# ATP-Induced Dissociation of Rabbit Skeletal Actomyosin Subfragment 1. Characterization of an Isomerization of the Ternary Acto–S1–ATP Complex†

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*Received May 23, 1986; Revised Manuscript Received August 21, 1986*

ABSTRACT: The adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) induced dissociation of actomyosin subfragment 1 (S1) has been investigated by monitoring the light scattering changes that occur on dissociation. We have shown that ATPγS dissociates acto–S1 by a mechanism similar to that of ATP but at a rate 10 times slower. The maximum rate of dissociation is limited by an isomerization of the ternary actin–S1–nucleotide complex, which has a rate of 500 $s^{-1}$ for ATPγS and an estimated rate of 5000 $s^{-1}$ for ATP (20 °C, 0.1 M KCl, pH 7.0). The activation energy for the isomerization is the same for ATP and ATPγS, and both show a break in the Arrhenius plot at 5 °C. The reaction between acto–S1 and ATP was also followed by the fluorescence of a pyrene group covalently attached to Cys-374. We show that the fluorescence of the pyrene group reports the isomerization step and not actin dissociation. The characterization of this isomerization is discussed in relation to force-generating models of the actomyosin cross-bridge cycle.

The ATP-induced dissociation of actomyosin is a principle step in the cross-bridge cycle of muscle contraction. Studies of this reaction both in solutions of purified proteins and in skinned muscle fibers have shown that the observed rate is linearly dependent upon the concentration of ATP over the measurable range. Observations are limited in fibers by the concentration of ATP that can be produced inside the fiber and in solution by the reaction becoming too fast to be observed by conventional rapid-flow equipment (Lymn & Taylor, 1971; White & Taylor, 1976; Goldman et al., 1982, 1984).

Millar and Geeves (1983) reinvestigated the ATP-induced dissociation of actomyosin subfragment 1 (S1)[1] in solution. They reported that at temperatures below 1 °C the observed rates became hyperbolically dependent upon the ATP concentration and that the maximum observed rate was markedly temperature sensitive. They interpreted their data in terms of Scheme I, where A = actin, M = myosin or its proteolytic subfragments, and T = ATP (adenosine 5′-triphosphate). In this model, ATP is in rapid equilibrium with the ternary complex A·M·T, and this can isomerize to a complex in which actin is more weakly bound to S1 (A–M·T). This complex is in rapid equilibrium with free actin.

Scheme I

$$A{\cdot}M + T \overset{K_1}{\rightleftharpoons} A{\cdot}M{\cdot}T \overset{K_2}{\rightleftharpoons} A\text{–}M{\cdot}T \overset{K_3}{\rightleftharpoons} A + M{\cdot}T$$

If $[T]k_1 + k_{-1}$ and $k_3 \gg k_2$ and $k_{-2}$ and the protein concentration is low such that $k_{-3}[A][MT]$ is $\approx 0$, then the observed rate of dissociation is given by

$$k_{obsd} = \frac{K_1 k_2 [T]}{K_1 [T] + 1} \qquad (1)$$

The isomerization (step 2) was not observed directly by Millar and Geeves (1983) but was inferred from the detailed balance of known equilibrium constants. The strong temperature dependence of the maximum observed rate of dissociation also suggested that the rate of actin dissociation was limited by a preceding conformational change. A similar isomerization of the ternary complex had been proposed earlier for the slower actomyosins (Eccleston et al., 1975; Marston & Taylor, 1980) and the dissociation of rabbit acto–S1 by ATP analogues (Goody & Hofmann, 1980).

Geeves et al. (1984) proposed a general model for the interaction between actin and myosin or myosin–nucleotide complexes (M·N) in which actin was bound in a two-step reaction (Scheme II).

