The Interaction of Chromophoric Nucleotides with Subfragment 1 of Myosin

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The interaction of a series of chromophoric nucleotides derived from 6-mercapto-9- β -ribofuranosylpurine (thioinosine, thioI) and 2-amino-6-mercapto-9- β -ribofuranosylpurine (thioguanosine, thioG) with myosin subfragment 1 isolated from rabbit skeletal muscle was investigated kinetically and spectroscopically. The Mg^{2+} -dependent hydrolyses of thioITP and thioGTP are catalysed by subfragment 1 and probably proceed by a similar mechanism as for ATP hydrolysis, although with different rate constants. For example, the binary thioGDP-protein complex only comprises 8% of the steady-state intermediate of the thioGTPase at 5°C and pH 6.5. Long-lived analogues of intermediates of the thioGTPase were generated by using thioGTP(γ S) [thioguanosine 5'-(3-thio)-triphosphate], thioGMP-P(NH)P (5'-thioguananylimidodiphosphate) and thioGDP. The near-u.v. spectra of the thioguanosine nucleotides bound to subfragment 1 were measured and showed that in all cases the purine ring is bound to the protein in a hydrophobic environment, although the pK of the purine thiol group only increases by 0.2–0.3. ThioGTP caused glycerinated rabbit psoas muscle to contract, but in contrast with thioITP was not able to relax muscle. The applications of these chromophoric nucleotides for investigating the mechanism of muscle contraction and other biological systems, particularly those involving guanosine nucleotide regulation, are discussed.

The characterization of the intermediates of the myosin and actomyosin ATPases* is important in order to relate them to the stages of cross-bridge movement in relaxed and contracting muscle and hence provide a basis of an understanding of muscle contraction at the molecular level. This paper reports a study of the interaction of subfragment 1 from rabbit skeletal myosin with a series of chromophoric nucleotides derived from thioinosine (I) and thioguanosine (II) which are modified in the 5'-triphosphate moiety. These thionucleosides have pK values near 8 and absorb in the near-u.v. (Fig. 1). Transient-kinetic techniques are used to elucidate the thioITPase and thioGTPase mechanisms. A spectral

characterization is made of the intermediates of the thioGTPase reaction.

Lynn & Taylor (1971) proposed a model designed to relate the biochemical events of the Mg^{2+} -dependent myosin and actomyosin ATPase mechanisms to the discrete steps of the cross-bridge cycle. A number of studies have been carried out to define the intermediates more explicitly. In particular it is evident on kinetic and structural grounds that at 20°C the steady-state intermediate of the myosin ATPase is predominantly a myosin-products complex which is different from the complex formed by the addition of ADP to the protein (Taylor, 1973). The latter complex is nevertheless an intermediate of the ATPase mechanism (Bagshaw & Trentham, 1974). On the basis of further kinetic studies a seven-step mechanism has been proposed (eqn. 1; Bagshaw *et al.*, 1974) where M represents myosin and $M^{**}\cdot ADP\cdot P_i$ is the myosin-products complex which is the predominate steady-state intermediate at 20°C.

ThioITP is a substrate for myosin. Its spectrum changes on binding to the protein (Murphy &

* Abbreviations: ATPase, adenosine triphosphatase; thioITP, 6-mercapto-9- β -ribofuranosylpurine 5'-triphosphate; thioGTP, 2-amino-6-mercapto-9- β -ribofuranosylpurine 5'-triphosphate; AMP-P(NH)P, [formerly ATP(β , γ -NH) (Trentham *et al.*, 1976)], 5'-adenylylimidodiphosphate; ATP(γ S), adenosine 5'-(3-thio)triphosphate; alternative symbol AMP-PP(S). Other nucleotide analogues derived from thioguanosine are similarly abbreviated. Mes, 2-(N-morpholino)ethanesulphonic acid; Pipes, piperazine-*NN'*-bis-(2-ethanesulphonic acid); butyl-PBD, 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole.

Structures (I) and (II)

Fig. 1. *U.v. spectrum of (a) 50 μ M-thioITP and (b) 50 μ M-thioGTP in 0.1 M-acetic acid adjusted to pH 4.5 with NaOH and in 0.1 M-sodium carbonate adjusted to pH 10.2 with HCl*

The solutions also contained 0.1 mM-dithiothreitol. Note the change of wavelength scale at 350 nm.

Morales, 1970) and it also quenches protein fluorescence (Bagshaw *et al.*, 1972). These properties were utilized by Trentham *et al.* (1972) to study the heavy-meromyosin ATPase. They showed that the dissocia-

tion rate constant of thioIDP release from a thioIDP-protein complex is greater than the turnover rate of the Mg^{2+} -dependent thioITPase and that the rate of the spectral change when thioITP was mixed with the

protein is much greater than the turnover rate. Eqn. (1) is a plausible mechanism for the thioITPase, where ATP and ADP are replaced by thioITP and thioIDP respectively. This proposed thioITPase mechanism is tested here by analysing the kinetics of [γ - 32 P]thioITP cleavage on the enzyme to see if this process is more rapid than the turnover rate, as is the case with the ATPase, and also by looking for evidence of a two-step nucleotide-binding process. On the basis of these studies and with additional kinetic analysis using thioGTP as substrate, a thioGTPase mechanism is proposed.

The absorption maximum of the neutral thione form of thioguanosine (Fox *et al.*, 1958; Broom & Milne, 1975) is at 342 nm and that of the anion ($pK_{8.24}$) is at 320 nm. This means that detailed analysis of the spectral changes associated with the binding of thioGTP to subfragment 1 is possible, since the major part of the chromophore is clear of protein absorption. Further, modification of the 5'-triphosphate moiety of thioGTP can alter the interaction of the nucleotide with the protein in such a way that analogues of intermediates of the thioGTPase which may normally only occur transiently are formed with a sufficient lifetime for a detailed spectral characterization to be made. In this way the spectral properties of different intermediates of the thioGTPase are compared. Comparisons are also made between the environment of the nucleotide-binding site on the protein and various solvent media based on the extent of the red shift of the chromophore and the perturbation of the thioguanosine pK .

A reason for using ATP analogues such as thioITP and thioGTP is that it provides one approach to study the ATPase mechanism in more organized systems such as glycerinated or skinned muscle fibres and to follow the relative population of the different intermediates during muscle contraction. The ability of the analogues to support contraction in fibres is tested.

Materials and Methods

Solvents

Diethyl ether (A.R. grade) and dimethylformamide were dried by storage over molecular sieves (4A; BDH Chemicals Ltd., Poole, Dorset, U.K.). Dioxan (A.R. grade) was passed through a column of Woelm basic alumina (activity grade 1) to remove organic peroxides which rapidly oxidize thionucleotides. It was then stored over molecular sieves 13X. Pyridine (A.R. grade) was heated under reflux over CaH_2 overnight and then distilled and stored over molecular sieves 4A. Triethylammonium bicarbonate was prepared as a 1 M solution by passing CO_2 through a mixture of 140 ml of freshly distilled triethylamine and 500 ml of water at 0°C until the pH fell to 7.6. The volume was then made up to 1 litre with water.

