for the preparation of N-4-azido-2-nitrophenyl-β-alanine (IV) and the physical characteristics of these derivatives are indicated below. The R_f values given are those obtained on silica gel plates with n-butanol saturated with water as the solvent.

N-4-azido-2-nitrophenyl-glycine (III): UV: $\lambda_{max}^{CH_3OH}$ 260, 280 (sh), and 460 nm; IR: λ_{max}^{KBr} 3, 3.5, 4.7, 5.70, and 6.12 μ; R_f: 0.31; MS: m/e 237.

N-4-azido-2-nitrophenyl-α-alanine (IVa): UV: $\lambda_{max}^{CH_3OH}$ 263, 284 (sh), and 460 nm; IR: λ_{max}^{KBr} 3.0, 3.5, 4.71, 4.78, 5.79, 6.12, and 6.38 μ; $R_f = 0.41$; MS: m/e 251.

N-4-azido-2-nitrophenyl-4-amino butyric acid (V): UV: $\lambda_{max}^{CH_3OH}$ 260, 280 (sh), and 460 nm; IR: λ_{max}^{KBr} 2.96, 3.36, 4.71, 5.88, 6.12, and 6.38 μ; $R_f = 0.63$; MS: m/e 265.

N-4-azido-2-nitrophenyl-5-amino valeric acid (VI): UV: $\lambda_{max}^{CH_3OH}$ 257, 282 (sh), and 460 nm; $R_f = 0.68$.

N-4-azido-2-nitrophenyl-6-aminocaproic acid (VII): UV: $\lambda_{max}^{CH_3OH}$ 259, 280 (sh), and 460 nm; IR: λ_{max}^{KBr} 2.96, 3.42, 4.75, 5.88, 6.13, and 6.38 μ; MS: m/e 293; $R_f = 0.68$.

N-4-azido-2-nitrophenyl-11-undecanoic acid (VIII): UV: $\lambda_{max}^{CH_3OH}$ 261, 285 (sh), and 460 nm; IR: λ_{max}^{KBr} 2.96, 3.43, 4.72, 5.90, 6.13, and 6.39 μ; MS: m/e 363; $R_f = 0.77$.

N-4-azido-2-nitrophenyl-12-aminododecanoic acid (IX): UV: $\lambda_{max}^{CH_3OH}$ 259, 282 (sh), and 460 nm; IR: λ_{max}^{KBr} 2.96, 3.44, 4.77, 5.87, 6.14, and 6.40 μ; $R_f = 0.77$; MS: m/e 377.

Preparation of 3′-0-{3-[N-(4-azido-2-nitrophenyl)amino] propionyl} adenosine-5′ triphosphate (X).

Carbodiimidazole (270 mg, 1.67 mmoles) and N-4-azido-2-nitrophenyl-β-alanine (125.5 mg, 0.5 mmole) dissolved in 0.5 ml of dimethylformamide (dried over molecular sieves) were stirred for 15 min prior to introducing 60.5 mg of ATP (0.1 mmole) dissolved in 2.5 ml of water. The reaction was allowed to proceed overnight in the dark at room temperature. Following the evaporation of the solvent to dryness, the residue was repeatedly washed with acetone followed by centrifugation. The acetone-washed residue obtained after drying under vacuum was redissolved in ca. 0.2 ml of water. This sample was then applied to Whatman 3 MM paper and eluted with n-butanol:water: acetic acid (5:3:2). Two major orange-colored UV-absorbing bands of R_f values 0.91 and 0.38, in addition to a colorless UV-absorbing band with an R_f value 0.05 were obtained. The front moving band (R_f = 0.91) and the slow moving band (R_f = 0.05) were identified as N-4-azido-2-nitrophenyl-β-alanine and ATP, respectively. The material (19.3 mg) recovered by eluting the band of R_f value 0.38 with water followed by lyophilization gave a spectrum with maxima at 480 (molar extinction coefficient 4.2×10^3), 290 (sh), and 260 nm (molar extinction coefficient 35.4×10^3) attributed to the addition of the ATP and azido-β-alanine molecules. Azido-4-aminobutyric ATP (XI) and azido-6-amino-caproic ATP (XII) were prepared according to the procedure described for the preparation of azido-β-alanine ATP (X). The R_f values given refer to paper chromatography. Attempts to prepare azido-glycine ATP and azido-α-alanine ATP by identical procedures gave no indication of product formation.

Azido-4-aminobutyric ATP (XI). R_f = 0.40 with n-butanol:water:acetic acid (5:3:2) as the solvent; R_f = 0.64 with isobutyric acid:0.5 N NH_3 (5:3) as the solvent; UV: $\lambda^{max}_{H_2O}$ 260, 290 (sh) and 480 nm.

Azido-6-aminocaproic ATP (XII). R_f = 0.49 (solvent; n-butanol:water:acetic acid, 5:3:2); R_f = 0.65 (solvent; isobutyric:0.5 N NH_3, 5:3); UV: $\lambda^{max}_{H_2O}$ 260, 290 (sh), 480 nm.

Preparation of Myosin Subfragment 1 ATPase

Myosin subfragment 1 was obtained by digesting rabbit skeletal muscle myosin with insoluble papain according to the procedure of Lowey (23) as described by Liu-Osheroff and Guillory (24). Protein was determined by the biuret and the Folin method and the calcium-dependent ATPase assays were carried out by published procedures (24). Phosphate was measured by the method of Lohmann and Jendrassik (25).

Norit Treatment of Myosin ATPase

Myosin subfragment 1 was partly freed of bound nucleotides by Norit treatment. Subfragment 1 (2 ml, 30 mg) was allowed to interact with acid-washed Norit (30 mg) (26) for 15 min at 0°C. The protein obtained following centrifugation at 20,000 rpm for 10 min had the ratio of absorbance at 280–260 nm increased from about 1.38 to 1.46 by the Norit treatment.

Preparation of Azido-1-^{14}C-β-Alanine-ATP (XIII)

(i) The β-alanine-1-^{14}C sample (0.05 mC, in 0.2 ml of 0.01 N HCl, 0.5 mg, 6.2 μmoles) was concentrated under vacuum and redissolved in 0.2 ml of water. To this, sodium carbonate (3.9 mg, 37.2 μmoles) was added followed by 0.2 ml of an ethanol solution containing

3.4 mg of 4-fluoro-3-nitrophenyl azide (18.6 μmoles). The resulting clear solution was maintained at 50°C in a sand bath for 5.5 hr.

The reaction mixture, after being evaporated to dryness, was extracted with acetone and then methanol and the combined solvents were concentrated down, yielding a solid residue. The residue was taken up in a minimal amount of methanol and chromatographed on a 20 X 20-cm silica gel plate with n-butanol saturated with water as the solvent. Three colored bands were observed visually (orange to yellow color) and radioactivity could be detected at the band with an R_f value of 0.41 (azido-1-^{14}C-β-alanine [IV]). The labeled azido-β-alanine was recovered from the plate by elution with methanol (27).

(ii) The azido-1-^{14}C-β-alanine diluted with 61.5 mg (0.25 mmole) of cold N_3-β-alanine was allowed to react with ATP (30.25 mg, 0.05 mmole) in the presence of carbodiimidazole (60.5 mg, 0.37 mmole) as a catalyst. The azido-1-^{14}C-β-alanine ATP obtained by paper chromatography separation had a specific activity of 20,000 cpm /μmole with UV maxima at 260, 290 (sh), and 474 nm.