Scheme II

$$A + M{\cdot}N \overset{K_a}{\rightleftharpoons} A\text{–}M{\cdot}N \overset{K_b}{\rightleftharpoons} A{\cdot}M{\cdot}N$$

They used this simple model to describe the overall actomyosin ATPase reaction and suggested that the isomerization step (b) was closely linked to the force-generating event in muscle contraction. In this model, step b is identical with step 2 of Scheme I. This implies that the binding of ATP to A·M results in a reversal of the isomerization step prior to the dissociation of actin. If this is correct, then a reversal of this step in an attached state poses difficulties for the contraction cycle. Two ways of dealing with these difficulties were discussed by Geeves et al. (1984). If the isomerization is not directly coupled to the force-generating event, as would be the case if, for example, the force-generating event was a helix–coil transition in S2 as envisaged by Harrington (1971), then reversal of the isomerization would not imply a reversal of the force-generating event. The alternative, as envisaged by Eisenberg and Greene (1980) and Eisenberg and Hill (1985), is that the reversal of the isomerization followed by dissociation takes place so quickly that any negative tension developed is very short lived and contributes very little to the overall tension. Characterization of the isomerization in the presence of ATP

† This work was financially supported by the Medical Research Council, U.K., and the Muscular Dystrophy Association Inc. M.A.G. is a Royal Society 1983 University Research Fellow.

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[1] Abbreviations: AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); DEAE, diethylaminoethyl; PEI, poly(ethylenimine); S1, actomyosin subfragment 1; TLC, thin-layer chromatography.

0006-2960/86/0425-8454$01.50/0 

is important in order to test the viability of these two models.

We report here a detailed characterization of this isomerization that Millar and Geeves (1983) were unable to observe directly. Two techniques have enabled characterization of this isomerization. First, we have used the ATP analogue ATPγS, which dissociates acto–S1 in a similar manner to ATP but at rates that are much slower (Goody & Hofmann, 1980). This allows observation of the dissociation reaction at ambient temperatures. Second, the fluorescence of a pyrene group covalently attached to Cys-374 of actin reports the isomerization step directly and independently of the dissociation step.

## Materials and Methods

*Proteins.* Myosin subfragment 1 was prepared by a chymotryptic digest of rabbit muscle myosin, as described by Weeds and Taylor (1975). The two isoenzymes S1.A1 and S1.A2 were not separated for use in these experiments. F-Actin was prepared according to the method described by Lehrer and Kerwar (1972). Protein concentrations were calculated with $M_r$ 115000 and $E_{280}^{1\%}$ = 7.9 $cm^{-1}$ for S1 (Margossian & Lowey, 1978) and $M_r$ 42000 and $E_{280}^{1\%}$ = 11.08 $cm^{-1}$ for actin (West et al., 1967). Pyrene-labeled actin was prepared as described by Criddle et al. (1985).

ATP and ATPγS were obtained from B.C.L., and the ATP was used without further purification. ATPγS was purified by ion-exchange chromatography on a DEAE-cellulose column, elution being with a linear gradient of 0–0.6 M triethylamine bicarbonate buffer, pH 7.6. The purity of the ATPγS was assayed qualitatively by thin-layer chromatography on PEI–MN-polygram cellulose 300 UV TLC sheets (Camlab, Cambridge) with a solvent of 0.75 M $KH_2PO_4$/HCl, pH 3.4 (Goody & Eckstein, 1971). The concentrations of the nucleotides were determined with the molar extinction coefficient at 259 nm of 15.4 $M^{-1}$ $cm^{-1}$.

The rapid-mixing experiments were carried out on a Hi-Tech Scientific SF3L stopped-flow spectrophotometer that was thermostated to within 0.1 °C with liquid nitrogen and a solid-state proportional temperature controller. The dead time of the apparatus was measured as 0.9 ms. The optical system consisted of a high-pressure mercury lamp with a Bausch and Lomb monochromator set at 365 nm, and the light was transmitted to the integral mixing and observation chamber by a quartz light guide. The 90° emitted light was detected by an EMI 9526B photomultiplier either directly for light-scattering measurements or indirectly through a Schott KV 393 glass filter for fluorescence. The signal from the photomultiplier was captured by a Datalab DL905 transient record and then analyzed on an ITT 2020 microcomputer using a nonlinear, least-squares routine (Edsall & Gutfreund, 1983).