Reagents

Diphenyl phosphorochloridate, phosphorus oxychloride, tri-*n*-butylamine and triethyl phosphate were obtained from BDH Chemicals Ltd. 1,1'-Carbonyldi-imidazole (R. N. Emanuel Ltd., Wembley, Middx., U.K.) is very susceptible to hydrolysis and batches varied considerably. The melting point (115.5–116°C; Staab, 1957) and i.r. spectrum in chloroform (carbonyl peak 1760 cm^{-1}) were checked before use. Tri-*n*-octylamine was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and any insoluble carbonates formed during storage were filtered off before use. Sodium imidodiphosphate was prepared as described by Yount *et al.* (1971) by using diphenylimidodiphosphoric acid previously recrystallized from ethanol/water. This was prepared from PCl_5 and diphenylphosphoroamidate (Koch-Light; recrystallized from chloroform) as described by Kirsanov & Zhmurova (1958).

Thioinosine and thioguanosine were purchased from Cambrian Chemicals Ltd., Croydon, U.K., papain from Sigma Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K., and other enzymes from Boehringer Corporation Ltd., Ealing, London W.5, U.K.

Analytical methods

Nucleotide preparations were checked as a routine in a high-voltage-electrophoresis system by using Whatman no. 1 paper, 0.5% pyridine/5% (v/v) acetic acid, pH 3.5, buffer and a coolant of white spirit. Satisfactory separation of nucleoside mono-, di-, and tri-phosphates was achieved in 90 min with a potential gradient of 60 V/cm. Electrophoresis was also performed in 0.1 M-sodium borate buffer, pH 9.2, on Whatman 3 MM paper by using a flat-bed high-voltage electrophoresis equipment (Locarte, London S.W.7, U.K.). Nucleotides were detected by spraying for phosphate with molybdate reagent after hydrolysis of nucleotides with $HClO_4$ (Brookes *et al.*, 1959) and for ribose with periodate followed by Schiff's reagent (Baddiley *et al.*, 1956). Thionucleotides were also detected with an iodine/ NaN_3 spray (Smith, 1960). Markers of Xylene Cyanol FF, Orange G and Acid Fuchsin (BDH Chemicals Ltd.) were run with all samples and electrophoretic mobilities calculated relative to Orange G being 1.00 (Table 1). T.l.c. on PEI[poly(ethyleneimine)]-cellulose plates (Polygram Cel 300 PEI/ U_{254} ; Macherey, Nagel and Co., Düren, Germany) was used for following the course of phosphorylation reactions by using suitable concentrations of LiCl (Randerath & Randerath, 1964), or 0.75 M- KH_2PO_4 adjusted to pH 3.4 with HCl for elution. Nucleotides were detected as dark spots on a fluorescent background.

Both thioIDP and thioGDP are substrates for pyruvate kinase and so could be assayed enzymically

by measuring the oxidation of NADH in a solution containing 50 mM-imidazole adjusted to pH 7.6 with HCl, 0.1 M-KCl, 1 mM-phosphoenolpyruvate, 0.25 mM-NADH, 10 μ g of pyruvate kinase/ml and 10 μ g of lactate dehydrogenase/ml. Nucleoside 5'-triphosphates were assayed in a similar manner after hydrolysis with myosin.

Concentrations of nucleotides were determined spectrophotometrically in a Zeiss PMQ 11 spectrophotometer (thioinosine, $\epsilon_{320} = 2.80 \times 10^4$ litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$; thioguanosine, $\epsilon_{342} = 2.48 \times 10^4$ litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ at pH 4.5; Fox *et al.*, 1958).

Preparation of nucleotides

ThioIMP and thioGMP were prepared by a direct phosphorylation method of F. Eckstein & R. S. Goody (personal communication) based on the method of Yoshikawa *et al.* (1969). Thioinosine or thioguanosine (1 mmol) was mixed with 5 ml of triethyl phosphate and the suspension was stirred and cooled in ice to 0°C. To the stirred suspension was added 0.6 ml of POCl₃. The reaction was allowed to proceed until t.l.c. showed that most of the nucleoside had reacted and only small amounts of di- and tri-phosphorylated material were present. POCl₃ was then removed *in vacuo* at 25°C for 15 min, excess of ice was added and the solution was stored at 0°C for 30 min. After the removal of water *in vacuo*, the nucleotide was precipitated by the addition of 5 ml of redistilled triethylamine. The white solid was filtered off, dissolved in 30 ml of water, adjusted to pH 7.6 with KOH and applied to a column (45 cm $\times$ 3.5 cm diam.) of DE52 DEAE-cellulose (bicarbonate form) (Whatman Ltd., Maidstone, Kent, U.K.). After washing with water, the column was eluted with a linear gradient of 0–0.3 M-triethylammonium bicarbonate, pH 7.6, total volume 3 litres. The nucleotide was detected by measuring the A_{266} , and the P_i peak, which was eluted just before the 5'-mononucleotide, was detected by the method of Fiske & Subbarow (1925). The major peak of 5'-monophosphate, free of P_i, was evaporated to dryness and the eluting solvent removed by repeated additions and evaporations of methanol. A better separation of thioIMP from P_i was obtained by using a linear gradient of 0–0.3 M-NH₄HCO₃, pH 7.6, as the eluting buffer. Yields of 45–65% were obtained, and the preparation could be increased to a 4 mmol scale without loss of yield. The electrophoretic mobilities of thioIMP and thioGMP are shown in Table 1. Both gave positive reactions for phosphate, ribose and thiol. The absence of any 2'- or 3'-phosphorylated material was indicated by the compounds running as single spots on electrophoresis in borate buffer.

ThioITP and thioGTP were prepared by the carbonyldi-imidazole procedure of Hoard & Ott (1965). A portion (1 mmol) of the monophosphate,

Table 1. *Electrophoretic mobilities of thioinosine and thioguanosine nucleotides at pH 3.5*

The electrophoretic mobilities in 0.5% pyridine/5% acetic acid buffer, pH 3.5, are calculated relative to the mobility of Orange G.