Preparation of Spin Label ATP, ADP, AMP, and NADP

3-Carboxy-2,2,5,5-tetramethylpyrroline-1-oxyl (XIV) (184 mg, 1 mmole) and carbodiimidazole (173 mg, 1.1 mmoles) dissolved in 0.5 ml of anhydrous dimethylformamide were reacted at room temperature for 10 min to form the imidazolide. ATP (60.5 mg, 0.1 mmole) in 2.5 ml of water was then added to the DMF solution and the mixture stirred at room temperature for 10 hr. The liquid sample obtained following lyophilization was washed with ether and methanol to remove the starting material. The solid residue (37.5 mg) was chromatographed with isobutyric acid:0.5 N NH_3 (5:3) as the solvent. Material (XV) recovered from a UV-absorbing band appearing at R_f 0.58 weighed 12.3 mg and had UV maxima at 259 and 208.5 nm. The electron spin resonance spectrum of this material gave three sharp N-oxide peaks. Spin label ADP, sping label AMP , and spin label NADP were prepared according to the same procedure. Products isolated utilizing the same chromatography system were found with R_f values of 0.74, 0.84, and 0.63, respectively, with all three compounds having strong ESR signals. The UV spectrum of the spin label ADP showed two maxima, one at 259 and the other at 208 nm.

Photolysis

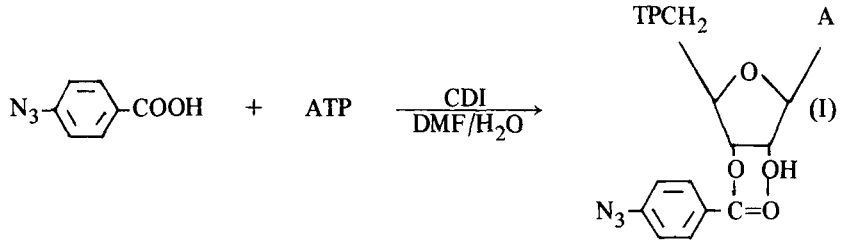
Photolysis was carried out in an air-cooled unit kept below 15°C. The samples in 13 X 100-mm culture tubes were located 12.5 cm distant from the light source, i.e. a tungsten halogen projector lamp (650 W, DVY, 3,400 K°) (28). The volume of the irradiation mixture varied from 0.1 to 3.2 ml. The sample was removed from the photolysis apparatus, chilled, and mixed well at 30-sec intervals during the irradiation in order to prevent overheating. The degree of photoinactivation of subfragment 1 at pH 6.7 by the azido ATP was followed by assaying its Ca^{++} ATPase activity. Dark controls of subfragment 1 in the presence of azido ATP were assayed simultaneously with the light irradiation samples.

RESULTS

Preparation of Arylazido ATP Analogs and Their Utilization

In view of the relative stability of nitrene precursors and the difficulties encountered in the final stages of synthesis of the diazo derivatives of ATP (Appendix) it was decided to investigate the synthesis of ATP analogs containing nitrene precursor groups. ATP was

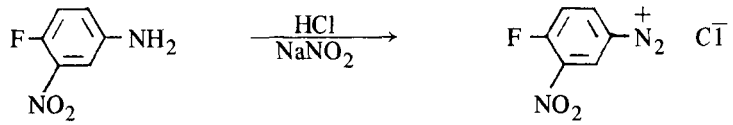
reacted with p-azido benzoic acid in the presence of carbodiimidazole. The reaction mixture was chromatographed and was shown to contain, in addition to the presence of ATP and p-azidobenzoic acid, a new UV-absorbing material. This band with an R_f of 0.35 in the solvent system n-butanol:water:acetic acid (5:3:2) was eluted with water. The UV spectrum of the material recovered following lyophilization showed a



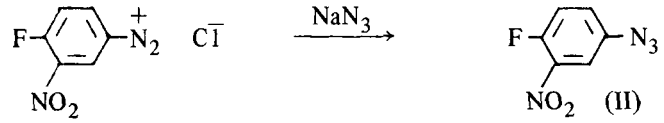
(1)

maximum at 266 nm and a shoulder at 285 nm. A similar reaction carried out with NADP replacing ATP showed that the azido pyridine nucleotide analog could be formed.

The successful synthesis of these compounds showed the feasibility of using the carbodiimidazole coupling reaction in the formation of arylazido ATP analogs. For the general synthesis of the arylazido carboxylic acids, 4-fluoro-3-nitroaniline is first diazotized in a concentrated hydrochloric acid medium in the presence of sodium nitrite (Eq. 2). The diazonium salt is subsequently treated with sodium azide which results in the formation of a light-sensitive 4-fluoro-3-nitrophenyl azide (II) (Eq. 3). The structure of (II) has been confirmed by its mp: 52–52.5°C (recrystallized from petroleum ether), its nmr spectrum: 7.47 ppm (2 protons), 7.78 ppm (1 proton), and its mass spectrum: m/e 182.

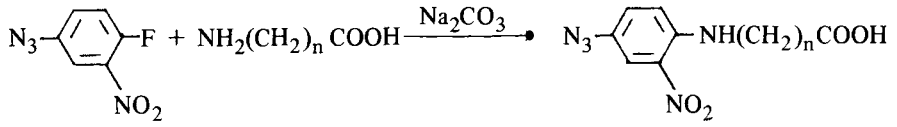


(2)



(3)

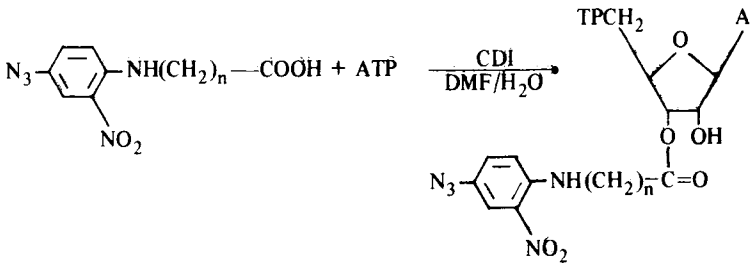
The resulting azide is then condensed with an amino carboxylic acid in an ethanolic aqueous solution containing sodium carbonate. N-4-azido-2-nitrophenyl amino carboxylic acids with varying hydrocarbon chain lengths separating the amino and carboxylic group have been synthesized (Eq. 4) and their structures identified via infrared and mass spectrometry and thin layer chromatography.



n = 1 (III) n = 5 (VII)
2 (IV) 10 (VIII)
3 (V) 11 (IX)
4 (VI)

(4)

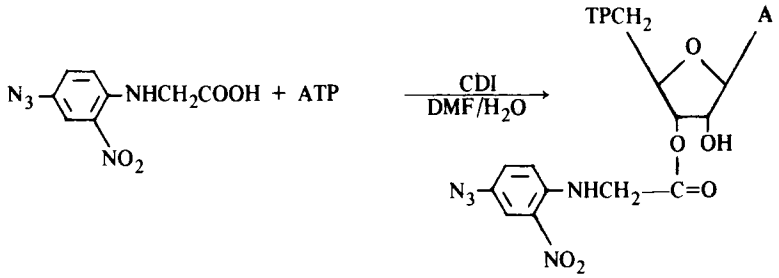
The N-(4-azido-2-nitrophenyl) amino carboxylic acids are then condensed with ATP by means of carbodiimidazole catalysis (Eq. 5).



$n = 2$, 3, 5
(X) (XI) (XII)

N $n = 2$, $-\overset{\overset{O}{\|}}{C}=O$ is ^{14}C labeled (XIII). (5)

Attempts to prepare an ATP analog by condensation of the azido derivative of glycine with ATP (Eq. 6) under our standard conditions were unsuccessful. Presumably the nucleophilic reaction center, i.e. $-\overset{\overset{O}{\|}}{C}-OH$, is sufficiently deactivated by its proximity to the α-amino group. The condensation reaction with the azido derivative of β-alanine, 4-aminobutyric acid, or 6-amino caproic acid (Eq. 5) in which the reaction center is not α to the amino deactivating group was found to proceed satisfactorily. In the case of the synthesis of azido-β-alanine ATP (X),



(6)

following the condensation with ATP, the crude reaction product was first dried under vacuum and then subjected to preliminary purification by repeated washing with acetone. This removes the excess carbodiimidazole and the azido derivative of the β-alanine (IV). The solid residue was taken up in water and applied to Whatman 3 MM paper (see Methods). The material from the band migrating at an R_f of 0.38 was recovered by elution with water and concentrated by lyophilization. This material (X) gave a UV spectrum with maxima at 260, 290 (sh), and 480 nm. The increase in 260 nm peak intensity as compared with the starting material is due to the presence of the adenine ring structure (Fig. 1).