## Results and Discussion

Millar and Geeves (1983) were unable to measure the maximum rate of the ATP-induced dissociation of acto–S1 at temperatures above 1 °C as the reaction became too fast to be observed in conventional stopped-flow equipment. However $K_1k_2$ (eq 1) was obtained from the concentration dependence of $k_{obsd}$ at low ATP concentrations (i.e., when $K_1[T] \ll 1$). The Arrhenius plot of $K_1k_2$ showed a sharp break at 5 °C, and Millar and Geeves were unable to assign the break unequivocally to a change in either $K_1$ or $k_2$ although they favored $k_2$.

ATPγS is an analogue of ATP that is hydrolyzed only slowly by S1 (Bagshaw et al., 1972). Its affinity for S1 cannot be measured directly but is $\geq 10^7$ $M^{-1}$ compared to $10^{11}$ for ATP (Bagshaw et al., 1974; Trentham et al., 1976). Goody and Hofmann (1980) reported that ATPγS dissociated acto–S1, that the observed rate of dissociation was hyperbolically dependent upon ATPγS concentration, and that the maximum observed rate was 380 $s^{-1}$ at 20 °C. We therefore examined the temperature dependence of this reaction over the range −10 to 20 °C to compare with the results obtained by Millar and Geeves for ATP. Studies at temperatures below 0 °C were made possible by the addition of 40% ethylene glycol to the solution.

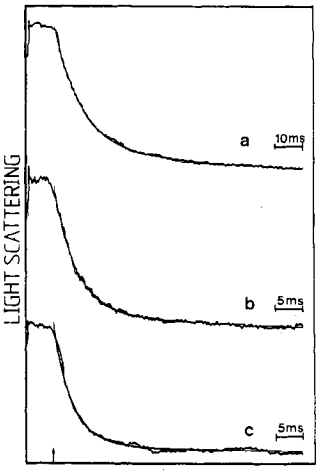


FIGURE 1: Stopped-flow light scattering records of ATPγS-induced dissociation of acto–S1 at 20 °C. The arrow indicates the time at which flow stops. The vertical axis represents light scattering on the same scale for each record. Each trace represents the average of five successive reactions in the stopped flow, and the best fit single exponential is superimposed. Conditions: 0.1 M KCl, 5 mM $MgCl_2$, 40% ethylene glycol, 200 mM cacodylate, pH 7.0, 20 °C, 5 μM S1, and 6 μM actin. The 90° light scattering was observed at 365 nm: (a) 100 μM ATPγS, $k_{obsd}$ = 93 $s^{-1}$; (b) 500 μM ATPγS, $k_{obsd}$ = 240 $s^{-1}$; (c) 1 mM ATPγS, $k_{obsd}$ = 270 $s^{-1}$.

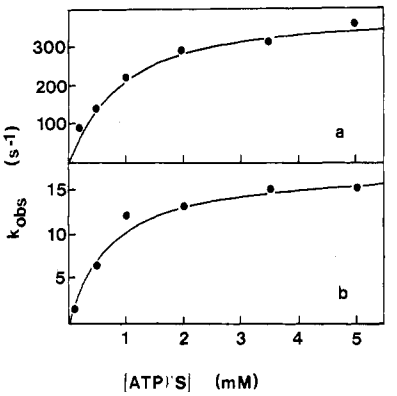


FIGURE 2: Temperature and concentration dependence of the ATPγS dissociation of acto–S1. The concentration dependence of the observed rates is plotted with the best fit to eq 1 superimposed: (a) aqueous, 10 °C, $K_1$ = 1.1 × $10^3$ $M^{-1}$, and $k_2$ = 415 $s^{-1}$; (b) 40% ethylene glycol, −10 °C, $K_1$ = 1.4 × $10^3$ $M^{-1}$, and $k_2$ = 18 $s^{-1}$. Other conditions are as for Figure 1.