	Mobility
Orange G	1.00
Acid Fuchsin	0.97
Xylene Cyanol FF	0.60
ThioIMP	0.69
ThioITP	1.11
ThioGMP	0.60
ThioGDP	0.88
ThioGTP	1.15
ThioGTP(γ S)	0.78
ThioGMP-P(NH)P	1.02

obtained as the triethylamine salt, was converted into the pyridinium salt by passage through a column (10 cm $\times$ 3 cm diam.) of Merck 1 (Anderman & Co. Ltd., London, S.E.1., U.K.) ion-exchange resin in the pyridinium form [prepared by washing the resin in the H⁺ form with 50% (v/v) pyridine followed by a short wash with water]. Although in principle Dowex 50 might be considered suitable, thio-nucleotides were found to interact with it and not be eluted cleanly from the column. After the nucleotide was eluted from this column, 1 mmol of tri-*n*-butylamine was added to the solution, which was then evaporated to dryness and dried by repeated additions and evaporations of pyridine (three times) and then dimethylformamide (three times). The residue was dissolved in 10 ml of dimethylformamide and 800 mg of 1,1'-carbonyldi-imidazole in 10 ml of dimethylformamide was added. The mixture was shaken for 30 min and stored in a desiccator for 4 h. Then 0.33 ml of methanol was added, and after 30 min, 5 mmol of tri-*n*-butylammonium pyrophosphate (prepared by conversion of tetrasodium pyrophosphate into the tri-*n*-butylammonium salt as described for the nucleoside 5'-monophosphate) in 50 ml of dimethylformamide was added. After 24 h at 20°C, the supernatant was decanted and the precipitate which had formed was washed with 20 ml of dimethylformamide. To the combined supernatant and washings, an equal volume of methanol was added, and the solution evaporated to dryness on a rotary evaporator. The residue was dissolved in 100 ml of water and purified on a DE52 DEAE-cellulose column as described for the monophosphate but with a linear triethylammonium bicarbonate gradient of 0–0.6 M. High-voltage electrophoresis at pH 3.5 showed a single spot for both compounds with a mobility (Table 1) consistent with a nucleoside 5'-triphosphate, and giving positive reactions for phosphate, ribose and thiol. Electrophoresis in borate buffer showed no material modified in the 2'- or 3'-positions. Enzymic analysis gave values of 93%

5'-triphosphate based on absorption measurements. Yields of 25–60% were obtained from the mono-phosphate.

[γ - 32 P]ThioITP was prepared by the method of Glynn & Chappell (1964) after demonstration that thioITP was a substrate for phosphoglycerate kinase. ThioITP (6 μ mol) was equilibrated with 10 mCi of [32 P]P_i for 1 h under the conditions described by Glynn & Chappell (1964), and the [γ - 32 P]thioITP purified in the same way as for [γ - 32 P]ATP. T.l.c. of the product on a PEI-cellulose plate eluted with 0.75 M-potassium phosphate adjusted to pH 3.4 with HCl showed that there was less than 3% [32 P]P_i (which has an identical mobility with AMP) contaminating the [γ - 32 P]thioITP.

ThioGDP and thioGMP-P(NH)P were both prepared by the method of Michelson (1964), although for the latter nucleotide some modifications had to be made to overcome solubility problems. To prepare thioGMP-P(NH)P 0.55 mmol of thioGMP was converted into the pyridinium salt as described above. Then 0.49 ml of tri-*n*-octylamine (a twofold molar excess) was added together with sufficient dimethylformamide to achieve solution. After evaporation to dryness, the residue was dried by repeated additions and evaporations of dimethylformamide (four times). The residue was then dissolved in 3.5 ml of dioxan and to this was added 0.15 ml of diphenyl phosphorochloridate and 0.15 ml of tri-*n*-butylamine. The solution was left for 3 h at 20°C, during which time a very faint precipitate formed. The solvent was then removed under reduced pressure and 25 ml of diethyl ether added. The solution was left at 0°C for 30 min, the ether was decanted off and the remainder removed under reduced pressure. The residue of activated nucleoside 5'-monophosphate was dissolved in 2 ml of hexamethylphosphorotriamide. Sodium imidodiphosphate (0.66 mmol) was converted into the pyridinium salt as described above by using a Merck 1 (pyridinium form) column. The eluate was collected directly into a solution of 1.57 ml of tri-*n*-butylamine in 20 ml of water at 0°C, evaporated to dryness under reduced pressure and dried by repeated additions and evaporations of chloroform. The residue was dissolved in the minimum volume of dimethylformamide (2 ml) and as much pyridine as possible was added without precipitation occurring (4 ml). The activated thioGMP solution was then added dropwise over a period of 10 min, taking precautions to keep the reaction mixture anhydrous. It was left stirring for a further 1 h. Solvents were removed under reduced pressure and the residue was taken up in 30 ml of cold water, adjusted to pH 7.6 with KOH and extracted with ether. The product in the aqueous solution was then purified as described for thioITP and thioGTP. A yield of 18% was obtained. The product ran as a single spot on high-voltage electrophoresis at pH 3.5 with a mobility of 1.02 and after

treatment with alkaline phosphatase ran with a mobility of 0.78, both spots giving positive reactions for phosphate, ribose and thiol. This decrease in mobility is consistent with the loss of a single phosphate group from thioGMP-P(NH)P to give thioGMP-P(NH₂) (Yount *et al.*, 1971). ThioGTP(γ S) was a gift from Dr. R. S. Goody.

Proteins

Preparation of subfragment 1 was based on that described by Lowey *et al.* (1969). Myosin from rabbit skeletal muscle was digested by papain in a solution containing 10 mg of myosin/ml, 0.1 mg of papain/ml, 0.6 M-KCl, 2 mM-EDTA, 1 mM-dithiothreitol, 10 mM-potassium phosphate, pH 6.5, for 7 min at 22°C. The reaction was stopped by the addition of iodoacetic acid at pH 6.5 to give a final concentration of 0.5 mM. Under these conditions no alkylation of myosin or its proteolytic fragments occurs (A. G. Weeds, personal communication). The solution was cooled and dialysed against 10 vol. of cold water overnight. The precipitate was removed and the supernatant applied to a column (25 cm $\times$ 3.5 cm diam.) of DEAE-cellulose (Cl⁻ form) and eluted with a linear gradient of 0–0.25 M-KCl in 50 mM-Tris adjusted to pH 8.0 with HCl. The initial protein peak was characterized as subfragment 1 (Lowey *et al.*, 1969). It was concentrated in an Amicon ultrafiltration cell fitted with a UM10 membrane to a concentration of about 10 mg/ml and dialysed against the appropriate buffer.

Heavy meromyosin was prepared by the method of Lowey & Cohen (1962).

Concentrations of subfragment 1 and heavy meromyosin were determined as described by Bagshaw & Trentham (1973).

Spectroscopic equipment

The stopped-flow spectrophotometer used for following absorption changes was the split-beam instrument described by Gutfreund (1972), and that for following protein fluorescence was the instrument described by Whitaker *et al.* (1974). Absolute spectra of nucleotides and protein-nucleotide complexes were measured with a Perkin-Elmer 402 u.v. scanning spectrophotometer and difference spectra were measured by using 2 $\times$ 4.5 mm-path-length tandem cells in a Unicam SP.1800 spectrophotometer. The p*K* determination described in Fig. 15 was carried out with the equipment described by Shore *et al.* (1975).

