Circular Dichroic Spectra of 6-Thioguanosine Nucleotides and Their Complexes with Myosin Subfragment 1†

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ABSTRACT: The circular dichroic spectra of the thione form of 2-amino-6-mercapto-9β-ribofuranosylpurine 5′-triphosphate (thioGTP), thioGDP, thioGTP(γS), thioGMP-P(NH)P, and 6-mercapto-9β-ribofuranosylpurine 5′-diphosphate (thioIDP) and their complexes with myosin subfragment 1 and heavy meromyosin have been measured between 300 and 400 nm. The free nucleotides have a weak negative circular dichroic spectral peak at their absorption maxima, with a longer wavelength shoulder. On binding to the proteolytic fragments of myosin, the negative peak is enhanced ~10-fold and a longer wavelength positive peak appears. These effects are attributed to a change in the stereochemical structure of the nucleotide and specific interactions within the nucleotide binding site. All four thioguanosine nucleotides give these spectral changes although minor significant differences do occur. The CD spectra of the subfragment 1 steady-state complexes with thioGTP and thioGTP(γS) are similar to each other but different from the complexes of subfragment 1 with thioGDP and thioGMP-P(NH)P. Interactions between nucleotides in the complexes are excluded by a study of their spectra in 2-propanol when base stacking occurs. This is the first investigation where circular dichroism has been used to determine structural differences between different myosin–nucleotide states and provides evidence that the nucleotide in the γS bound state is similar to that of the triphosphate bound state.

Purines and pyrimidines containing sulfur atoms show absorption bands at longer wavelengths than the corresponding compounds containing oxygen (Cheong et al., 1969). Nucleotides can therefore be synthesized containing a thiopurine or thiopyrimidine which have an absorption band well removed from the region of protein absorption. Perturbation of the spectra of this single chromophore may be readily measured during nucleotide–protein interactions. Following the observations of Murphy & Morales (1970) that there is a change in the absorption spectrum of thioITP[1] on binding to myosin (M), Trentham et al. (1972) used this compound to study certain partial reactions of myosin ATPase by using stopped-flow spectrophotometry. Hilborn & Hammes (1973) have performed active-site titrations of mitochondrial ATPase with thioIDP, and the perturbations of the absorption and circular dichroism spectra of nucleotides derived from 4-thiouridine, 6-thioguanosine, and 6-thioinosine on binding to pancreatic and T_1 ribonuclease have been reported by Samejima et al. (1969) and Sawada et al. (1973). Murphy (1971, 1974) has used circular dichroism studies of the binding to actin and myosin of thioITP and thioIDP. In this paper we report studies on the interactions of thioguanosine and thioinosine nucleotides with proteolytic fragments of myosin.

A mechanism has been proposed for myosin ATPase by Bagshaw et al. (1974) in which several steps involve conformational changes of a protein–nucleotide complex (eq 1). The

$$\mathrm{M + ATP \rightleftharpoons M{\cdot}ATP \rightleftharpoons M^{*}{\cdot}ATP \rightleftharpoons M^{**}{\cdot}ADP{\cdot}P_i \rightleftharpoons M^{*}{\cdot}ADP{\cdot}P_i \rightleftharpoons M^{*}{\cdot}ADP \rightleftharpoons M{\cdot}ADP \rightleftharpoons M + ADP \quad (1)}$$
$$\mathrm{+\ P_i}$$

nature of these conformational changes is not known and, as pointed out by Huxley (1974), may involve anything from the movement of whole regions of peptide chain down to a change in electron distribution within a certain group. It is necessary to investigate these changes in more detail before the mechanism of ATP hydrolysis by myosin in solution can be related to discrete steps of the cross-bridge cycle in muscle.

Eccleston & Trentham (1977) synthesized a series of nucleotides derived from thioinosine and thioguanosine and investigated the mechanism of the myosin thioITPase and thioGTPase in order to determine whether this was the same as the myosin ATPase. In addition to monitoring the binding of thionucleotides by absorption changes, they found that these compounds caused a quenching of the protein fluorescence of myosin subfragment 1 which was a more sensitive signal than absorption changes of the nucleotide. They also showed that the mechanism of the thioITPase was essentially the same as that of the ATPase although rate constants for particular steps were different, the important features being a two-step binding of thioITP to myosin followed by a rapid cleavage step and then a rate-limiting conformational change of this myosin–product complex. ThioGTP is also hydrolyzed by myosin although Eccleston & Trentham (1979) have showed that the steady-state intermediate consists predominantly of the analogue of $M^{*}{\cdot}ATP$ rather than $M^{**}{\cdot}ADP{\cdot}P_i$. Eccleston & Trentham (1977) also synthesized thioGDP, thioGMP-P-(NH)P, and thioGTP(γS) and studied their interaction with subfragment 1 by myosin. ThioGMP-P(NH)P binds to subfragment 1 and is not hydrolyzed. ThioGDP also forms a binary complex with subfragment 1. ThioGTP(γS) is hydrolyzed by subfragment 1, and we assume that the cleavage step is rate limiting by analogy with the mechanism of ATP-

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[1] Abbreviations used: thioITP, 6-mercapto-9β-ribofuranosylpurine 5′-triphosphate; thioGTP, 2-amino-6-mercapto-9β-ribofuranosylpurine 5′-triphosphate; thioIDP and thioGDP are the corresponding 5′-diphosphates; AMP-P(NH)P, 5′-adenylyl imidodiphosphate; ATP(γS), adenosine 5′-(3-thio)triphosphate; nucleotide analogues derived from thioguanosine are similarly abbreviated; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ATPase, adenosine 5′-triphosphatase; thioGTPase, thioguanosine 5′-triphosphatase; thioITPase, thioinosine 5′-triphosphatase; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide.