Under all conditions used, the observed dissociation fitted a single exponential (Figure 1), and the observed rate of the exponential reaction was hyperbolically dependent upon ATPγS concentration (Figure 2). Table I lists the kinetic constants obtained from analysis of the data according to eq 1. The presence of ethylene glycol and changes in ionic strength altered $K_1$ by less than a factor of 2, which is the limit of the accuracy of the experiment. This is in contrast to the situation found by Millar and Geeves for ATP where a more detailed series of experiments showed that $K_1$ was increased by a factor of 5 in the presence of 40% ethylene glycol. Temperature had little effect upon $K_1$ in all of the solvents used, as was observed for ATP. The value of $k_2$ was relatively

Table I: Values of $K_1$ and $k_2$ as a Function of Temperature and Solvent for ATPγS-Induced Dissociation of Acto–S1

| conditions | temp (°C) | $K_1$ ($M^{-1}$) | $k_2$ ($s^{-1}$) |
|---|---|---|---|
| 50 mM cacodylate, pH 7.0, | 20.0 | $1.0 \times 10^3$ | 480 |
| 0.1 M KCl, 5 mM $MgCl_2$ | 0.1 | $2.1 \times 10^3$ | 105 |
| 200 mM cacodylate, pH 7.0, | 20.0 | $1.2 \times 10^3$ | 490 |
| 0.1 M KCl, 5 mM $MgCl_2$ | 15.0 | $1.5 \times 10^3$ | 440 |
| | 10.0 | $1.1 \times 10^3$ | 415 |
| | 5.0 | $1.6 \times 10^3$ | 235 |
| | 1.5 | $0.94 \times 10^3$ | 195 |
| 200 mM cacodylate, pH 7.0, | 20.0 | $0.71 \times 10^3$ | 570 |
| 0.1 M KCl, 5 mM $MgCl_2$, | 10.0 | $0.83 \times 10^3$ | 295 |
| 40% ethylene glycol | 0.0 | $1.0 \times 10^3$ | 111 |
| | −5.0 | $1.8 \times 10^3$ | 38.5 |
| | −10.0 | $1.4 \times 10^3$ | 18 |

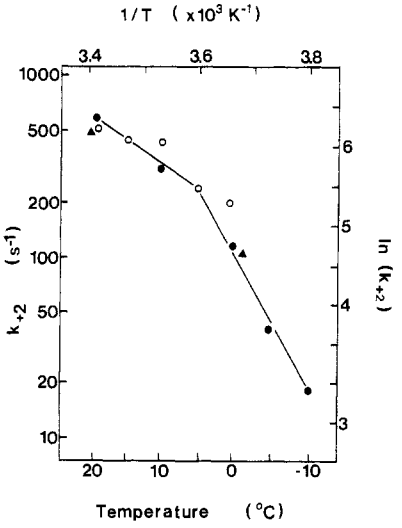


FIGURE 3: Arrhenius plot of the maximum observed rate of dissociation ($k_2$): (▲) aqueous 50 mM cacodylate; (○) aqueous 200 mM cacodylate; (●) 40% ethylene glycol and 200 mM cacodylate. Other conditions are as for Figure 1. Activation is energy 32 kJ·mol$^{-1}$ above 5 °C and 104 kJ·mol$^{-1}$ below 5 °C.

independent of the solvent conditions for both ATP and ATPγS, but it was very temperature sensitive for both nucleotides.

An Arrhenius plot of $k_2$ for ATPγS is shown in Figure 3, and this shows that there is a change in the activation energy of $k_2$ at about 5 °C, similar to that observed for $K_1k_2$ when ATP was used. The activation energies above and below the break (32 and 104 kJ·mol$^{-1}$, respectively) are very close to those observed for $K_1k_2$ of the ATP reaction (35 and 117 kJ·mol$^{-1}$). The activation energy of $k_2$ for ATP was only obtained below 0 °C and was 110 kJ·mol$^{-1}$. This suggests that the same phenomenon is being observed in both cases. This supports the suggestion of Millar and Geeves that $K_1$ is relatively constant over the temperature range −10 to 25 °C. The rate of the isomerization controlled by $k_2$ in the presence of ATP at 20 °C would therefore be 5000 $s^{-1}$ as suggested by Millar and Geeves. The molecular interpretation of the break in the Arrhenius plots was discussed previously by these authors.