The quenched-flow apparatus used has been described by Gutfreund (1969). Measurements of the effect of thionucleotides on mechanical properties of glycerinated rabbit psoas-muscle fibres were carried out in the A.R.C. Unit of Insect Physiology, Department of Zoology, Oxford, U.K., by using the apparatus described by Ruegg & Tregear (1965).

Results

ThioITPase and thioGTPase mechanisms of subfragment 1

A central feature of the Mg^{2+} -dependent ATPase mechanism of subfragment 1 is that the predominant intermediate in the steady state is a protein-products complex. An experiment was performed to find out if the corresponding intermediate was present in the Mg^{2+} -dependent thioITPase mechanisms. $[\gamma\text{-}^{32}\text{P}]$ -

Fig. 2. Rate of transient production of $M^{**}\cdot\text{thioIDP}\cdot P_i$ followed by measurement of $[\text{}^{32}\text{P}]P_i$ production in the reaction of $10\mu\text{M}$ -subfragment 1 with $50\mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{thioITP}$ in the quenched-flow apparatus at 20°C

Both syringes contained 0.1M-KCl , 5mM-MgCl_2 , 0.1mM -dithiothreitol, 50mM -Tris adjusted to $\text{pH}8.0$ with HCl . The reaction mixture was quenched into an equal volume (3.3ml) of ice-cold 10% (w/v) HClO_4 containing $200\mu\text{M}$ - P_i to act as a carrier and to prevent $[\text{}^{32}\text{P}]P_i$ adsorption on to denatured protein. The separation of $[\text{}^{32}\text{P}]P_i$ was based on the method of Martin & Doty (1948), except that a double extraction was performed. All the extraction steps were carried out at 0°C and within 5 min to prevent $[\text{}^{32}\text{P}]P_i$ production arising from thioITP hydrolysis in the acid medium. Then 5ml of the final 2-methylpropan-1-ol/benzene extract was mixed with 10ml of butyl-PBD scintillator and $[\text{}^{32}\text{P}]P_i$ was counted by using a liquid-scintillation counter. The butyl-PBD scintillation fluid was as described by Bagshaw *et al.* (1972). The points are the experimental values of $[\text{}^{32}\text{P}]P_i$ production over the range $0\text{--}40\text{ms}$. The solid lines represent theoretical time-courses of $[\text{}^{32}\text{P}]P_i$ production if it is limited by the second-order binding process (this process had a pseudo-first-order rate constant of 20s^{-1} determined by following an identical reaction mixture in a stopped-flow apparatus) and the stoichiometry of transient $[\text{}^{32}\text{P}]P_i$ production is 1.0 (upper line) and 0.8 (lower line) mol of $[\text{}^{32}\text{P}]P_i$ /mol of subfragment 1. ----, Steady-state rate of hydrolysis of thioITP obtained from measurements of $[\text{}^{32}\text{P}]P_i$ production over the time-range $0\text{--}8\text{s}$.

ThioITP was mixed with subfragment 1 in the quenched-flow apparatus and the reaction was sampled at different times. Fig. 2 shows the rate of P_i formation. This rate is too rapid to be accounted for by the steady-state rate (see dashed line in Fig. 2). It does, however, follow the time-course expected if the P_i production occurred at the same rate as the thioITP absorption change on mixing thioITP with the protein and if there was transient P_i formation equivalent to between 0.8 and 1.0 mol of P_i /mol of subfragment 1 (see solid lines in Fig. 2). The value of 0.8 is typical of the transient amplitude observed in the heavy-meromyosin ATPase (Lymn & Taylor, 1971). The stoichiometry is likely to be less than 1 because of the possible presence of some catalytically inactive protein and because of the reversibility of the cleavage step (Bagshaw & Trentham, 1973; Trentham *et al.*, 1976). It is probable that a subfragment 1-products complex analogous to $M^{**}\cdot\text{ADP}\cdot P_i$ is the predominant intermediate of the thioITPase steady state.

It has been shown kinetically that the binding of ATP to subfragment 1 is a two-step process (Bagshaw *et al.*, 1974; Koretz & Taylor, 1975). The main evidence for this is that the rate constant of the observed protein-fluorescence enhancement which is an exponential process does not increase linearly with substrate or substrate-analogue concentration. It was difficult to test for the same binding model by using thioITP and following protein fluorescence quenching, because of the inner filter effects generated by the high thioITP concentrations required for the experiment. However, the observed rate constant of the fluorescence quenching did not increase linearly with thioITP concentration (Fig. 3), and so a two-step binding mechanism is likely for this substrate also. Nevertheless this conclusion is made with reservations because of the restriction on the maximum thioITP concentration that could be used and the lower signal-to-noise ratio of the fluorescence change at high thioITP concentration. It was not possible to characterize spectrally an intermediate analogous to $M\cdot\text{ATP}$, because the inner-filter effect prevented accurate absolute protein-fluorescence measurements at high thioITP concentrations.

These results, together with the earlier finding (Trentham *et al.*, 1972) that the thioIDP dissociation rate from a binary protein-thioIDP complex is more rapid than the thioITPase catalytic-centre activity, indicate that the mechanism of the thioITPase is similar to that of the ATPase (eqn. 1). A two-step binding process for thioITP, and hence probably thioIDP, is also indicated. The presence or otherwise of weakly bound intermediate complexes analogous to $M\cdot\text{ATP}$ and $M\cdot\text{ADP}$ is not crucial to the difference-spectra work described below.

The steady-state parameters of the Mg^{2+} -dependent subfragment-1 thioGTPase were measured by using a

Fig. 3. Stopped-flow records of the fluorescence of subfragment 1 when mixed with thioITP at 20°C

One syringe contained 4 μM -subfragment 1 and the other thioITP at (a) 54 μM and (b) 275 μM (reaction-chamber concentrations). Both syringes contained 50 mM-KCl, 2 mM-MgCl₂, 0.3 mM-dithiothreitol, 20 mM-Tris adjusted to pH 8.0 with HCl.

Fig. 4. Hydrolysis of thioGTP catalysed by subfragment 1 at 20°C in 50 mM-Tris adjusted to pH 8.0 with HCl

From a plot of velocity (v) against $v/[\text{thioGTP}]$, the catalytic-centre activity and K_m for thioGTP were calculated. The reaction mixture contained 74 μM -NADH, 0.37 mM-phosphoenolpyruvate, 0.1 mg of pyruvate kinase/ml, 0.1 mg of lactate dehydrogenase/ml, 5 mM-MgCl₂, 0.1 M-KCl and 1.3 μM -subfragment 1. The decrease in A_{340} was measured and the reaction was initiated by the addition of thioGTP to a final concentration of 3.1–100 μM .