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(γS) hydrolysis by myosin (Bagshaw et al., 1972).

The long-wavelength absorption maximum of thioguanosine nucleotides (342 nm) and the kinetic characteristics of their interaction with subfragment 1 make them suitable compounds for studying the spectral characterization of the intermediates of the reaction mechanism of subfragment 1. The absorption spectra of such intermediates have been described by Eccleston & Trentham (1977).

This paper describes the circular dichroism spectra of the thionucleotides in solution and bound to subfragment 1. Circular dichroism spectra respond in a sensitive way to changes in molecular conformation and to the orientation of a chromophore relative to other structures in its immediate environment. Thus differences in the glycosidic angle of the thionucleotide on complex formation or conformational changes in the protein–nucleotide complexes should be detected by using CD spectroscopy. These may therefore help to characterize further the conformational changes inferred in the kinetic mechanism of the myosin ATPase.

Materials and Methods

Proteins and Nucleotides. Myosin subfragment 1 was prepared from rabbit skeletal muscle as described by Eccleston & Trentham (1977) and heavy meromyosin by the method of Lowey & Cohen (1962).

ThioGTP, thioGMP-P(NH)P, and thioITP were synthesized as described by Eccleston & Trentham (1977). ThioGDP was prepared from thioGTP by hydrolysis with subfragment 1. ThioGTP(γS) was prepared by the reaction of thioGDP and ATP(γS) in the presence of nucleoside diphosphate kinase (Goody et al., 1972). ThioGDP and thioGTP(γS) were both then purified by using a DEAE-cellulose (Whatman DE-52) column in the bicarbonate form and eluting with a linear gradient of 0–0.6 M triethylammonium bicarbonate, pH 7.6. The nucleotide peak was evaporated to dryness and the eluting solvent removed by repeated additions and evaporations of methanol.

Spectroscopic Measurements. Circular dichroism spectra were recorded on a Jouan dichrograph, Model IIB, fitted with a 150-W xenon arc lamp. Solutions in silica cuvettes were thermostatically maintained at 5 °C for measuring the spectra of thionucleotides bound to protein and at 20 °C for measuring the effects of 2-propanol. A_{342} of the thionucleotide solutions was kept at <1.5. The operating voltage was 560 V. Spectra were measured between 300 and 400 nm with a nominal time constant of 10 s and scanning at 3.75 nm/min, and 2 points/nm were recorded in digital form [cf. Chen et al. (1977)].

The data were processed numerically, averaging multiple scans where necessary and subtracting the instrumental base line (see Figure 1). The spectra were smoothed to remove instrumental noise by serial application of a third-order polynominal algorithm and plotted via Calcomp routines. Three or four spectra of each subfragment 1–thionucleotide complex were measured and plotted as average spectra (except Figure 1). A fresh solution was used for each spectrum to overcome problems of decomposition of complexes (see Discussion). All spectra recorded were shown to be stable over the time course of the measurement. CD spectra are reported as the experimentally observed dichroic absorbance (ΔA) for free nucleotides and mixtures with the protein. The derivation of molar circular dichroism for the components of the mixture is described below.

Absorption spectra were recorded on Unicam SP1800 or Cary 118 spectrophotometers. All solutions of thionucleotides contained 0.1 mM dithiothreitol.

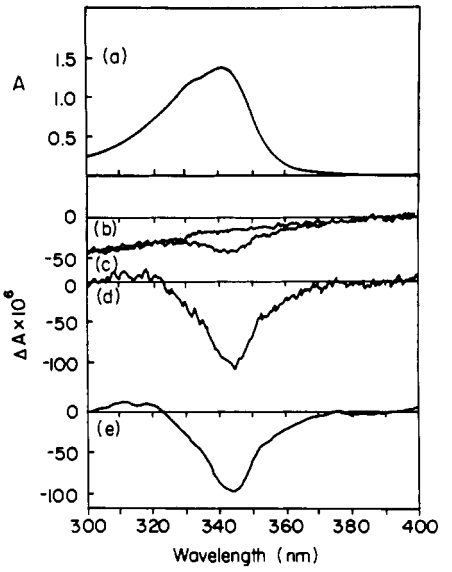

FIGURE 1: The absorption and CD spectrum of thioGTP at pH 6.5 and 20 °C. (a) Absorption spectrum of 111 μM thioGTP; (b) CD spectrum instrument base line; (c) single-scan CD spectrum of 111 μM thioGTP; (d) the sum of four CD spectra scans with base line subtracted; (e) as in (d) but after smoothing. The solutions in an 0.5 cm path length cell also contained 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.1 M Mes adjusted to pH 6.5 with KOH.

Measurement of Equilibrium and Kinetic Constants. The dissociation constants of thioGDP and thioGMP-P(NH)P from subfragment 1 at 5 °C were determined by measuring the second-order binding rate constants and dissociation rate constants using the same stopped-flow spectrofluorometric techniques described by Eccleston & Trentham (1977). The K_m of subfragment 1 for thioGTP at 5 °C was measured by using a linked-assay system containing 50 mM Mes adjusted to pH 6.5 with KOH, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.25 mM NADH, 10 μg of pyruvate kinase/mL, and 10 μg of lactate dehydrogenase/mL, and NADH oxidation was followed at 366 nm by using $\epsilon_{366} = 3.3 \times 10^3$ mol^{-1} cm^{-1} for NADH in order to avoid excessively high absorbances since thioGTP absorbs maximally at 342 nm.