Hydrolysis of the ATP Azido Analogs at Neutral and Alkaline pH

Samples of the azido-β-alanine ATP analog were dissolved in (a) 0.5 M NH_4HCO_3, (b) 1.0 M NH_4HCO_3, (c) H_2O, and (d) 5 mM Tris-HCl, pH 6.7, and chromatographed

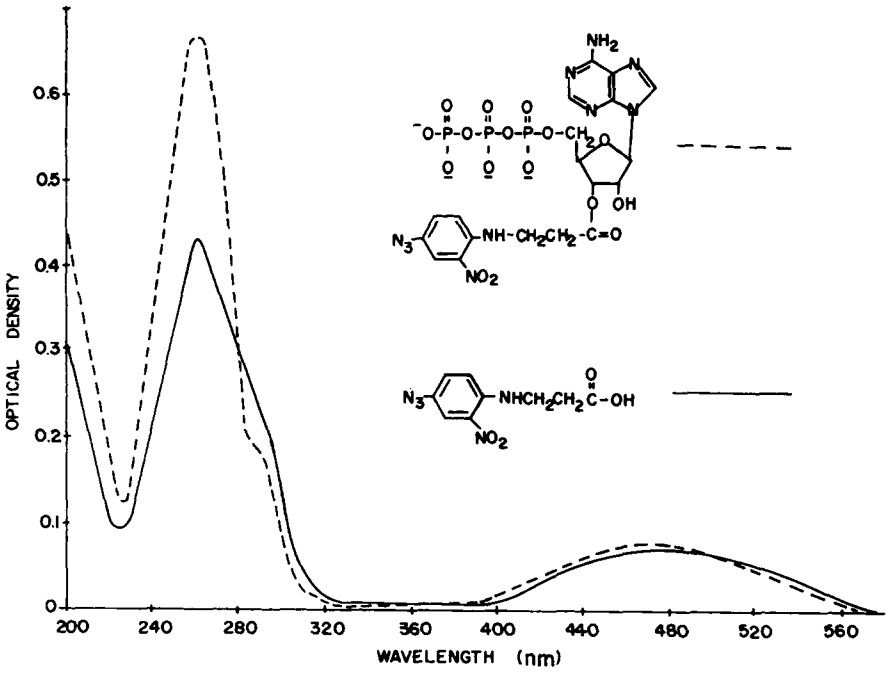


Fig. 1. A comparison of the absorption spectra of N-(4-azido-2-nitrophenyl)-β-alanine (IV) with 3'-0-{3-[N]4-azido-2-nitrophenyl)amino] propionyl} adenosine-5'-triphosphate (X). The N-(4-azido-2-nitrophenyl)-β-alanine was at 0.015 mM and the 3'-0-{3-[N-4(4-azido-2-nitrophenyl)amino] propionyl} adenosine-5'-triphosphate was 0.018 mM.

on Whatman 3 MM paper following varied periods of incubation at 20°C. All four samples immediately upon mixing showed a single clear UV-absorbing spot with an R_f of 0.38 corresponding to the azido-β-alanine ATP analog and indicating that no hydrolysis had taken place. One hr later sample (a) gave one major spot at R_f 0.90 corresponding to the azido-β-alanine and two spots at R_f 0.38 and 0.05 which correlated with the azido-β-alanine ATP and ATP. Sample (b) was completely hydrolyzed and only ATP and azido-β-alanine could be detected. Overnight storage of sample (a) at room temperature resulted in complete hydrolysis to ATP and azido-β-alanine. Samples (c) and (d) remain resistant to degradation following overnight incubation at 20° or at 4°C (Fig. 2).

Inhibition of Myosin Subfragment 1 ATPase with Azido-β-Alanine ATP (X)

The aromatic azido group is known to be capable of undergoing photolysis forming nitrene at wavelengths in a region where proteins do not denature. Nitrenes, being extremely reactive species, can carry out a wide range of chemical reactions including insertion into chemically very stable carbon hydrogen bonds. The photolability coupled to the otherwise fairly stable characteristics of the azido derivatives are highly desirable properties for a photoaffinity label. As can be seen in Fig. 3, subfragment 1 ATPase activity was maintained both in the dark and in the light for at least 5 min under the conditions of the experiment. When subfragment 1 was irradiated in the presence of azido-β-alanine ATP (X), a 67% loss in ATPase activity was observed within 60 sec, while the control dark sample with azido ATP remained stable. The extent of photoinactivation has been found to approach maximal at irradiation of 2-min duration. It is reasoned that

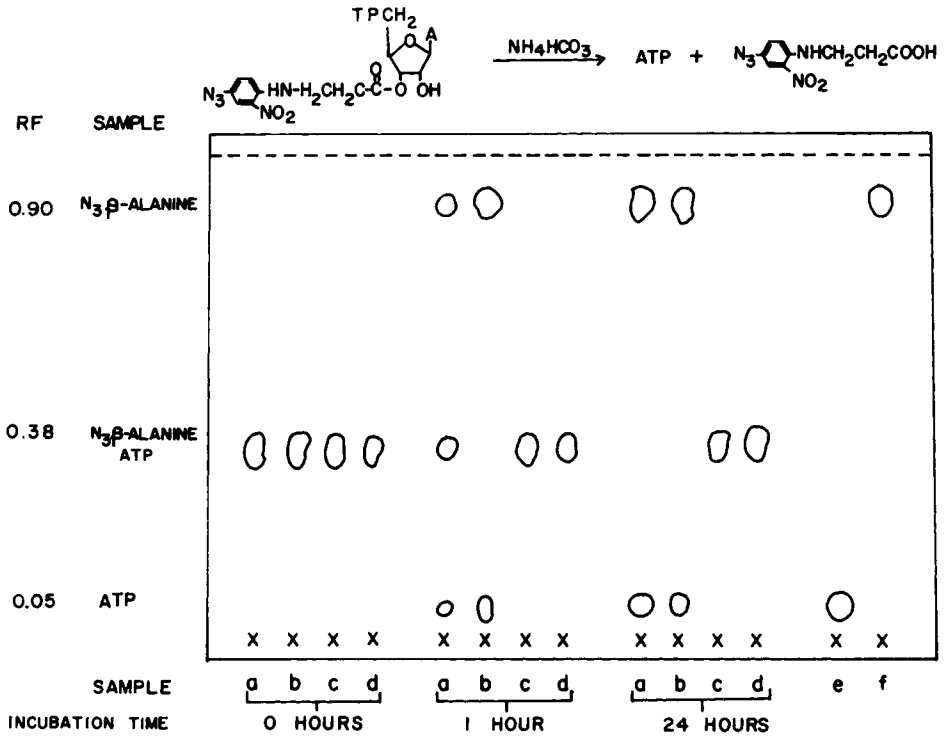


Fig. 2. The stability of 3'-0-{3-[N-(4-azido-2-nitrophenyl)amino] propionyl} adenosine-5'-triphosphate (X). The azido-ATP analog was tested in neutral and basic conditions at 20°C. Small amounts of the reagent were dissolved in (a) 0.5 M NH_4HCO_3; (b) 1.0 M NH_4HCO_3; (c) H_2O, and (d) 5 mM Tris-HCl, pH 6.7. Incubation was allowed to take place at 0, 1, and 24 hr at which time a small aliquot of the reaction mixture was spotted on Whatman 3 MM paper. Chromatography was conducted with n-butanol:water:acetic acid (5:3:2) as the solvent. The paper was dried and examined for UV-absorbing material. Samples (e) and (f) represent pure ATP and azido-β-alanine (IV), respectively.

the adenine nucleotide with the aryl azido group attached on the 3′ hydroxy position is directed to the ATP-binding site via the 6-amino group and the triphosphate group positioned by the ribose ring structure. Irradiation of ATPase enzyme-azido ATP mixture causes inhibition of ATPase activity due to the photogenerated nitrene which chemically modifies the nucleotide-binding site.