linked-assay system (Fig. 4). At pH 8 and 20°C the K_m for thioGTPase was 22 μM and the catalytic-centre activity was 0.35 s^{-1} . As with thioITP (Trentham *et al.*, 1972) intermediate states of the thioGTPase were observed by following nucleotide-absorption changes or protein-fluorescence changes during a single turnover of subfragment 1 (Fig. 5) (i.e. the protein concentration was greater than that of the nucleotide). To relate the mechanism of the thioGTPase to the spectral characterization of intermediates described below, the kinetic parameters were measured at pH 6.5 at both 5° and 20°C. These results are summarized in Table 2. Apparent second-order association rate constants, k_f , were determined from the slope of graphs of the observed exponential rate constant versus the nucleotide concentration. Values for k_r , the apparent dissociation rate constants, could be determined for the non-hydrolysable substrates thioGDP and thioGMP-P(NH)P from the ordinate intercept of the above graphs. k_r was, however, determined with more precision by measuring the rate at which ATP displaced the thionucleotide under conditions for which the rate was independent of ATP concentration (Fig. 6). Equating the rate constant for this process (Fig. 6) with k_r is further justified because the amplitude of the signal indicated total thionucleotide displacement. The catalytic-centre activity, k_{cat} , of thioGTP was calculated from

Fig. 5. Stopped-flow record of the fluorescence of subfragment 1 when mixed with thioGTP at 20°C

One syringe contained 10 μM -subfragment 1 and the other 8 μM -thioGTP (reaction-chamber concentrations). Both syringes contained 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol, 50 mM-Tris adjusted to pH 8.0 with HCl.

steady-state measurements by using the linked-assay system (Table 2). With thioGTP(γ S) the linked-assay system was not appropriate since thioGTP would be generated during the course of the assay. So for thioGTP(γ S) the rate of the slower exponential

Table 2. Kinetic parameters of the interaction of thioguanosine nucleotides with subfragment 1 at pH6.5

At 5°C the kinetics were followed in the absorption stopped-flow spectrophotometer monitoring nucleotide absorption at 352nm. At 20°C measurements were carried out in the stopped-flow fluorimeter and protein fluorescence was monitored. The solvent was 0.1 M-KCl/5 mM-MgCl₂/0.1 mM-dithiothreitol/100 mM-Mes adjusted to pH6.5 with KOH at 20°C. Methods for calculating k_f and k_r , the apparent association and dissociation rate constants, and k_{cat} , are described in the text.

Temperature	Analogue	$10^{-4} k_f$ (M ⁻¹ ·s ⁻¹)	k_r (s ⁻¹)	$K_{diss.} (=k_r/k_f)$ (μM)	$k_{cat.}$ (s ⁻¹)
5°C	ThioGTP	1.0			0.04
	ThioGTP(γS)	1.6			<0.0006
	ThioGDP	1.0	0.5	50	
20°C	ThioGTP	16			0.6
	ThioGTP(γS)	17			0.009
	ThioGMP-P(NH)P	0.8	0.055	6.7	
	ThioGDP	16	6.0	38

Fig. 6. Spectrophotometric records of protein fluorescence during the binding of thioGMP-P(NH)P to subfragment 1 and the displacement of thioGMP-P(NH)P from subfragment 1 with ATP at 20°C

(a) One syringe contained 2 μM-subfragment 1 and the other 51 μM-thioGMP-P(NH)P. (b) One syringe contained 2 μM-subfragment 1 and 13 μM-thioGMP-P(NH)P and the other 250 μM-ATP. All syringes contained 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol and 50 mM-Mes adjusted to pH 6.5 with KOH.

process in a single turnover experiment (e.g. as in Fig. 5) was used to evaluate k_{cat} . The rate constant of this process probably equals the rate of cleavage of $M^* \cdot \text{thioGTP}(\gamma S)$ [by analogy with ATP(γS); Bagshaw *et al.*, 1972] and is likely to equal k_{cat} , since the subsequent thioGDP dissociation process is controlled by a larger rate constant (see Table 2). Another approach used to calculate k_{cat} of both thioGTP and thioGTP(γS) was by measuring the rates of total dis-

Fig. 7. Stopped-flow spectrophotometric record of protein fluorescence during the displacement of thioGTP and thioGTP(γS) from their steady-state intermediates with subfragment 1 by ATP at 20°C

(a) One syringe contained 1.2 μM-subfragment 1 and 100 μM-thioGTP, and the other 1 mM-ATP. (b) One syringe contained 1.2 μM-subfragment 1 and 50 μM-thioGTP(γS) and the other 1 mM-ATP. The syringes also contained 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol and 50 mM-Mes adjusted to pH 6.5 with KOH. Both reactions were started within 2 min of mixing thioGTP or thioGTP(γS) with subfragment 1. In (b) the oscilloscope was allowed to run in continuous mode after the initial triggering in order that the complete reaction could be observed.

placement of thionucleotides from the proteins when present as their steady-state intermediates by the addition of excess of ATP (Fig. 7). In principle the observed rate constants will be the sum of k_{cat} , and an

apparent dissociation rate of uncleaved thioGTP or thioGTP(γ S). In practice the observed rate constants equalled the previously determined $k_{\text{cat.}}$ values, indicating that the apparent dissociation rate constants of uncleaved nucleotides are smaller than $k_{\text{cat.}}$. It is noteworthy how small $k_{\text{cat.}}$ of the thioGTP(γ S)-catalysed hydrolysis is at 5°C (Table 2). In the work described below this property is exploited, since the easiest way to complex all the nucleotide with subfragment 1 and have virtually no free nucleotide in the medium for times up to 5 min is through the use of thioGTP(γ S).

Spectrum of thioguanosine and its nucleotides

The pK of the thiol group of thioguanosine and its nucleotides was determined by measuring their spectra over a range of pH values and calculating the relative concentrations of the neutral (R-SH) and anionic (R-S⁻) forms present at each pH from the absorption at 342 nm and 320 nm and plotting $\log([R-S^-]/[R-SH])$ against pH (Fig. 8). The results are shown in Table 3 for the compounds both alone and in the presence of 5 mM-MgCl₂. The pK of the thiol group of thioguanosine was calculated to be

Fig. 8. Determination of the pK of thioGTP in the absence (●) and presence (○) of 5 mM-MgCl₂ at 20°C

The spectra of identical concentrations of thioGTP were measured in 50 mM-Tris/HCl/0.1 M-KCl/0.1 mM-dithiothreitol between pH 7.8 and 8.7. The ratio of neutral to anionic form was calculated from a knowledge of the absorption of the same concentration of thioGTP in 0.1 M-acetic acid adjusted to pH 4.5 with NaOH, and 0.1 M-Na₂CO₃ adjusted to pH 10.2 with HCl.

8.24, compared with the value determined by Fox *et al.* (1958) of 8.35 in different salt conditions. The intensity of the 342 nm peak decreased below pH 4.5, indicating the existence of a second pK presumably associated with protonation of the thioguanine ring. The spectra of the neutral and anionic forms of the thioguanosine nucleotides showed no differences from those of thioguanosine itself, although the pK values were higher (Table 3). MgCl₂ did not affect the spectrum of the neutral forms of thioguanosine or its nucleotides, or the pK of thioguanosine. However, it did decrease the pK of the thiol group of thioguanosine nucleotides. The effect of MgCl₂ on the anionic forms of the nucleotides was not studied because of Mg(OH)₂ precipitation.