Evaluation of Molar Circular Dichroism Parameters for Complexes of Thioguanosine Nucleotides. The molar circular dichroism of each complex has been evaluated at the maxima of the negative band (335 nm) and the positive band (357 nm) (Table I). Corrections were made for the calculated concentration of free nucleotide by using the values of $\Delta\epsilon_{335} = -0.22$ M^{-1} cm^{-1} and $\Delta\epsilon_{357} = -0.13$ M^{-1} cm^{-1} obtained from Figure 1. Since errors are introduced in this calculation by uncertainties in calculating the concentration of bound nucleotide (see Discussion), the ratios of the CD intensities of the positive and negative bands have also been calculated for each complex. This value is much less affected by the absolute concentration of bound nucleotide (see Table II).

Results

CD Spectra of Thioguanosine Nucleotides in Solution. Figure 1 shows the spectrum for thioGTP at pH 6.5, obtained by a single scan, multiple scans, and smoothing of the weak CD signal from the free nucleotide. A single negative peak is observed with maximum at $\lambda = 342$ nm ($\Delta\epsilon = 0.45$ M^{-1} cm^{-1}; $[\Theta] = 1.5 \times 10^3$ deg cm^2 dmol^{-1}). This may be compared with the value reported by Cheong et al. (1969) for the corresponding nucleoside ($\Delta\epsilon_{345} = 0.76$ M^{-1} cm^{-1}; $[\Theta]_{345} = 2.5 \times 10^3$ deg cm^2 dmol^{-1}). The multiple scanning shows the presence of a shoulder at 353 nm.

Table I: Molar Circular Dichroism of Thioguanosine Nucleotide Complexes with Myosin Subfragment 1 Derived from Experimental Spectra of Figures 2 and 3

complex	figure	concn of bound nucleotide (μM)	concn of free nucleotide (μM)	$-\Delta A_{335}$ of complex ($\times 10^6$)	$-\Delta\epsilon_{335}$ of complex (M^{-1} cm^{-1})	ΔA_{357} of complex ($\times 10^6$)	$\Delta\epsilon_{357}$ of complex (M^{-1} cm^{-1})	$\Delta\epsilon_{357}/-\Delta\epsilon_{335}$
subfragment 1-thioGDP	2a	31	30	184	5.94	56	1.81	0.30
subfragment 1-thioGTP	2b	42	21.5	277	6.60	123	2.93	0.44
subfragment 1-thioGMP-P(NH)P	3a	45.5	4.5	167	3.68	41	0.90	0.24
subfragment 1-thioGTP(γS)	3b	20	40	77	3.85	32	1.60	0.42

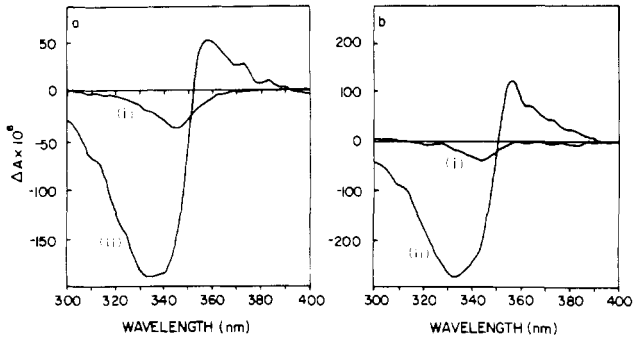

FIGURE 2: The CD spectrum of the subfragment 1–thioGDP complex and subfragment 1–thioGTP steady-state intermediate at pH 6.5 and 5 °C. (a) The solution contained (i) 61 μM thioGDP and (ii) 83 μM subfragment 1 and 61 μM thioGDP. (b) The solution contained (i) 63.5 μM thioGTP and (ii) 83 μM subfragment 1, 5 μM pyruvate kinase, 5 mM phosphoenolpyruvate, and 63.5 μM thioGTP. Solvent conditions are as described in Figure 1. Spectra were recorded with a 1 cm path length cell.

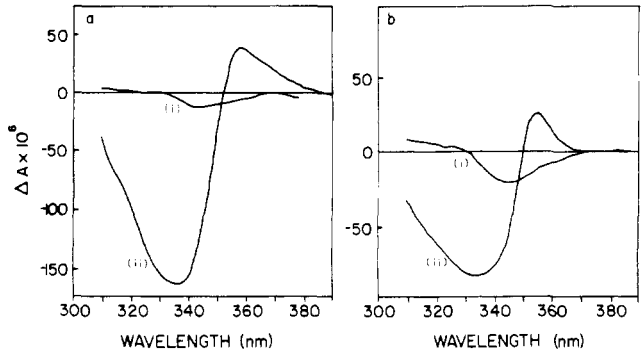

FIGURE 3: The CD spectrum of the subfragment 1–thioGMP-P(NH)P complex and the subfragment 1–thioGTP(γS) steady-state intermediate at pH 6.5 and 5 °C. (a) The solution contained (i) 58 μM thioGMP-P(NH)P and (ii) 198 μM subfragment 1 and 50 μM thioGMP-P(NH)P. (b) The solution contained (i) 68 μM thioGTP(γS) and (ii) 20 μM subfragment 1 and 60 μM thioGTP(γS). Solvent conditions are as described in Figure 1. Spectra were recorded with a 1 cm path length cell.

Qualitatively similar weak CD properties were observed for thioGDP, thioGMP-P(NH)P, and thioGTP(γS) which are shown in Figures 2 and 3. In making the small correction for the relatively weak contributions from free nucleotide, the more accurately determined values for thioGTP were used throughout.