The effectiveness of azido-β-alanine ATP (X) as a photoinactivator of ATPase activity is influenced greatly by the presence of the natural substrate ATP. Non-Norit-treated myosin subfragment 1 was maximally inhibited only 20%, while Norit-treated preparations have been maximally inhibited 72%. In other experiments, when equivalent amounts of ATP and azido-β-alanine ATP were added to the Norit-treated subfragment, irradiation induced no inhibition. The additional fact that azido-β-alanine (IV) without the nucleotide adjunct is not an effective photoinactivator strongly indicates that the azido ATP photoinactivation is due to its active site directing ability.

A variable degree of inhibition has been obtained with different protein preparations. As can be seen in Table I, a 2-min photolysis of myosin subfragment 1 in the presence of azido-β-alanine ATP (X) resulted in a 45%–72% decrease in ATPase activity. There are indications that the age of the protein preparation influences the degree of

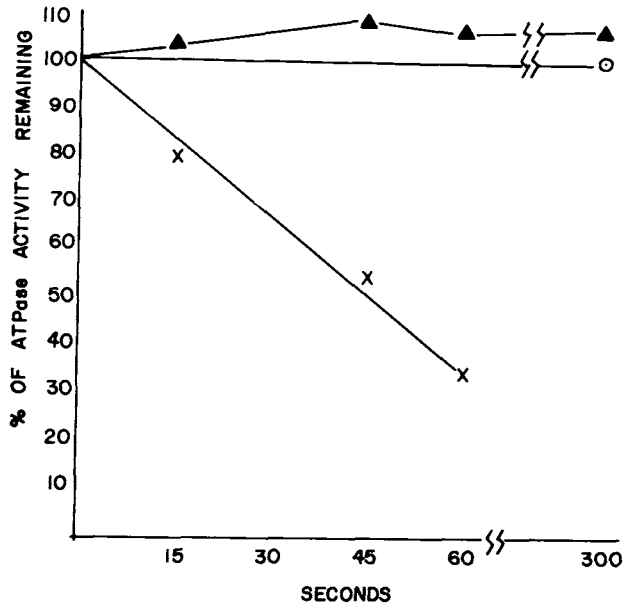


Fig. 3. Photoinactivation of myosin subfragment 1 ATPase activity. 3.2 ml containing 4 mg subfragment 1 in 5 mM Tris-HCl, pH 6.7, were irradiated for the appropriate time. A 0.35-ml vol was removed and assayed for Ca^{++}-dependent ATPase activity. The procedure for photoirradiation and the assay of Ca^{++} ATPase activity is described under Methods. ▲, Subfragment 1 ATPase activity measured under photoinactivation conditions but in the absence of N_3-β-alanine ATP. ⊙, Subfragment 1 ATPase activity in the presence of N_3-β-alanine ATP at 0.374 mM in the dark. X, Subfragment 1 ATPase activity following irradiation in the presence of azido-β-alanine ATP (0.374 mM).

photoinactivation. In Table I sample 18a is shown to be initially inhibited 72%; while 16 days later, (sample 18b), under otherwise identical conditions only 46% inhibition was obtained.

TABLE I. Photoinhibition of the ATPase Activity of Myosin Subfragment 1 Preparations

Subfragment 1 preparation	Protein (mM) [X 10^{-2}]	Azido ATP (mM)	Maximal % photo-inactivation
15	1.25	0.374	67
16	0.80	0.240	62
18a	1.40	0.420	72
18b	1.40	0.420	46
20	0.55	0.280	45

See Methods for inhibition and assay conditions.

Covalent Labeling of Myosin Subfragment 1 with Azido-1-^{14}C-β-Alanine ATP (XIII)

Photoinactivation of myosin subfragment 1 with azido-1-^{14}C-β-alanine ATP (XIII) resulted in the labeling of the protein with approximately stoichiometric amounts of radioactivity. The protein inhibited with ^{14}C azido ATP was dialyzed first against 5 mM Tris-HCl, pH 6.7, and a second time against 25 mM Tris-HCl, pH 7.6, each for 24 hr. This was followed by Norit treatment (Methods) prior to Sephadex G-75 gel filtration with 25 mM Tris-HCl, pH 7.6, as the eluent. Protein fractions emerging at the void volume contained radioactivity (Fig. 4). These were combined and the combined sample assayed for protein and radioactivity.

Reactivity of Other Arylazido ATP Analogs

Two additional azido ATP analogs, azido-4-aminobutyric ATP (XI) and azido-6-aminocaproic ATP (XII) in which there are three and five methylene groups, respectively, bridging the distance between the ribose portion of the adenine nucleotide and the aryl nitrene have been tested on myosin subfragment 1 ATPase activity. All three arylazido ATP analogs inhibited enzymatic activity, the extent of photoinactivation being dependent upon the concentration of the azido ATP and showing saturation kinetics (Fig. 5).

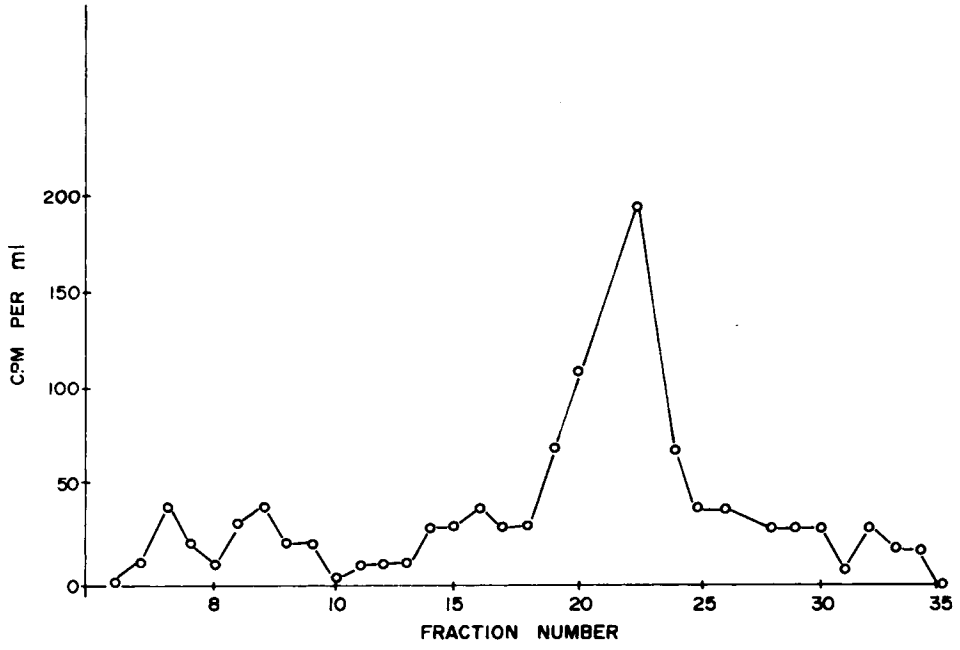


Fig. 4. The labeling of myosin subfragment 1 with azido-1-^{14}C-β-alanine ATP (XIII). Subfragment 1 (2.6 mg, 2×10^{-2} μmoles) in 5 mM Tris-HCl, pH 6.7, was incubated with azido-1-^{14}C-β-alanine ATP (0.925 mg, 2.2×10^{4} cpm, 1.1 μmoles) for 15 min at 0°C followed by 3 min of irradiation. The protein was dialyzed against 5 mM Tris-HCl, pH 6.7, and then against 25 mM Tris-HCl, pH 7.6, for 24 hr, respectively. The dialyzate was then treated with 2.6 mg of Norit. The Norit-treated preparation (1.5 ml) was passed through a Sephadex G-75 column (1 × 15 cm) and 0.35-ml fractions were collected. Protein eluted at the void volume (fractions 20–23 inclusive) contained radioactivity. These fractions were combined and total protein was determined at 6.8×10^{-3} μmoles by the Folin procedure. Radioactive measurements showed 7.1×10^{-3} μmoles of the azido compound to be covalently bound to the protein. Such measurements indicate a labeling-protein ratio of 1.04.