The effect of solvent on the spectra of the thioguanosine nucleotides was then studied in order to understand the spectral perturbation of the nucleotides on binding to subfragment 1 in terms of the environment of the chromophore. Two effects were measured, spectral perturbations of the neutral and anionic forms of the nucleotide, and pK perturbations of the thiol group.

The thione form of thioguanosine has an absorption peak at 342 nm, with a slight shoulder at 335 nm (Fig. 1). In increasing concentrations of propan-2-ol, the absorption peak undergoes a red shift, the difference spectrum showing peaks at 328, 338 and 352 nm. The intensity of these peaks is approximately proportional to the propan-2-ol concentration. Although the thioguanosine nucleotides undergo a similar red shift in increasing concentrations of propan-2-ol, this is not a linear effect and the intensity of the 338 nm difference-spectrum peak undergoes relatively large changes over small changes in the fraction of propan-2-ol, particularly in the range 60–80% (Figs. 9 and 10).

The anionic form of thioguanosine and its nucleotides also undergoes a red shift with increasing proportions of propan-2-ol, although this is more of a broadening than a simple shift of $\lambda_{\text{max.}}$

Methanol was used to study solvent effects on the pK of thioguanosine. This solvent was chosen because its effect on the pH of standard buffers has been documented by Bates *et al.* (1963). The pK increased

Table 3. pK of the thiol group of thioguanosine nucleotides (solvent as in legend to Fig. 8)

	pK in the absence of Mg ²⁺	pK in the presence of 5 mM-Mg ²⁺
Thioguanosine	8.24	8.24
ThioGMP	8.68	8.60
ThioGDP	8.73	8.52
ThioGTP	8.57	8.18
ThioGMP-P(NH)P	8.72	8.55

Fig. 9. Change in the molar extinction coefficient of thioGMP-P(NH)P with increasing propan-2-ol concentration

The values for changes in molar extinction coefficients at 328 (○), 338 (●) and 352 (◐) nm were calculated from difference spectra of a 37 μM solution of thioGMP-P(NH)P in solutions containing 10 mM-acetic acid adjusted to pH 4.5 with NaOH, 0.1 mM-dithiothreitol and increasing fractions of propan-2-ol at 20°C.

approximately linearly over the range 0–68% (w/w) methanol by 0.7 pH unit (Fig. 11).

Absorption spectra of the thioguanosine nucleotide-subfragment 1 complexes

ThioGMP-P(NH)P binds to subfragment 1 to form a binary complex. The absorption spectrum of the thioguanosine chromophore in this complex was measured at pH 6.5 (when over 99% of thioguanosine is in the thione form) by mixing equal volumes of subfragment 1 and thioGMP-P(NH)P in a tandem cell. Fig. 12(a) shows the spectrum before and after mixing these solutions. The observed red shift is shown as a difference spectrum in Fig. 12(b). Under the conditions of this experiment, 92% of the thioGMP-P(NH)P is bound to subfragment 1.

Although thioGTP(γS) is hydrolysed by subfragment 1, the turnover rate ($<6 \times 10^{-4} \text{ s}^{-1}$) at 5°C is sufficiently slow for the spectrum of the steady-state intermediate to be measured. Fig. 13 shows the

Fig. 10. Difference spectrum between 37 μM-thioGMP-P(NH)P in water and in 70% propan-2-ol. Solvent conditions are as described in Fig. 9.

Fig. 11. Effect of methanol on the pK of thioguanosine

The pK values were determined by measuring the spectra of a 30 μM solution of thioguanosine in the methanolic Tris, acetate and carbonate buffers described by Bates *et al.* (1963).

spectrum of this intermediate formed after mixing 30 μM-subfragment 1 with 27 μM-thioGTP(γS) at 5°C. After the solution had been allowed to warm up to 20°C, the spectrum was identical with that formed on mixing subfragment 1 with thioGDP. Identical

Fig. 12. Spectral perturbation on binding thioGMP-P(NH)P to subfragment 1

(a) Spectrum of thioGMP-P(NH)P before (A) and after (B) binding to subfragment 1 at pH 6.5 at 20°C. One side of a 2×4.5 mm-path-length tandem cell contained 106 μM-subfragment 1 and the other side 33 μM-thioGMP-P(NH)P (concentrations after mixing). Solvent conditions were 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol, 0.1 M-Mes adjusted to pH 6.5 with KOH. The spectra were measured with water in the reference beam. The baseline is the absorbance of the protein alone. (b) Difference spectrum between the solutions before and after mixing the contents of the tandem cell. Note the change in wavelength scale at 350 nm in (a).

Fig. 13. Difference spectrum between thioGTP(γS) and the subfragment-1-thioGTP(γS) steady-state intermediate at 5°C and pH 6.5

One side of a 2×4.5 mm-path-length tandem cell contained 30 μM-subfragment 1 and the other side 27 μM-thioGTP(γS) (concentrations after mixing). Spectra were recorded (a) before and (b) after mixing contents of cell, (c) after leaving the cell at 25°C for 5 min. Solvent conditions were as in Fig. 12.

spectra were obtained when subfragment 1 was replaced by heavy meromyosin containing the same concentration of subfragment-1 heads.

ThioGTP is hydrolysed too rapidly even at 5°C for the steady-state intermediate spectrum to be measured by conventional means. However, by using a regenerating system of pyruvate kinase and phosphoenolpyruvate, this intermediate could be formed in a high enough concentration and for a sufficient time for measurements to be made (Fig. 14). The same difference spectrum was found on repeating the experiment at 5°C. After total thioGTP hydrolysis the spectrum reverted to that formed on mixing thioGDP with subfragment 1. No spectral changes were found on mixing thioGTP with pyruvate kinase and phosphoenolpyruvate.

The perturbation in the pK of the thiol group of thioGMP-P(NH)P on binding to subfragment 1 was measured by following the absorbance at 344 nm with change in pH (Fig. 15). Under the reaction conditions 83% of the nucleotide was bound to the protein at pH 8, since the dissociation constant, determined kinetically as in Table 2, was 19 μM. The nucleotide binding caused a pK increase of between 0.2 and 0.3. The experiment described in Fig. 15 has the technical problem that the protein is unstable at high pH values. Consequently the relatively high absorbance of the protein plus nucleotide solution at pH 10 is probably due in part to turbidity of the protein solution. The spectra of thioGMP-P(NH)P bound to

Fig. 14. Difference spectrum between thioGTP and the subfragment 1-thioGTP steady-state intermediate at 20°C and pH 6.5

One side of a 2×4.5 mm-path-length tandem cell contained 30 μM-subfragment 1 and 3 μM-pyruvate kinase, and the other side 38 μM-thioGTP. Both sides also contained 1 mM-phosphoenolpyruvate, 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol and 50 mM-Mes adjusted to pH 6.5 with KOH. Spectra were recorded (a) before and (b) immediately after mixing contents of cell.