CD Spectrum of the Subfragment 1–ThioGDP Complex. ThioGDP binds to subfragment 1 to form a binary complex with a K_d of 50 μM at pH 6.5 and 5 °C (Eccleston & Trentham, 1977). Figure 2a shows that on binding to subfragment 1, the CD spectrum of thioGDP has a negative peak at 335 nm, a positive peak at 357 nm, and a crossover point at 352 nm. At the concentrations of protein and nucleotide used, 51% of the thioGDP is bound to subfragment 1. A correction was made for the contribution of free nucleotide to this spectrum, and the results are shown in Table I for $\Delta\epsilon_{357}$ and $\Delta\epsilon_{335}$ for the subfragment 1–thioGDP complex. The ratio of these two values was calculated to be 0.30.

At 20 °C, spectroscopic measurements show that the subfragment 1-thioGDP complex is not stable. For example, in a solution containing 85 μM subfragment 1 and 102 μM thioGDP, the difference absorption spectrum between the subfragment 1–thioGDP complex and free thioGDP decreased with time until after 30 min no difference spectrum could be detected. Similarly, at 20 °C, the circular dichroism spectrum of the complex decayed with time until a spectrum identical with the free nucleotide was formed. Thin-layer chromatography showed that the thioGDP had been hydrolyzed. It has previously been shown that GDP is slowly hydrolyzed by myosin (Eccleston & Trentham, 1979).

CD Spectrum of the Subfragment 1–ThioGTP Steady-State Intermediate. ThioGTP is hydrolyzed by subfragment 1. At pH 6.5 and 5 °C we determined the K_m for the hydrolysis to be 21 μM with a V_{max} of 0.025 s^{-1}. This rate is too fast to allow the direct measurement of the CD spectrum of the steady-state intermediate of the thioGTPase. However, in the presence of phosphoenolpyruvate and pyruvate kinase, thioGDP is immediately regenerated to thioGTP, and so the steady-state intermediate can be maintained for sufficient time for the CD spectrum to be measured. In a solution containing 83 μM subfragment 1 and 5 mM phosphoenolpyruvate, the steady-state intermediate should be maintained for 40 min at 5 °C when the thioGTPase rate is 0.025 s^{-1}. Figure 2b shows the CD spectrum of this intermediate. The intensity of the circular dichroism at 335 and 357 nm did not change during the time required to scan the spectrum. No perturbation of the CD spectrum of thioGTP was observed on adding it to the regenerating system without subfragment 1. The values for $\Delta\epsilon_{357}$ and $\Delta\epsilon_{335}$ for the intermediate are shown in Table I after correcting for 35% free thioGTP. The ratio $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ is 0.44.

CD Spectrum of the Subfragment 1–ThioGTP(γS) Steady-State Intermediate. ThioGTP(γS) binds tightly to subfragment 1. The first-order binding rate constant at pH 6.5 and 5 °C is 1.6×10^4 M^{-1} s^{-1}, and the rate of displacement from the steady-state intermediate by the addition of a large excess of ATP is $<6 \times 10^{-4}$ s^{-1}. This displacement rate is the sum of k_{cat} and the dissociation rate of uncleaved thioGTP(γS), although k_{cat} makes the major contribution to this rate (Eccleston & Trentham, 1977). These rate constants give an effective K_d of thioGTP(γS) from subfragment 1 of $<3.8 \times 10^{-8}$ M. This binding is sufficiently tight for subfragment 1 to be saturated with thioGTP(γS) under the conditions of the spectroscopic measurement shown in Figure 3b. By use of the upper value of k_{cat} of 6×10^{-4} s^{-1} [i.e., assuming that the dissociation rate of uncleaved thioGTP(γS) is zero], 62% of the thioGTP(γS) will be hydrolyzed to thioGDP in 25 min, the time course of the measurement. Since thioGTP(γS) binds much more tightly to subfragment 1 than does thioGDP, the thioGTP(γS) steady-state intermediate should be maintained for this time. In fact, no change in the CD at 335 or 357 nm

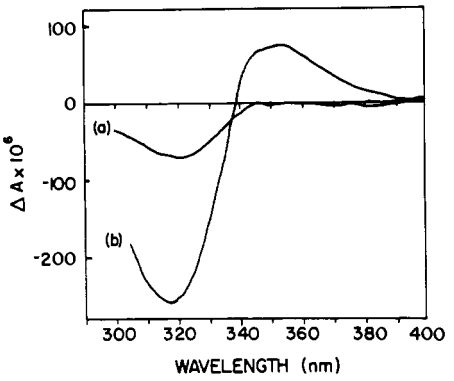

FIGURE 4: The CD spectrum of thioITP and the subfragment 1–thioIDP complex at pH 6.5 and 5 °C. The solutions contained (a) 94 μM thioITP and (b) 53 μM subfragment 1 and 94 μM thioITP, which was then allowed to hydrolyze to thioIDP. The spectrum was recorded before hydrolysis of the thioIDP occurred. Solvent conditions are as described in Figure 1. Spectra were recorded with a 0.5-cm cell.

was observable during the measurement. After correction for the presence of 67% free nucleotide, the ratio of $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ for the intermediate was 0.42 (Table I).