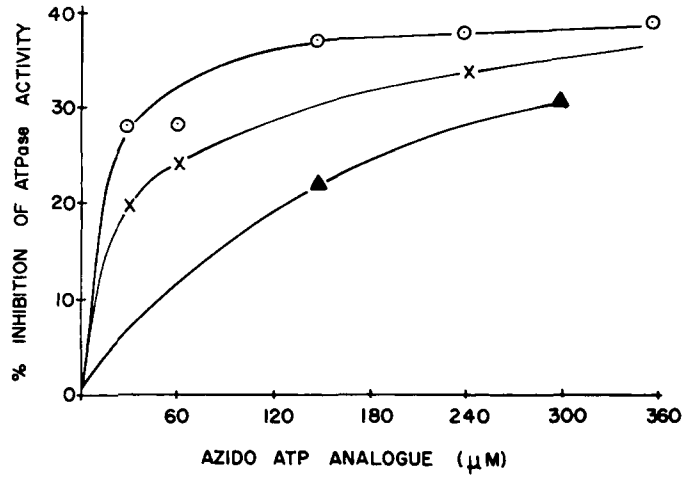


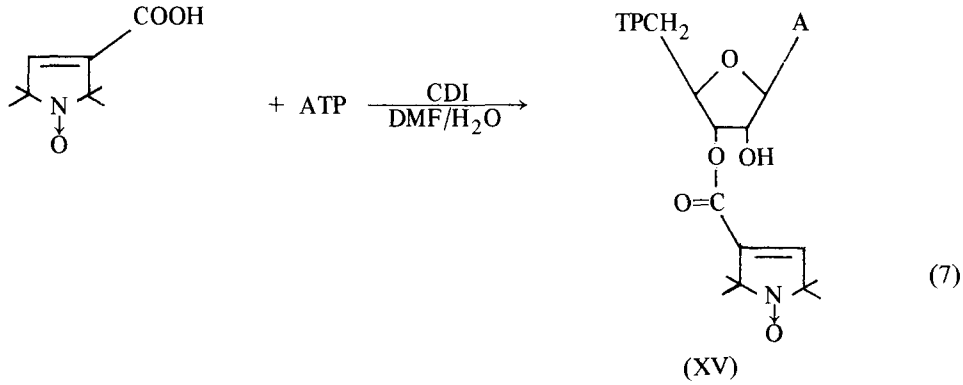
Fig. 5. Photoinactivation with aryl azido ATP analogs. Photoinactivation of subfragment 1 (2.75 μM) was carried out as described under Methods. Irradiation was for 2 min at 10°C. ▲, Azido-β-alanine ATP; ⊙, azido-4-aminobutyric ATP; X, azido-6-aminocaproic ATP.

Azido-β-Alanine ATP as a Substrate for Myosin Subfragment 1 EDTA-ATPase

The possibility that the arylazido analog could act as a substrate for ATPase activity in the dark was tested with the EDTA-stimulated ATPase activity of subfragment 1. The control assay system in 0.5 ml contained 71 mM EDTA, 0.41 M NH_4OH, 86 mM Tric-HCl, pH 8.0, and 10 mM ATP (29). In the experimental vessel 3 mM azido-β-alanine ATP replaced the 10 mM ATP. Dephosphorylation was allowed to proceed for 20 min at 30°C in the dark and the degree of ATPase activity determined by the amount of inorganic phosphate liberated (24). The azido analog of ATP was dephosphorylated at a rate equivalent to 17% of the control ATPase activity. (Control ATPase activity was 230 μmoles of phosphate liberated per mg protein per hr.)

Synthesis of Spin-Labeled Derivatives of Nucleotides

The product isolated from the reaction 3-carboxy-2,2,5,5-tetramethyl-pyrroline-1-oxyl (XIV) and ATP in the presence of carbodiimidazole described under Methods gave a UV spectrum with UV maxima at 258.5 and 210 nm corresponding to adenine nucleoside derivatives and an α, β-unsaturate carbonyl group, respectively. In addition, the



(XV) (7)

product has an electron spin resonance spectrum with three sharp peaks at slightly narrowed spacing compared to the starting N-oxide. This procedure has as well been used for the synthesis of 3′ spin-labeled analogs of ADP, AMP, and NADP.

DISCUSSION

The utilization of carbodiimidazole to facilitate the formation of activated carboxylic acids was elegantly applied by Gottikh and his group (30, 31) to the synthesis of a wide spectrum of amino acyl nucleosides, nucleotides, nucleoside di- and triphosphates, and tRNA derivatives. The formation of an imidazolide intermediate has been used in our work in the synthetic schemes leading to the esterification of adenosine and diphosphopyridine nucleotides. Such esterification resulted in the formation of nucleotide analogs containing a spin label or azido function on the ribose molecule.

It is known that the amino acylation can take place at the 2′ or 3′ hydroxy position or at the 6-amino group of the adenine ring structure. The solvent systems varying in polarity and solvation capacity play an important role in orienting precisely where the reaction occurs. The dimethylformamide:water (1:5) solvent system we have adopted has been shown by Gottikh (31) to be most advantageous in restricting the reaction center to the ribose hydroxyl groups rather than at the 6-amino group.

The esterification step provides 2′ or 3′ isomers which in the case of amino acid esters of nucleotides can be resolved chromatographically. In the esterification of ATP with N-t-butoxycarbonyl-α-alanine (Appendix, Eq. 8), the reaction products could be shown to be resolved into three components by paper chromatography using n-butanol: water:acetic acid (5:3:2) as eluent. The two minor components with R_f values of 0.45 and 0.69 were converted to the major component (R_f 0.37) upon standing at room temperature. We assume that the component with R_f 0.69 because of its lesser polarity represents the 2′,3′-di-substituted ATP analog. The component with R_f 0.45 represents the 2′ isomer and the component with R_f 0.37 is the 3′ isomer. The possibility of conversion of the 2′ to the 3′ isomer by acyl migration is well supported by a number of studies (32, 33). Evidence in the literature indicates that the 2′ hydroxyl groups of both adenosine and uridine are more open to electrophilic attack than the 3′ hydroxyl group (34). Thus the 2′ hydroxyl group is kinetically more reactive for substitution. However, such substitution is relatively less stable in comparison to the thermally more favorable esterification at the 3′ hydroxyl group (32). From this accumulation of data we consider the 3′ isomers to be the specific component isolated under our synthetic conditions. Further support for this conclusion is seen in the recent observation that the esterification of adenosine with fluorosulfonylbenzoyl chloride results in the preferential formation of 3′-p-fluorosulfonylbenzoyl adenosine (35).

The imidazolide method did not result in the formation of ester linkages to ATP with azido glycine (Eq. 6) or the diazotized p-nitrophenyl ester of α-alanine (Appendix, Eq. 13) This lack of reactivity can be rationalized by the assumption that the diazo functional group as well as the amino group α to the carbonyl has deactivated the reaction center via resonance effects. In support of this possibility is our finding that the presence of an α-amino group next to the ester bond induces a higher hydrolysis rate of this ester linkage than in the case where the amino group was more remote. When the N-t-BOC-α-alanine ATP (XIX) is left in aqueous solution at room temperature for 24 hr the formation of the cleavage products ATP and N-t-BOC-α-alanine is readily demonstrated. The corresponding N-t-BOC-β-alanine ATP under identical conditions remains unchanged.