Fig. 15. Titration curve of thioGMP-P(NH)P in the absence and presence of subfragment 1 at 20°C

The 1 cm-path-length cuvette contained 5 mM-Tris/HCl, 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol and 24 μM-thioGMP-P(NH)P (lower curve). The pH was increased by the addition of KOH. The upper curve is an identical experiment in the presence of 203 μM-subfragment 1.

subfragment 1 at pH 8.22 and 8.48 are also consistent with a pK increase of 0.2 of the thionucleotide on forming the binary complex (Fig. 16). In addition these spectra show an overall red shift after complex-formation, which indicates that the active site retains

Fig. 16. Spectrum of thioGMP-P(NH)P before and after binding to subfragment 1 at pH 8.22 and 8.48 at 20°C

One side of a 2×4.5 mm-path-length tandem cell contained 106 μM-subfragment 1 and the other side 33 μM-thioGMP-P(NH)P. (a) Spectrum of subfragment 1 and buffer; (b) spectrum of subfragment 1 and thioGMP-P(NH)P before mixing; (c) spectrum of subfragment 1 and thioGMP-P(NH)P after mixing. Solvent conditions were 0.1 M-KCl, 5 mM-MgCl₂, 0.1 mM-dithiothreitol and 0.1 M-Tris adjusted to pH 8.22 or 8.48 with HCl. No change in pH occurred on mixing thioGMP-P(NH)P with subfragment 1.

its hydrophobic environment when the pH changes from 6.5 to 8.

Effect of thioGTP on glycerinated muscle fibres

The ability of thioGTP to relax and contract glycerinated rabbit psoas-muscle fibres was measured in order to assess the practicability of extending the kinetic and spectral work described above to this system. Measurements were made on bundles of approx. 20–30 fibres in a buffer containing 50 mM-KCl, 5 mM-MgCl₂, 0.5 mM-EGTA, 1.5 mM-Na₃N and 5 mM-Pipes adjusted to pH 7.1 with HCl. CaCl₂ was added to give a μM solution of free Ca²⁺.

In the presence of Ca²⁺, 1 mM-thioGTP caused an increase in tension of the fibres to a value of 50% of that when the same fibres were placed in 1 mM-ATP. However, in the absence of Ca²⁺, unlike ATP, 1 mM-thioGTP did not cause a decrease in the stiffness of the fibres. Further, rapidly decreasing the length of these fibres by 10% was followed by an increase in tension. This effect could be comparable with that described by Bremel & Weber (1972), who showed that when some actin molecules in a thin filament containing the regulatory proteins have formed rigor complexes, then the remaining actin monomers not combined with myosin are altered so that they are able to interact with myosin in the presence of ATP at concentrations of Ca²⁺ much lower than 1 μM. They found that this occurred at very low concentrations of ATP. That these results are applicable to the events

in muscle contraction was supported by the studies of Godt (1974) on the tension development of skinned frog fibres at low ATP concentrations. The much greater (>100-fold) K_m value of thioGTP for myosin than of ATP may cause the same effect at even 1 mM-thioGTP.

Discussion

The intermediates involved in the hydrolysis of ATP by subfragment 1 of myosin have been characterized kinetically (Bagshaw & Trentham, 1974). The results described above suggest that the hydrolyses of thioITP and thioGTP proceed by the same mechanism, although rate constants of interconversion between intermediates are different. Modification of the triphosphate moiety enables intermediates of the reaction mechanism to be formed at high enough concentrations and for sufficient time for the spectral characterization of these intermediates to be made.

The steady-state intermediates formed during the hydrolysis of thioITP by subfragment 1 has been shown to be a protein-products complex analogous to the $M^{**}\cdot ADP\cdot P_i$ intermediate of the scheme proposed by Bagshaw & Trentham (1974). Since the cleavage step of the hydrolysis of ATP has an equilibrium constant of 9, the species $M^*\cdot ATP$ is also present in the steady state at 10% of the concentration of $M^{**}\cdot ADP\cdot P_i$ (Bagshaw & Trentham, 1973). Similarly it is probable that $M^*\cdot thioITP$ and $M^*\cdot thioGTP$ are present at significant concentration during the hydrolysis of thioITP and thioGTP. In fact, transient kinetic studies at pH 6.5 with $[\gamma\text{-}^{32}P]$ thioGTP show that $M^*\cdot thioGTP$ is the predominant steady-state intermediate of the thioGTPase (J. F. Eccleston & D. R. Trentham, unpublished work, note added in proof 6 December 1976). At 5°C and pH 8 the ratio of the rate constants controlling the transformations $M^{**}\cdot ADP\cdot P_i$ into $M^*\cdot ADP\cdot P_i$ and $M^*\cdot ADP$ into $M\cdot ADP$ were such that $M^*\cdot ADP$ was present as a significant component of the steady-state intermediate. However, comparable rates for thioGTP hydrolysis (Table 2) showed that $M^*\cdot thioGDP$ is not a significant intermediate even at 5°C.

Subfragment 1 catalyses the hydrolysis of $ATP(\gamma S)$ and the rate-limiting step is that involving cleavage of $M^*\cdot ATP(\gamma S)$ (Bagshaw *et al.*, 1972). We assume that the catalysis by subfragment 1 of thioGTP(γS) hydrolysis is also rate-limited by the cleavage reaction.

Considering the subfragment-1-catalysed hydrolysis of thioGTP in terms of eqn. (1), chromophoric analogues of three of these intermediates (namely $M^*\cdot ATP$, $M^{**}\cdot ADP\cdot P_i$ and $M^*\cdot ADP$) can be studied. ThioGTP(γS) and thioGMP-P(NH)P bound to the protein probably form analogues of the $M^*\cdot ATP$ intermediate. The steady-state intermediate of thioGTP hydrolysis probably simulates a mixture

of $M^*\cdot ATP$ and $M^{**}\cdot ADP\cdot P_i$, and the binary thioGDP-subfragment-1 complex corresponds to $M^*\cdot ADP$. The spectral characteristics of the thioguanosine moiety in these intermediates in the 300–400 nm range then allows one in principle to deduce something of the nature of the subfragment-1 ATPase active site.

Fig. 12 shows that a red shift in the spectrum of the thione occurs when thioGMP-P(NH)P binds to subfragment 1 and this is characterized by negative difference-spectrum peaks at 328 nm and 338 nm and a positive difference-spectrum peak at 352 nm. Similar spectra are seen when thioGDP binds to subfragment 1 and in the thioGTP and thioGTP(γS) steady-state intermediates. The most accurate measurement of the molar extinction changes of this spectral perturbation can be obtained from the thioGTP(γS)-subfragment-1 complex, since this nucleotide is bound sufficiently tightly so that corrections owing to unbound nucleotide need not be made. From Fig. 13, the molar extinction changes are 1.8×10^3 , 1.5×10^3 , and $3.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 328, 338 and 352 nm respectively.