CD Spectrum of the Subfragment 1-ThioGMP–P(NH)P Complex. ThioGMP-P(NH)P binds to subfragment 1 but is not hydrolyzed. We measured the second-order binding rate constant and dissociation rate constant at pH 6.5 and 5 °C by using stopped-flow spectrofluorometic techniques and obtained values of 1.5×10^3 M^{-1} s^{-1} and 0.23 s^{-1}, respectively, giving K_d to be 15.4 μM under these conditions. The circular dichroism spectrum of the subfragment 1–thioGMP-P(NH)P complex shown in Figure 3a and the values of $\Delta\epsilon_{357}$ and $\Delta\epsilon_{335}$ after correction for 9% free thioGMP-P(NH)P are given in Table I. The ratio of $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ is 0.24.

CD Spectrum of the Subfragment 1–ThioIDP Complex. Although the absorption band of thioinosine nucleotides is at a shorter wavelength than those of thioguanosine, it is still sufficiently far removed from protein absorption for its circular dichroism spectrum to be measured readily in the presence of protein (Murphy, 1974). Figure 4 shows the circular dichroism spectrum of thioITP in solution and of the subfragment 1–thioIDP complex. Quantitative measurements were not made on this complex. It is notable that there is again an intensification of negative CD and the appearance of the positive band of CD in the lowest energy absorption region ($\lambda > 340$ nm). This spectrum, like that of the subfragment 1–thioGDP complex, reverted to that of free nucleotide after 1 h at 20 °C.

Effect of 2-Propanol on Spectra of Thionucleotides. A comparison of the absorption spectra of thioinosine and thioguanosine nucleotides in 2-propanol and when bound to myosin has previously been made by Murphy & Morales (1970) and Eccleston & Trentham (1977). They concluded that the nucleotide binding site of myosin has a hydrophobic environment, similar to that of 70% 2-propanol. ThioGTP in solutions containing up to 70% 2-propanol has a characteristic absorption difference spectrum compared to a solution in water alone. This consists of a positive peak at 352 nm and a negative peak at 328 nm with a shoulder at 338 nm. Below 70% 2-propanol the relative intensities of these peaks vary, but the general shape of the difference spectrum remains the same. However, above 75% 2-propanol, large changes occur in the absorption difference spectrum. Figure 5 shows the difference spectra of thioGTP in 75%, 85%, and 90% 2-propanol. A new negative peak at 345 nm has formed in 90% 2-propanol.

The CD properties of thioGTP in various concentrations of 2-propanol were investigated, in order to test whether these

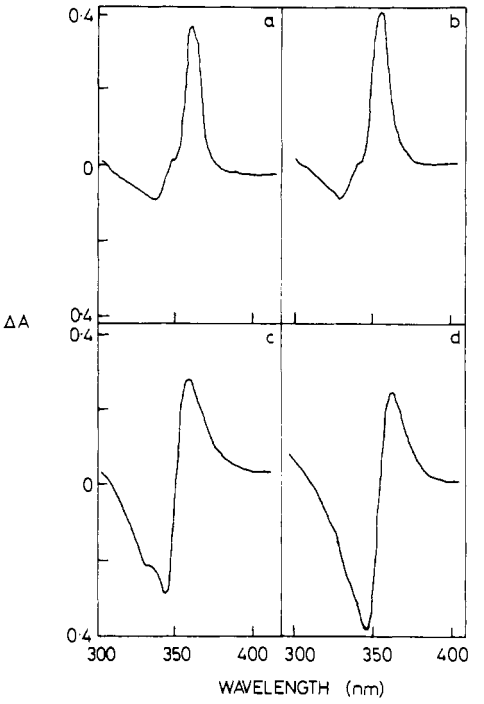

FIGURE 5: The effect of 2-propanol and $MgCl_2$ on the absorption difference spectrum of thioGTP at pH 4.5 and 20 °C. The 1 cm path length reference cell contained 55 μM thioGTP in water, and the 1 cm path length sample cell contained 55 μM thioGTP in (a) 75% 2-propanol, (b) 85% 2-propanol, (c) 85% 2-propanol and 5 mM $MgCl_2$, and (d) 90% 2-propanol. All solutions also contained 0.1 mM dithiothreitol and 1 mM acetic acid adjusted to pH 4.5 with NaOH.

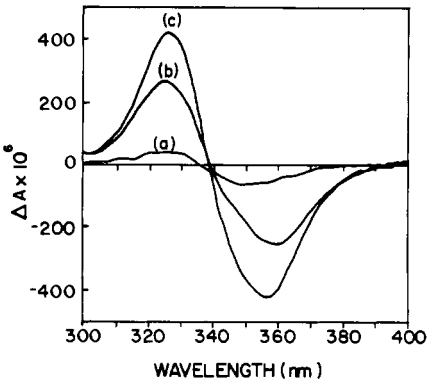

FIGURE 6: The effect of 2-propanol and $MgCl_2$ on the CD spectrum of thioGTP at pH 4.5 and 20 °C. The solutions contained 57.5 μM thioGTP in (a) 75% 2-propanol, (b) 95% 2-propanol, and (c) 95% 2-propanol and 5 mM $MgCl_2$. Other conditions are as in Figure 5.

spectroscopic features correlate with the positions of the extrema observed in CD upon subfragment 1–nucleotide complex formation. Up to 70% 2-propanol, the spectrum remains identical with that in water. At higher concentrations of 2-propanol, a highly characteristic bimodal spectrum is formed with a "conservative" band system composed of a positive peak at 325 nm and a negative peak at 358 nm (Figure 6). The addition of a large excess of $MgCl_2$ (5 mM) reduces the intensity of this peak somewhat, although the form of the spectrum is unchanged.