The design of the photoreactive ATP analogs described in this paper specifically utilizes what might appear to be at first a rather bulky arylazide. On the other hand, these substituents are chemically stable at 25°C, not drastically susceptible to photochemical rearrangement and when substituted with a nitro group can be photolyzed to the aryl nitrene at wavelengths above 350 nm, i.e. at wavelengths clear of the protein absorption.

That the azido ATP analogs discussed in this paper have properties consistent with the requirements of photoactive site-directed reagents as discussed by Knowles (13) is evident from a number of our experimental observations. The photoactive species azido-β-alanine (IV) does not inhibit myosin subfragment 1 ATPase activity in comparison to the extensive photodependent inhibition observed in the presence of azido-β-alanine ATP (X). The ATP moiety is thus required to direct the nitrene precursor to the active site of the enzyme. In addition, the dependency of azido ATP photoinhibition upon prior treatment of the subfragment 1 with Norit is taken as an indication of the necessity for removal of bound nucleotide in order to allow the azido ATP to approach the active site and act effectively in photoinactivation. The Norit treatment of subfragment 1 preparations results in protein with a 280:260 nm optical density ratio ranging from 1.41 to 1.50. The variability of the Norit treatment in clearing bound nucleotide from the protein may explain the variability in the extent of photoinactivation of different protein preparations. A complicating factor is that the methodology involved in our photolysis experiments has not been optimized with respect to azido concentration and proper mixing during irradiation.

Even after extensive treatment of the protein following photolysis in the presence of azido-1-^{14}C-β-alanine ATP (XIII), 7.1×10^{-3} μmole of the analog remained per 6.8×10^{-3} μmole of subfragment 1. In addition to the clear indication of covalent bindings a single binding site for the azido analog per protein molecule is indicated. These results indicate the extreme effectiveness and specificity of the analogs as site-directed reagents for the myosin protein. This effectiveness is seen as well with respect to azido-β-alanine ATP inhibition of the mitochondrial F_1 ATPase (21).

The relative stability of the aryl azido group and the reactivity of the aryl nitrene coupled to the binding site-seeking properties of the ATP portion of the nucleotide analog prevents rapid reaction with solvent and allows for concentration at the binding site prior to covalent insertion. These are exactly the characteristics required for a site-directed photoaffinity label (13). A competitive binding mechanism is indicated for myosin subfragment 1 inhibition by the requirement for Norit treatment and by the saturation kinetics observed in the concentration dependency for photoinhibition by azido-ATP analogs. Work is presently directed towards evaluating the precise region of insertion on the ATPase proteins of both subfragment 1 and the ox heart mitochondrial F_1 ATPase protein.

The preparation procedures outlined in this paper have great potential use in that they may be used as the basis for the synthesis of a wide variety of different nucleotide probes. In addition to photoaffinity labels, spin labels and fluorescent adjuncts may also be coupled to the reactive ribose while still retaining the enzymatic functioning of the nucleotide. Variation in the distance of the azido group from the 3′ hydroxy position using azido-β-alanine, azido-4-aminobutyric, and azido-6-aminocaproic ATP and other potential analogs is anticipated to be a powerful tool in the mapping of nucleotide-binding regions of proteins (Fig. 6). Rossmann et al. (36) have shown that the three-dimensional alignment of the nucleotide-binding structure in dehydrogenases, kinases,

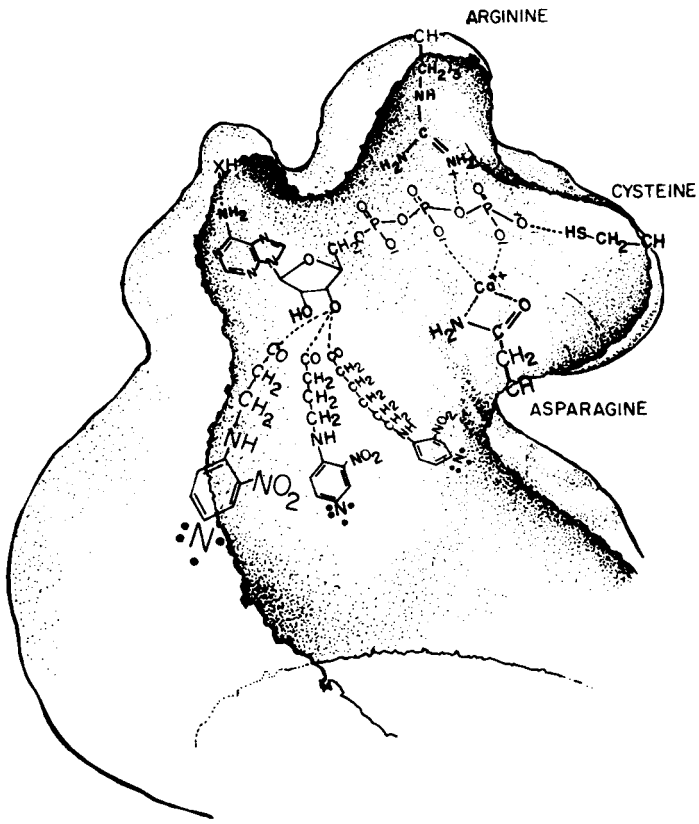


Fig. 6. Schematic representation of the positioning of aryl azido ATP analogs at the myosin subfragment 1 ATP binding site. An attempt is made to indicate in this figure the manner in which the topographical region about the binding site may be scanned by the nitrene analog as a function of the methylene units separating the active nitrene from the nucleotide-binding region.

and flavodoxins indicates a common structural domain whose function is to bind nucleotides (37). The availability of the nucleotide photoaffinity reagents described in this paper represents a chemical tool for the seeking out of such regions on proteins without the necessity of a knowledge of the complete primary structure of the protein.

ACKNOWLEDGMENTS

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REFERENCES

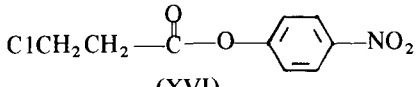
1. Carlson, F. D., and Wilkie, D. R., "Muscle Physiology," Prentice-Hall, Inc., New York (1974).
2. Means, G. E., and Feeney, R. E., "Chemical Modification of Proteins," Holden-Day, Inc., New York (1971).
3. Shimada, T., J. Biochem. 67:185 (1970).