An alternative to monitoring actin–S1 interactions with light scattering is the use of actin that has been labeled at Cys-374 with a pyrene group (actin*). This fluorescent group is quenched by 70% when S1 binds to actin*. Criddle et al. (1985) showed that the presence of pyrene on actin had almost no effect upon the equilibrium and dynamics of actin–S1 interactions. Coates et al. (1985) studied the dynamics of

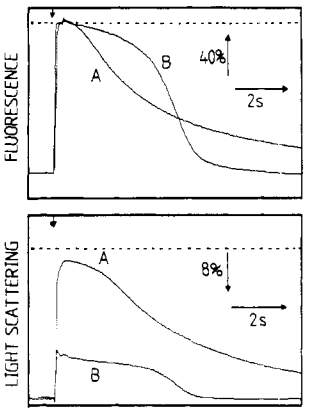


FIGURE 4: Light scattering and fluorescence changes in actin*–S1 on addition of a small excess of ATP. In experiment A 6 μM S1 and 5 μM actin* were mixed with 10 μM ATP (reaction chamber concentrations). The light scattering signal showed a rapid change consistent with >90% dissociation of actin. This was followed by reassociation, as the ATP was hydrolyzed, back to the original light scattering signal. The broken line represents the amplitude expected for 100% dissociation and was obtained from the amplitude of the same reaction performed in the presence of 0.1 M KCl. The fluorescence signal showed the same features; in this case the broken line is the observed fluorescence in the absence of S1. In experiment B the actin concentration was held constant and the [S1] increased to 50 μM. A small excess of ATP was added (75 μM). In this case a rapid change in light scattering was observed corresponding to 30% of the original amplitude followed by reassociation to the starting signal. The fluorescence signal had the same amplitude as in experiment A. In experiment B the return to the original signal is faster because of the higher ATPase rate of this solution. Reaction conditions: 2 mM $MgCl_2$, 10 mM imidazole, pH 7.0, and 20 °C. The 90° light scattering was observed at 430 nm in order to be free of interference from the pyrene signal. Actin* fluorescence was excited at 365 nm and observed through a 393-nm cutoff filter.

actin*–S1 interactions in the absence of nucleotide. They showed that the association reaction took place in two steps (eq 3 of their paper) and that light scattering monitored the association step and pyrene fluorescence the subsequent isomerization. The use of pyrene–actin therefore allows independent observation of the isomerization and dissociation steps on adding ATP to acto–S1.

Under conditions where ATP induced complete dissociation of acto–S1, Scheme I (where $k_3 \gg k_2$) would predict that the rate of the observed dissociation would be the same, monitored with either light scattering or pyrene fluorescence. Criddle et al. (1985) have previously shown that at 20 °C the rates of the ATP-induced dissociation of native acto–S1 (monitored with light scattering) and actin*–S1 (monitored with fluorescence) are identical. We repeated this experiment but with actin* for both light scattering and fluorescence measurements, and once again at 20 °C the rates were identical. At 1 °C the rates were sufficiently slow for the maximum rate of dissociation to be estimated. At this temperature light scattering measurements gave a maximum rate of 605 $s^{-1}$ for actin* and 710 $s^{-1}$ for native actin, while the fluorescence of the signal with actin* gave a maximum rate of 325 $s^{-1}$. The difference of a factor of 2 in the maximum rates of the light scattering and fluorescence signals is at the level at which it is difficult to decide if these rates are significantly different. If the fluorescence were slower than the light scattering, then it would suggest a change takes place in the actin structure after the S1 has dissociated. However, a lag phase would be expected in the fluorescence signal, and this has not been observed [see, for example, Figure 6b of Criddle et al. (1985)]. We therefore believe these rates to be essentially the same.

At very low ionic strengths the affinity of actin for S1 and S1–nucleotide complexes is greatly increased. In a buffer of

Table II: Affinities of Nucleotides for S1 and Acto–S1

| nucleotide | affinity for S1 ($M^{-1}$) | affinity for A·M, $K_1$ ($M^{-1}$) | max actin dissociation rate, $k_2$ ($s^{-1}$) |
|---|---|---|---|
| ATP[a] | $10^{11}$ | $0.5 \times 10^3$ | 5000 |
| ATPγS[b] | $\geq 10^7$ | $1.0 \times 10^3$ | 500 |
| AMP-PNP[c] | $10^7$ | $0.5 \times 10^3$ | 14 |

[a] Trentham et al. (1976) and Millar and Geeves (1983). [b] Bagshaw et al. (1974) and this work. [c] Konrad and Goody (1982).