With both absorption difference spectra and circular dichroism spectra (Figure 7), the effect of 2-propanol concentration is dependent on the magnesium ion concentration. In absorption spectra, a sharp transition is seen between 85% and 90% 2-propanol in the absence of magnesium, but this occurs at lower 2-propanol concentration in the presence of 5 mM $MgCl_2$. The greatest differential effect occurs at ~85% 2-

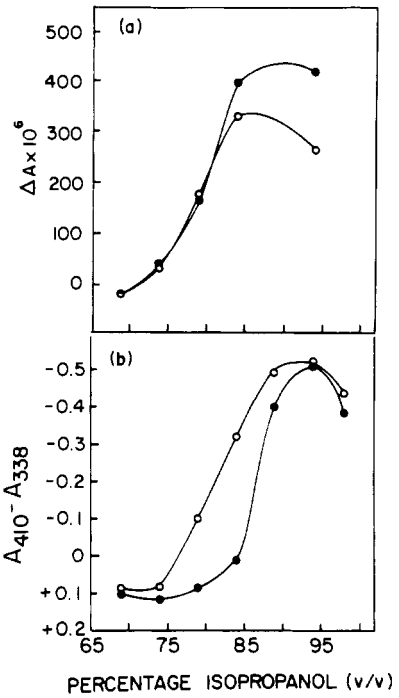

FIGURE 7: The effect of increasing concentrations of 2-propanol on the absorption difference spectrum and CD spectrum of thioGTP in the absence and presence of $MgCl_2$ at pH 4.5 and 20 °C. Absorption difference spectra and CD spectra were recorded as described in Figures 5 and 6 in the presence (O) and absence (●) of 5 mM $MgCl_2$.

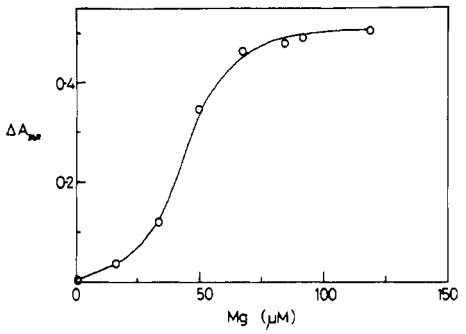

FIGURE 8: Titration of thioGTP in 85% 2-propanol with $MgCl_2$. The 1 cm path length reference cell contained 55 μM thioGTP in 85% 2-propanol, and the 1 cm path length sample cell contained an identical solution to which aliquots of 1 M $MgCl_2$ were added.

propanol. The CD spectra show qualitatively similar effects but with a different sensitivity to magnesium ion.

The stoichiometry of Mg^{2+} to thioGTP in the transition was investigated by titrating $MgCl_2$ into a solution of thioGTP in 85% 2-propanol and measuring the difference spectrum of this solution compared to the same concentration of thioGTP in 85% 2-propanol to which $MgCl_2$ was not added. A plot of ΔA_{348} against $MgCl_2$ shows that the stoichiometry was 1:1 and K_D = 50 μM (Figure 8). No further change in the spectrum occurred at higher magnesium concentrations.

Discussion

The circular dichroism spectra of thioGDP, thioGMP-P-(NH)P, thioGTP, and thioGTP(γS) are all weak but qualitatively appear very similar, being derived from the thioguanosine chromophore. The maximum in absorption and CD spectra coincide at 342 nm; however, the CD spectra show a distinct shoulder to the long-wavelength side of the maximum and the negative dichroism extends to at least 360 nm. This is presumably due to a long-wavelength transition ($n \rightarrow \pi^*$) which is very weak in intensity in absorption, as discussed by Cheong et al. (1969).

The spectra of the nucleotides complexed with subfragment 1 show certain well-defined common features. Thus the CD spectrum undergoes an intensification (~10-fold) in the negative band associated with the absorption maximum. The long-wavelength band is now inverted in sign (i.e., positive) and also intensified. It reaches to nearly 400 nm. The same basic line shape is seen for all the complexes but the relative intensities of positive to negative bands show significant differences.

The appearance of oppositely signed effects in CD may in certain cases be indicative of the asymmetric association of two (or more) chromophores in generating an exciton. However this mechanism in its simplest form generates a "conservative" band system (equal bands of positive and negative CD) with a crossover close the absorption maximum associated with a strongly allowed electronic system. In the case of the complexes of thionucleotides with subfragment 1, we can confidently exclude such a mechanism for the following reasons. All spectrophotometric binding data are consistent with a 1:1 stoichiometry for nucleotide/subfragment 1. The crossover does not coincide with the absorption maxima, and the bands are not conservative. Other CD evidence to be discussed (for thioGTP in 2-propanol) shows the characteristic intensity and appearance of the true exciton phenomena.

Thus, for a single molecule of bound nucleotide, the possible mechanisms can be distinguished conceptually as (a) immobilization of the nucleotide and (b) interaction of the nucleotide with the binding site, although in practice the two mechanisms will overlap. It is known from model compound studies that immobilization of the base with respect to the sugar moiety, thereby fixing the glycosidic angle χ, can enhance the CD by up to 10-fold over that in an unrestricted nucleotide for which a range of χ values may obtain (Teng et al., 1971; Miles et al., 1971). The transition dipole associated with the allowed transition (λ_{max} = 342 nm) interacts with the polarizable electron density of the chiral ribose moiety in an asymmetric fashion to generate strong CD effects. This may be expected to be dependent upon χ, possibly in a very sensitive fashion.