4. Kuba, S., Tokura, S., and Tonomura, Y., J. Biol. Chem. 235:2835 (1960).
5. Tonomura, Y., Kubo, S., and Imamura, K., in "Molecular Biology of Muscular Contraction," S. Ebashi, F. Oosawa, T. Sekine, and Y. Tonomura (Eds.). Igaku Shoin, Tokyo (1965).
6. Tonomura, Y., "Muscle Proteins, Muscle Contraction and Cation Transport," Chapter 9, University Park Press, Baltimore, Md. (1973).
7. Azuma, N, Ikegara, M., Otsuka, E., and Tonomura, Y., Biochim. Biophys. Acta. 60:104 (1962).
8. Yamashita, T., Soma, Y., Kobayashi, S., Sekine, T., Titani, K., and Narita, K., J. Biochem. 55: 576 (1964).
9. Singh, A., Thornton, E. R., and Westheimer, F. H., J. Biol. Chem. 237: PC3006 (1962).
10. Shafer, J., Baronowsky, P., Laursen, R., Finn, F., and Westheimer, F. H., J. Biol. Chem. 241:421 (1966).
11. Vaughan, R. J., and Westheimer, F. H., J. Amer. Chem. Soc. 91:217 (1969).
12. Converse, C. A., and Richards, F. F., Biochemistry 8:4431 (1969).
13. Knowles, J. R., Accts. Chem. Res. 5:155 (1972).
14. Fleet, G. W. J., Knowles, J. R., and Porter, R. R., Biochem. J. 128:499 (1972).
15. Winter, B., and Goldstein, A., Mol. Pharmacol. 6:601 (1972).
16. Hixson, S. S., and Hixon, S. H., Photochem. Photobiol. 18:135 (1973).
17. Levy, D., Biochim. Biophys. Acta 322:329 (1973).
18. Perry, M. B., and Heung, L. L. W., Can. J. Biochem. 50:510 (1972).
19. Schwyzer, R., and Caviezel, M., Helv. Chim. Acta 54:1395 (1971).
20. Haley, B. E., and Hoffman, J. F., Proc. Natl. Acad. Sci. 71:3367 (1974).
21. Jeng, S. J., Russell, J., and Guillory, R. J., submitted to Biochimica et Biophysica Acta.
22. Paul, R., and Anderson, G. W., J. Amer. Chem. Soc. 82:4596 (1960).
23. Lowey, S., Slater, H. S., Weeds, A. G., and Baker, H., J. Mol. Biol. 42:1 (1969).
24. Liu-Osheroff, P., and Guillory, R. J., Biochem. J. 127:419 (1972).
25. Lohmann, K., and Jendrassik, L., Biochem. Z. 178:419 (1926).
26. Kornberg, A., and Stadtman, E. R., Methods Enzymol. III:909 (1957).
27. Smith, R. A. G., and Knowles, J. R., Biochem. J. 141:51 (1974).
28. Hanstein, W. G., and Hatefi, Y., J. Biol. Chem. 249:1356 (1974).
29. Yount, R. G., Frye, J. S., and O'Keefe, K. R., Symp. Quant. Biol., Vol. XXXVII, p. 113 (1972).
30. Gottikh, B. P., Kraevskii, A. A., Purygin, P. P., Tsilevich, T. L., Belova, Z. S., and Rudzite, L. N., Izv. Akad. Nauk SSSR, Ser. Khim. (Engl.) 2453 (1967).
31. Gottikh, B. P., Krayevsky, A. A., Tarussova, N. B., Purygin, P. P., and Tsilevich, T. L., Tetrahedron 26:4419 (1970).
32. Zamecnik, P. C., Biochem. J. 85:257 (1962).
33. McLaughlin, C. S., and Ingram, V. M., Biochemistry 4:1442, 1448 (1965).
34. Griffin, B. E., Jarman, M., Reese, C. B., Sulston, J. E., and Trenthan, D. R., Biochemistry 5: 3638 (1966).
35. Pal, P. K., Wechter, W. J., and Colman, R. F., Biochemistry 14:707 (1975).
36. Rossmann, M. G., Moros, D., and Olsen, K. W., Nature 250:194 (1974).
37. Wootton, J. C., Nature 252:542 (1974).
38. Secrist, J. A., Barrio, J. R., and Leonard, N. J., Chem. Ind. 1960 (1967).
39. Žemlička, J., and Chladek, S., Coll. Czech. Chem. Commun. 33:3293 (1968).

APPENDIX

Chemical Reactivity of the 3′Hydroxyl Group of Adenosine Triphosphate: Attempted Synthesis of Diazo Derivatives of ATP

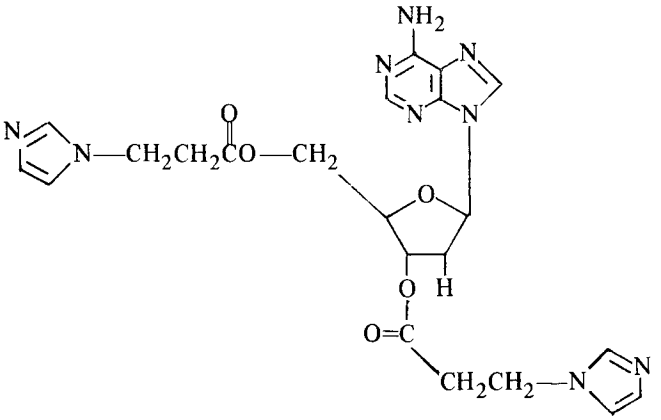
I. Condensation with p-nitrophenyl ester of 3-chloropropionic acid (XVI). Our initial experiments utilized 2′ deoxy adenosine in attempting to acquire knowledge of the reactivity of the 3′ hydroxyl group for condensation reactions with the p-nitrophenyl ester of 3-chloropropionic acid (XVI)



(XVI)

with free imidazole as catalyst (38). The choice of the 3-chloropropionic acid rested upon the feasibility of converting the halide first into an azide, then an amine, and finally a diazo derivative.

When this reaction was carried out according to the procedure outlined below, three products were detected by paper chromatography using UV light as a detecting agent. The major component was isolated by chloroform extraction of the reaction mixture followed by evaporation. The IR spectrum of this sample showed the characteristic ester band at 5.76 μ. Its UV maximum at 260 nm revealed that no acylation had taken place at the 6-amino group of adenosine. The nuclear magnetic resonance spectrum indicates the presence of imidazole protons. Further purification by rechromatography of this sample did not remove those low-field proton signals at 6.9, 7.0, and 7.5 ppm. The mass spectrum of this sample gave a fragmentation pattern devoid of chlorine. These findings led to the postulate that, during the process of the reaction, imidazole displaced the chlorine atom from the 3-chloropropionyl moiety. Structure (XVII) has been tentatively assigned to this product on the basis of mass spectrum data and produt solubility in chloroform. It was thus concluded that esterification of the 3′ OH of the ribose using imidazole catalysis can take place

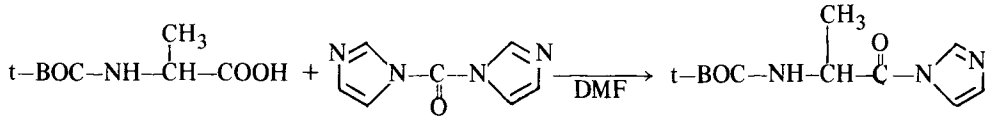


(XVII)

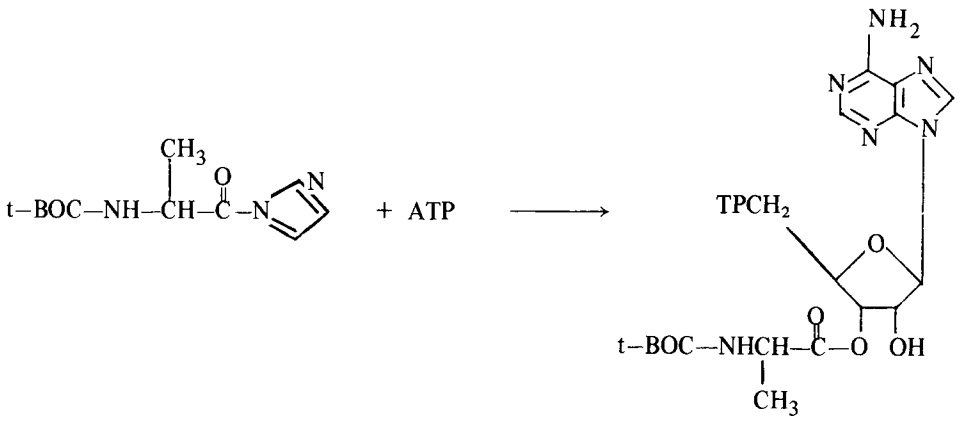
with good yields. On the other hand, our expectation that chlorine would be present in the product for subsequent transformation into the diazo compound was frustrated.