Perturbation of the spectrum of thioguanosine nucleotides in propan-2-ol gives a similar difference spectrum at about 70% propan-2-ol. It should be noted that the relative intensity of the 328 and 338 nm difference-spectrum peaks is very dependent on the fraction of propan-2-ol in this range. At lower fractions of propan-2-ol, the 338 nm difference peak rapidly diminishes to zero and at higher fractions this peak increases to a value greater than the 328 nm peak. The spectrum of the thioguanosine nucleotides when bound to subfragment 1 is similar to that when they are dissolved in 70% propan-2-ol. In both cases a red shift of the spectrum occurs, which is a result of increased polarizability of the environment of the chromophore, whether by the solvent effect of propan-2-ol or by a hydrophobic binding site for the nucleotide on the protein (Yanari & Bovey, 1960). However, this red shift is not linear with increasing proportion of propan-2-ol (Fig. 9) and other effects are likely to contribute to the spectral perturbation. Phenomenologically the results are similar to those of Murphy & Morales (1970), who studied the heavy-meromyosin-thioITP interaction. Cheong *et al.* (1969) have proposed that the absorption band of thioguanosine in the region of 340 nm is the result of at least two transitions, a $\pi \rightarrow \pi^*$ intramolecular charge-transfer transition and a weaker $n \rightarrow \pi^*$ transition. A detailed analysis of the effect of propan-2-ol on the spectrum of thioguanosine must take solvent effects of both of these transitions into account, together with the possibility of other tautomeric structures which may become stabilized in propan-2-ol solutions and of base stacking of the nucleotides. These effects have been studied by using circular-dichroism measurements, and have shown

that at the nucleotide concentrations used, base stacking is only important in solutions containing over 70% propan-2-ol (J. F. Eccleston & P. M. Bayley, unpublished work).

Sawada *et al.* (1973) have made spectral measurements of the complex between ribonuclease T_1 and a series of thioguanosine nucleotides and observed similar difference spectra to those of thioGMP-P(NH)P, thioGTP(γ S), thioGTP and thioGDP bound to subfragment 1. Ribonuclease T_1 is highly specific for guanosine residues and the replacement of an oxygen atom at the 6-position with a sulphur atom prevents hydrolysis of the nucleotides by this enzyme. It is perhaps surprising that such a binding site gives a similar spectral perturbation on binding nucleotide to that of subfragment 1, which has a low specificity for the purine moiety of nucleoside triphosphates in the hydrolysis reaction, though the specificity for ATP is greater in the functioning of muscle contraction.

Although the spectrum of the thione form of thioguanosine nucleotides on binding to subfragment 1 is similar to that in 70% propan-2-ol, the pK of the thiol group of those nucleotides is smaller than would be expected from the experiment showing the effect of methanol on the pK of thioguanosine. One possible explanation of this result is that a cationic group is present in the active site of myosin which suppresses the expected increase in the pK of the thiol group of the nucleotide on binding to subfragment 1. Takashina (1970) claimed to have demonstrated the presence of a lysine residue in the active site of myosin which could give rise to this effect. Nevertheless, although it is noteworthy that the red shift in the thioguanosine chromophore occurs at both pH 6.5 and 8, no firm conclusions can be drawn about the nature of the purine-binding domain in the protein.

For the most part changes induced by binding ATP, ADP or their analogues to myosin and its proteolytic subfragments have concentrated either on structural perturbations of the protein, as reflected by protein spectral changes (Morita, 1967; Gratzer & Lowey, 1969; Werber *et al.*, 1972; Mandelkow & Mandelkow, 1973; Seidel & Gergely, 1973; Murphy, 1974) or changes in chemical reactivity of amino acid side chains (Watterson & Schaub, 1973) or kinetic characteristics of the reaction (Bagshaw & Trentham, 1974; Koretz & Taylor, 1975). When comparisons of tri- and di-phosphate interactions have been made, the structural perturbations have in most cases shown differences and occasionally this has led to proposals in which the ligand-protein isomerizations as identified in eqn. (1) have been ascribed important roles in terms of conformation changes in the contractile cycle. Although this may be true, the spectral evidence so far obtained provides little evidence for or against, since it does not provide information about the spatial separation of components of the myosin

molecule (but cf. Bagshaw & Reed, 1976; Burke *et al.*, 1976).

The kinetic association rate constants of di- and tri-phosphates recorded here and previously (Bagshaw *et al.*, 1974) show that the protein recognizes both classes of compound equally well, but having done so has a very much greater affinity for the triphosphate than for the diphosphate (Mannherz *et al.*, 1974). The identical purine-spectral perturbation induced by the protein in all the thioguanosine nucleotides tested indicates that the protein-binding domain for the purine does not alter dramatically during the course of the thioGTPase and hence the ATPase reaction between the $M^* \cdot \text{ATP}$ and $M^* \cdot \text{ADP}$ states.

One of the principal aims of this and related work is to measure the concentration of intermediate states of the myosin and actomyosin ATPase in more organized myofibrillar systems. For example one would like to know how the concentrations of nucleotide-bound states vary according to tension generated or the work done by a skinned muscle fibre. No difference could be detected in the spectra of protein-bound thioguanosine nucleotides. Thus in principle thionucleotides might be considered useful for measuring concentrations of bound nucleotides in the myofibril, particularly since thioITP (Murphy & Morales, 1970) and thioGTP in the presence of Ca^{2+} cause contraction of glycerinated muscle fibres. Further, in the absence of Ca^{2+} , thioITP caused complete relaxation of muscle fibres (J. Lebacqz, personal communication), in contrast with our results with thioGTP. However, two factors suggest that further work in this direction using thionucleotides will be unprofitable. First, the high (above 1 mM) concentrations of thionucleotide required render the fibres opaque in the near u.v. Secondly, the u.v. light required to excite protein fluorescence and its quenching by thionucleotides damages muscle fibres (J. Lebacqz, personal communication). However, fluorescent ATP analogues such as formycin 5'-triphosphate may be used to advantage (Trentham *et al.*, 1976).

Perhaps the most promising prospect for using thioguanosine nucleotides is in those systems in which a guanosine nucleotide is the biologically active reagent. It is becoming increasingly apparent that guanosine nucleotides play an important role in metabolic regulation (Pogson, 1974; Rodbell *et al.*, 1975; Nicholls, 1976). Further, in addition to their chromophoric properties, the thioguanosine nucleotides or their derivatives in which the thiol is modified are, like the thioinosine nucleotides (Yount, 1975), potential affinity-labelling reagents. Fluorescent guanosine nucleotide analogues which are potential photo-affinity labelling reagents add to the range of compounds available (Wiegand & Kaleja, 1976).

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