It is not clear that mechanism (a) could account for the appearance of the oppositely signed longer wavelength band observed in the complexes. If, as seems likely, this positive CD band is associated with $n \rightarrow \pi^*$ transitions and these derive from the thione moiety, the alternative mechanism (b) is more plausible. Thus, while it is not strictly valid to consider the low energy $n \rightarrow \pi^*$ transitions in isolation (since they mix with the $\pi \rightarrow \pi^*$ transitions and may derive CD intensity indirectly from them), this approximation is not unreasonable in the present case given the orthogonality of the $n \rightarrow \pi^*$ (out of plane) and $\pi \rightarrow \pi^*$ (in plane) transitions in nucleotides and the possibility indicated by the work of Hug & Tinoco (1973) of treating portions of the complex nucleotide chromophore as separate contributors to the absorption and hence CD spectra. Given also in this case the marked enhancement as well as inversion of the observed long-wavelength CD, it is reasonable to suggest a specific asymmetric interaction involving the thione group. An intramolecular interaction (i.e., within the nucleotide) appears unlikely from molecular models, and no such CD effect appears to have been observed with other thiopurine nucleotide derivatives. The likely candidate would therefore be a group or groups in the protein, specific interactions with which may cause the characteristic effects. Since the absorption spectrum shifts are those for the transfer of the thioguanine chromophore to a hydrophobic environment,

Table II: Effect of the Percentage of Bound Nucleotide on $\Delta\epsilon$ and the Ratio $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ in a Solution of 61 μM ThioGDP and 83 μM Subfragment 1[a]

concn of bound nucleotide (μM)	K_D (μM)	% bound	total CD at 335 nm, $-\Delta A \times 10^6$	$-\Delta A_{335} \times 10^6$ for complex	$-\Delta\epsilon_{335}$ complex (M^{-1} cm^{-1})	total CD at 357 nm, $\Delta A \times 10^6$	$\Delta A_{357} \times 10^6$ for complex	$\Delta\epsilon_{357}$ complex (M^{-1} cm^{-1})	$\Delta\epsilon_{357}/-\Delta\epsilon_{335}$
9.15	420	15	191	180	19.67	52	59	6.45	0.33
18.30	150	30	191	182	9.95	52	58	3.17	0.32
27.45	68	45	191	184	6.70	52	56	2.04	0.30
36.60	31	60	191	186	5.08	52	55	1.50	0.30
45.75	12	75	191	188	4.11	52	54	1.18	0.29

[a] This calculation takes values of K_d ranging from 12 to 420 μM.

it appears reasonable that the spectral properties of the thione group reflect a specific asymmetric interaction within this environment. It may be noticed that the CD intensity of the strongly allowed transition (λ_{max} = 342 nm) could also be affected by interaction with local groups in the protein or with the bulk polarizability of the protein structure.

If these are indeed the mechanisms of the enhancement of the mononucleotide CD, it would appear that the CD should be a sensitive indicator of changes of conformation of the bound nucleotide or changes in the relative orientation of the nucleotide with respect to the protein as might occur with conformational changes located predominantly in the protein. Sawada et al. (1973) also observed an intensification of the CD peak associated with the absorption maximum of the thioguanine moiety of thioguanosine 2′(3′),5′-diphosphate on binding to RNase T_1 and the appearance of a long-wavelength positive CD peak. Although the values of $\Delta\epsilon$ for these peaks are different from those measured in this work, the effect may be ascribed to the same mechanisms discussed above. Murphy (1971, 1974) has reported that the CD spectrum of thioITP bound to actin or heavy meromyosin only shows an intensification of the negative band without any accompanying formation of a long-wavelength positive band. Although the absence of specific interactions of the thione group with amino acid residues in the nucleotide binding site of actin would account for this result in the case of actin, we cannot explain the lack of a positive peak in his experiment with heavy meromyosin and thioITP, since this is clearly visible in our experiment with subfragment 1 (Figure 4). We repeated the measurement with heavy meromyosin and observed the same CD spectrum as with subfragment 1.

The overall consistency of the CD spectra of the four complexes of subfragment 1 studied here shows that the geometry of the nucleotide and its immediate protein environment is similar in these complexes. However, the differences noticed in Table I are small, but we believe they are significant. The major uncertainty in determining the values of molar circular dichroism for the complexes is the concentration of the bound nucleotide in a particular solution. Errors in the value for the dissociation constant K_D (or the K_m for the thioGTP) will be reflected in the concentration of bound nucleotide. Perhaps more importantly, the discrepancies between the K_m values for myosin and ATP and other nucleoside 5′-triphosphates calculated from the rate constants of elementary steps in the mechanism and the measured value for the K_m may indicate that the current mechanism does not fully describe the myosin ATPase reaction. Also, it appears that GDP does not bind to subfragment 1 to form a single protein–nucleotide complex. Both of these phenomena are discussed by Eccleston & Trentham (1979).

Table II shows that, although calculations of absolute values of $\Delta\epsilon_{335}$ and $\Delta\epsilon_{357}$ are highly dependent on the dissociation constant and hence on the calculated concentration of bound nucleotide, the ratio of these values is relatively constant for a wide range of K_d values. This ratio, while clearly empirical and without structural significance, provides a useful measure of comparison for the typical CD spectra of different nucleotide complexes. It is also known that, when thioGDP binds to elongation factor Tu, a CD spectrum is obtained similar to those described for subfragment 1 except that the 335-nm peak is positive and the 357-nm peak is negative (J. F. Eccleston, unpublished experiments). This suggests that similar electronic factors are involved, but with completely different symmetry. While the ratio is relatively independent of K_d, it is somewhat dependent on the base line of the spectrum. However, we estimate that the uncertainty in our measured values is $\pm 2.5 \times 10^{-6}$ M^{-1} cm^{-1} and so that errors in the base line value would not significantly affect the $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ ratio. We therefore interpret the range of values for this ratio for the four thioguanosine nucleotide complexes as being due to minor but significant stereochemical differences in the nucleotide and its binding site in individual complexes.