II. Condensation with N-t-butoxycarbonyl α-alanine imidazole (XVIII). An alternative procedure attempted was the utilization of N-t-butoxycarbonyl amino acids for the esterification of the 3′ (or 2′) hydroxyl group of ATP with carbodiimidazole catalysis. Products of a similar esterification of the hydroxyl groups at the 2′ or 3′ positions of nucleosides and nucleotides by amino acids have been investigated as analogs for an intermediate step in protein synthesis (30, 31).

Experimentally, N-t-butoxycarbonyl-α-alanine imidazole (XVIII) formed in anhydrous dimethylformamide was allowed to react with ATP in



(XVIII) (8)

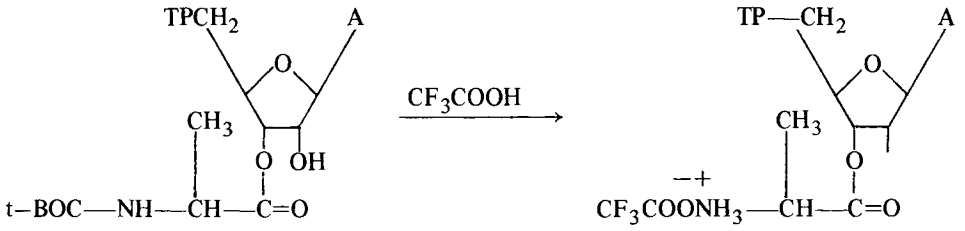


(XIX) (9)

aqueous solvent at room temperature. Descending preparative paper chromatography using n-butanol:water:acetic acid (5:3:2) as the migrating solvent afforded a well-defined, UV-absorbing band, a rather diffused band, and a narrow UV-absorbing front with R_f values of 0.37, 0.45, and 0.69, respectively. The fractions isolated by eluting each band from the paper with water were stored in powder form dessicated under vaccum at $-20°C$. Fractions with R_f values of 0.45 and 0.69 were re-examined after storage and were found to have been partially converted to that fraction with an R_f value of 0.37. Acyl migration to the thermally more stable 3′-OH substituted structure (33) is felt to be responsible for this interchange reaction. It was also noticed that, during storage in an aqueous medium at room temperature, all three fractions produced ATP.

A potassium bromide pellet prepared from the major component of R_f value 0.37 indicated infrared peaks at 5.74, 5.86, 5.91, and 6.06 μ. Although not well resolved, these peaks suggest the formation of an ester linkage, the presence of a phosphate grouping, and the N-t-butoxycarbonyl group. A UV spectrum with a maximum absorption at 257.5 nm identical to that of ATP eliminated the possibility that amino-acylation may have taken place at 6-amino group of nucleotide. A favorable mass spectrum was not able to be obtained due to the extremely low volatility of the sample. Nuclear magnetic resonance spectroscopy demonstrated the presence of tertiary butyl protons at ca. 2 ppm in addition to other pertinent proton signals. On the basis of the above evidence, structure (XIX) has been assigned to this product.

The cleavage of the N-t-butoxycarbonyl group from the N-t-butoxycarbonyl amino-α-alanyl ATP (XIX) was performed by treating with trifluoroacetic acid briefly at ice bath temperature (Eq. 10).

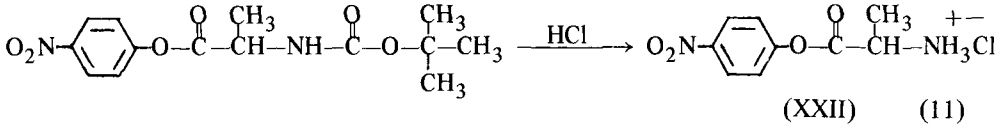


(XIX) (XX) (10)

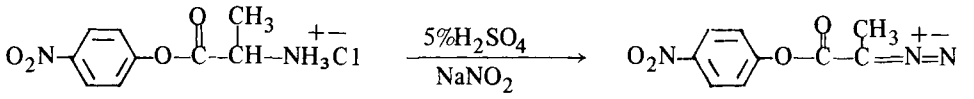
Trifluoroacetic acid was evaporated under vacuum and the residue subjected to paper chromatography. Chromatography in isobutyric acid:0.5 N NH_3 (5:3) was able to resolve a UV-absorbing product from the reactants. Indications that this product (XX) was the aminoacylated ATP were obtained from the observation that alanine and ATP could be formed from this material during basic hydrolysis in 0.5 N NH_3. The rapid ester bond hydrolysis is attributed to the presence of the amino group at the carbon α to the ester linkage (31).

Diazotization of the aminoacyl derivative of ATP (XX) using sodium nitrite in 5% sulfuric acid was not successful. Successful diazotization is known to depend upon being able to extract the product into a water-immiscible phase immediately upon formation. The water solubility of the nucleotide would thus appear to limit the formation of the diazotization product.

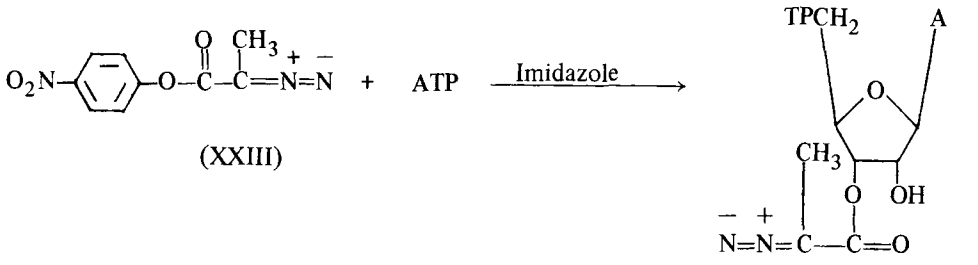
III. Condensation with N-t-butoxycarbonyl α-alanine p-nitrophenyl ester and N-t-butoxycarbonyl β-alanine p-nitrophenyl ester. An additional method attempted for the synthesis of diazo derivatives of ATP was via the condensation of diazotized α-alanine p-nitrophenyl ester (XXIII) with ATP under imidazole catalysis (Eq. 13). The N-protecting group from N-t-butoxycarbonyl α-alanine p-nitrophenyl ester was removed by acid (Eq. 11) and the product diazotized (Eq. 12) prior to attempted condensation with ATP (Eq. 14):



(XXII) (11)



(XXII) (XXIII) (12)



(XXIII)

(13)

The first step worked out with quantitative yields. However, yeilds for the formation of the diazo ester (XXIII) vary from 8% to near 0 depending to a great extent upon how rapidly and how thoroughly the methylene chloride extraction of the diazo ester can be managed. The diazo ester once isolated from the reaction mixture by preparative thin layer chromatography can be stored indefinitely at 0°C without decomposition. The infrared spectrum of the purified sample showed 4.78 and 5.84 μ absorption bands which are characteristic of, respectively, a diazo group α to an ester linkable and the p-nitrophenyl ester. The mass spectrum of this sample gave the molecular ion peak at m/e 221 and fragment m/e 193 by expulsion of the N_2 moiety.

The condensation of the diazo ester and the ATP triethylammonium salt (39) was attempted by allowing the reaction to proceed at room temperature for 4 hr in dimethyl sulfoxide under imidazole catalysis. However, no products other than ATP and unchanged diazo ester were recovered. The lower activity of the diazo p-nitrophenyl esters are explained by the electron distribution resulting from the presence of the α-diazo group. This arrangement makes it difficult for the carbonyl carbon to assume the partial positive charge required for the nucleophilic attack.

The difficulty encountered in carrying out the final step in the synthesis of the precursors of active carbene analogs of ATP directed us towards investigations of the synthesis of the diazo derivative of β-alanine p-nitrophenyl ester, a compound in which the diazo group is not conjugated to the active ester group. Diazotization of β-alanine p-nitrophenyl ester hydrochloride salt has, however, not been able to be accomplished. Since the diazo group is not conjugated with a carbonyl it is very reactive and tends to decompose and undergo further reaction as soon as it is formed.