2 mM $MgCl_2$ and 10 mM imidazole, pH 7, the affinity of actin for S1–ATP is of the order of 15 μM. Thus, addition of ATP to a solution of acto–S1 at protein concentrations in excess of 15 μM will produce incomplete dissociation. The results of such an experiment are shown in Figure 4. These results show that at 5 μM actin* and 6 μM S1 addition of a small excess of ATP causes a change in light scattering that is compatible with >90% dissociation of actin*. At 5 μM actin* and 50 μM S1, the amplitude of the change in light scattering is reduced by two-thirds, suggesting that only ≈30% of the actin* is dissociated. However the fluorescence signal changes for actin*, at both low and high protein concentration, remain the same within the limits of experimental accuracy. This result is compatible with Scheme I if pyrene fluorescence is monitoring step 2 and $K_2([A–M·T]/[A·M·T]) \geq 100$; i.e., only the high fluorescence, weakly attached actin state is occupied in the presence of ATP.

In summary, the results presented here are compatible with the model of Scheme I with the following assignment of steps. Step 1 is a rapid reversible binding of ATP with $K_1 = 10^3$ $M^{-1}$. Step 2 is the isomerization of the ternary complex where $k_2 = 5000$ $s^{-1}$ and $K_2 = 10^2 - 10^5$, and this step is reported by a change in pyrene fluorescence. Step 3 is a rapid reversible dissociation of actin, $K_3 \geq 10^{-4}$ M, and is reported by light scattering. The figures all refer to 20 °C, 0.1 M KCl, and pH 7.

The results presented here clearly demonstrate that at low ionic strength the isomerization observed by pyrene fluorescence (step 2) is reversed before actin dissociates, and this transition takes place at a rate of approximately 5000 $s^{-1}$. Step 3 (the dissociation step) must take place at a rate that is at least as fast as this, and we believe therefore that the A–M·T state would not contribute significantly to tension generation. Even under conditions where the local actin concentration is sufficiently high to prevent dissociation of actin, the A–M·T state will be in rapid equilibrium with free actin on a $10^{-4}$-s time scale, allowing rapid dissipation of any negative tension. We therefore conclude that in practice there can be coupling between the isomerization monitored by pyrene fluorescence and force generation, without significant reversal of the power stroke. However, the question of the exact relationship between the isomerization and the force-generating event remains open.

It is of interest to compare the isomerization characterized here with the work of Coates et al. (1985) in the absence of nucleotide. In terms of their model, the equilibrium constant for the isomerization $[A–M]/[AM] = 3 \times 10^{-3}$ while the data presented here show that in the presence of ATP the equilibrium constant $[A–MT]/[AMT] = 10^2–10^5$. The rate of the isomerization is very fast in both the presence and absence of ATP. Coates et al. were unable to measure the rate of the isomerization in the absence of ATP as the reaction was complete within the relaxation time of their apparatus (100 μs). This suggests a rate ($k_2 + k_{-2}$) in excess of 4000 $s^{-1}$. The results presented here are compatible with $k_2 = 5000$ $s^{-1}$ in the presence of ATP.

Complete analysis of the ATPγS data is not possible as the affinity of ATPγS for S1 and the affinity of actin for M·ATPγS remain undefined. It is however interesting to note the relationship between the affinity of a nucleotide for S1, its affinity for A·M (the complex in which actin is tightly bound) defined by $K_1$ of Scheme I, and the maximum rate of the nucleotide-induced dissociation of actin defined by $k_2$. These are shown in Table II for ATP, ATPγS, and AMP-PNP. This shows that $K_1$ is independent of the nature of the nucleotide, while $k_2 + k_{-2}$ is markedly changed by the nucleotide in a way that reflects the affinity of the nucleotide for S1 alone. It is a basic feature of the model of Geeves et al. (1984) that the affinity of a nucleotide for S1 is closely related to the equilibrium constant for the isomerization step (A·M·N → A–M·N). The tighter the nucleotide binds to S1 the more the equilibrium moves toward weaker actin binding. The data in Table II suggest that the change in the equilibrium constant is largely a result of the change in the rate defined by $k_2$.