The most notable features of these results are the higher values of the $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ ratio of the steady-state intermediates of subfragment 1 with thioGTP and thioGTP(γS) compared to the complexes of subfragment 1 with thioGDP and thioGMP-P(NH)P. Although comparisons of the effect of analogues on subfragment 1 with the intermediates of the ATPase mechanism must obviously be made with caution, there are sufficient studies in the literature to allow meaningful comparisons to be made.

The structure of AMP-P(NH)P suggests that it is an analogue of ATP and in certain systems it does have some of the properties of ATP not involving cleavage between the β and γ phosphate groups (Yount, 1975). It may therefore be considered to form an analogue of the M*·ATP state with subfragment 1. However, Mannherz & Goody (1976) and Trentham et al. (1976) have discussed the possibility that it may be more correct to regard it as an analogue of ADP in this system. For example, the X-ray diffraction pattern obtained from glycerinated muscle fibers is similar whether the fibers are soaked in ADP or AMP-P(NH)P and different from that obtained with ATP. Also, Seidel & Gergely (1973) found that AMP-P(NH)P gave the same ESR spectrum as ADP on binding to myosin containing a spin-label. It may be, therefore, that the state reached depends on the binding constant of the nucleotide to myosin rather than simply the presence of two or three phosphate groups. The values obtained by Bagshaw et al. (1974) by kinetic methods for the dissociation constant of ADP and AMP-P(NH)P from myosin are 3.3×10^{-7} M and 9.4×10^{-7} M, respectively. The CD spectra of the thioguanosine nucleotide complexes indicate a correlation between thioGDP and thioGMP-P(NH)P, as judged by the characteristic ratio $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ for the complexes.

ATP(γS) binds to myosin and, since the cleavage step is rate limiting, the steady-state intermediate may also be regarded as an analogue of the M*·ATP state. Bagshaw et al. (1974) showed that the binding of ATP and the binding of

ATP(γS) to subfragment 1 were both two-step processes with similar equilibrium constants for the biomolecular process and forward rate constants for the unimolecular process. The effective dissociation constant of ATP(γS) was not measured, but a higher limit of 1.7×10^{-7} M was determined. This compares with a value of 6×10^{-11} M for ATP (Cardon & Boyer, 1978). The X-ray diffraction data of Mannherz & Goody (1976) show that ATP(γS) induces the same changes in relaxed, glycerinated fibers as ATP does, although the two states formed, as determined by kinetic studies of subfragment 1 in solution, are M*·ATP(γS) and 10% M*·ATP–90% M**·ADP·P_i, respectively. Chock et al. (1979) and Chock (1979) have argued that the subfragment 1–ATP(γS) steady-state intermediate may be different from the M*·ATP state. However, the formation of the tightly bound ATP(γS) intermediate occurs at the same rate as the tightly bound ATP intermediate, as determined by a "cold chase" technique (D. R. Trentham, personal communication). The CD spectra, which are a sensitive probe of the state of the protein-bound nucleotide, do show that the M*·thioGTP and M*·thioGTP-(γS) intermediates are very similar, again as judged by the value of the ratio $\Delta\epsilon_{357}/-\Delta\epsilon_{335}$ for the complexes.

It would be of interest to compare differences between the CD spectra of analogues of the M*·ATP and M**·ADP·P_i states. In principle, this could be done by using thioITP since the equilibrium constant, K_3 (eq 1), can be varied by a suitable choice of solvent conditions to give predominantly either one of these intermediates (Eccleston & Trentham, 1979). However, the available data show that pH 8.0 is required for formation of the M**·ADP·P_i analogue as the steady-state intermediate of the subfragment 1–thioITPase mechanism. Under these conditions, an appreciable amount of the 6-mercapto group of thioITP would be ionized. This would complicate the spectral analysis since the ionized species has a different absorption spectrum from the thione.

These results and interpretations are to be contrasted to those of Murphy (1974), who claimed that the CD spectrum of the heavy meromyosin–thioIDP complex above 300 nm was identical with that of free thioIDP. Our results show a marked difference between the CD spectra of the subfragment 1–thioGDP, subfragment 1–thioIDP, and heavy meromyosin–thioIDP complexes and free nucleotides at 5 °C. We have found that both the absorption difference spectra and circular dichroism spectra of complexes of subfragment 1 with 6-thionucleoside 5′-diphosphates decay with time to give spectra identical with the free nucleotide. This is probably due to an analogous reaction to the slow GDPase activity of subfragment 1 (Eccleston & Trentham, 1979).

Finally the results with thioGTP in the presence of high concentrations of 2-propanol provide confirmation that interactions between thionucleotides are not responsible for any of the effects observed in the presence of subfragment 1. The appearance of the characteristic spectrum of an almost symmetrical CD complex (Figure 6) is a strong indication of the effects to be expected from direct interactions between thioguanine chromophores. A stacked structure which is still sensitive to magnesium ion is the most likely explanation of these effects with free nucleotides. The absorption difference spectra show that a 1:1 stoichiometry exists in the binding of Mg^{2+} and that the associated complex is formed more readily in the presence of the ion. The absence of these effects in the absorption and CD spectra of complexes with subfragment 1 supports the original interpretation that these spectra arise from a single nucleotide binding site.

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