The work presented here shows that pyrene is sensing an isomerization of the acto–S1 complex, and the data in Table II show that the isomerization is dependent upon the nature of the occupancy of the nucleotide binding site as proposed by Geeves et al. (1984). Ligand-induced conformational changes in proteins are a common feature of ligand binding reactions, and evidence of nucleotide-induced changes in both myosin (Bagshaw et al., 1974; Johnson & Taylor, 1978; Trybus & Taylor, 1982; Shriver & Sykes, 1981) and actomyosin (Geeves & Gutfreund, 1982; Konrad & Goody, 1982; Biosca et al., 1984; Rosenfeld & Taylor, 1984; Smith & White, 1985) structure has been available for some time. However, the majority of this evidence comes from changes in the spectral properties of myosin or the spectral properties of nucleotides and nucleotide analogues. It is not clear therefore if these spectral changes reflect a major structural change in the protein or a perturbation of the local environment around the probe being monitored. The fluorescence energy-transfer measurements of Bhandari et al. (1985) are an exception to this. The work presented here uses a probe attached to Cys-374 of actin, and this probe is responding to nucleotide binding to myosin. The current view of actomyosin structure suggests that Cys-374 is some 1–2 nm away from the myosin nucleotide binding site (Botts et al., 1984) and so the probe is not just reflecting some local conformational change. The idea that this is a major structural rearrangement is supported by the report of Coates et al. (1985) that the isomerization in the absence of nucleotide involves a volume change of 110 $cm^3 \cdot mol^{-1}$.

## ACKNOWLEDGMENTS

We thank Professor H. Gutfreund for his support and encouragement.

**Registry No.** ATP, 56-65-5; ATPγS, 35094-46-3.

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# Structure of a Stable Form of Sulfheme†

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*Received June 20, 1986; Revised Manuscript Received September 3, 1986*

ABSTRACT: A stable green heme was extracted from ferric cyanosulfmyoglobin after it had undergone an internal conversion reaction. After iron removal and conversion to the methyl ester, the resulting green porphyrin was purified by high-pressure liquid chromatography. Visible, $^1$H NMR, and mass spectrometric studies provided evidence to identify the substituents of the porphyrin. Nuclear Overhauser enhancements enabled an assignment of the single modified pyrrole. Substituent positions 1, 2, 5, 6, 7, and 8 have the original protoporphyrin IX substituents. At ring B, the 4-vinyl group has cyclized with a single sulfur atom to form a fifth ring with a 2,5-dihydrothiophene type of structure.

The sulfglobins are unusual derivatives of hemoglobin and myoglobin with the striking characteristic of possessing a strong green color. They are formed as hemoprotein degradation products in vivo and can also be synthesized in high yield in vitro. They have been the subject of intensive biochemical and physiological scrutiny because of their potential pathological/toxicological importance [see the bibliographic literature citations given in Park and Nagel (1984) and Timkovich and Vavra (1985)]. Sulfhemoglobin may be considered by some as the physiologically more important derivative, but because of size it is easier for NMR experiments to be conducted on sulfmyoglobin. Because of the similarity in crystal structures, there seems to have been an implicit assumption that the sulfheme in each protein is the same. This is a reasonable hypothesis, but it must be admitted that the correspondence in visible spectra and synthesis in vitro are about the only evidence to support this. A critical question has been what is the structure that gives rise to the dramatic change in the visible spectrum. Over time various structures (**1**-**6**) have been proposed. The Fe-S ligand structure **1** was ruled out because in model systems this ligand structure was insufficient to cause the visible spectrum actually seen and because the sulfglobins were capable of binding a range of ex-

† Financial support was provided in part by Grant GM37870 from the National Institutes of Health.

0006-2960/86/0425-8458$01.50/0 