Wang, C.-K., & Cheung, H. C. (1986) *J. Mol. Biol. 191*, 509–521.
Wells, J. A., & Yount, R. G. (1982) *Methods Enzymol. 85*, 93–116.
Wells, A. C., & Bagshaw, C. R. (1984) *J. Muscle Res. Cell Motil. 5*, 97–112.
Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2882.

Effect of Nucleotide Structure on Cardiac Myosin Subfragment 1 Transient Kinetics†

Jo H. Hazzard, Deborah H. Tollin, and Michael A. Cusanovich*
Department of Biochemistry, University of Arizona, Tucson, Arizona 85721
Received May 17, 1988; Revised Manuscript Received August 31, 1988

ABSTRACT: Transient kinetic data of the hydrolysis of several nucleotides (TTP, CTP, UTP, GTP) by cardiac myosin subfragment 1 (S1) were analyzed to obtain values for the equilibrium constant for nucleotide binding and rate constants for the S1–nucleotide isomerization and the subsequent nucleotide hydrolysis as well as the magnitudes of the relative fluorescence enhancements of the myosin that occur upon isomerization and hydrolysis. These data are compared with data from a previous study with ATP. Nucleotide binding is found to be relatively insensitive to nucleotide ring structure, being affected most by the group at position C6. Isomerization and hydrolysis are more sensitive to nucleotide structure, being inhibited by the presence of a bulky group at position C2. Kinetic parameters decrease as follows: for binding, GTP > UTP ~ TTP > ATP > CTP; for isomerization, ATP > UTP ~ TTP ~ CTP > GTP; for hydrolysis, ATP > TTP > CTP ~ UTP > GTP. Fluorescence enhancements appear to be most dependent upon the relative values of the individual rate constants.

Muscle myosin catalyzes the hydrolysis of ATP as the primary event in energy transduction during muscle contraction. The conversion of chemical energy to mechanical work takes place in vivo in the highly structured environment of the myofibril in which actin is in close association with myosin. In vitro myosin will catalyze ATP hydrolysis in the absence of other contractile proteins, and this simple system has been quite useful for focusing on the interaction of myosin with nucleotide. The sequence of events by which ATP is hydrolyzed by myosin consists of a minimum of three steps (Bagshaw & Trentham, 1974; Johnson & Taylor, 1978)

$$M_0 + T \underset{}{\overset{K_0}{\rightleftharpoons}} M_0{\cdot}T \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} M_1{\cdot}T \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} M_{12}{\cdot}D{\cdot}P_i \qquad (1)$$

where M_0 denotes myosin, M_1 and M_{12} are myosin intermediates and T, D, and P_i refer to ATP, ADP, and inorganic phosphate, respectively. The myosin intermediates are characterized by an enhanced intrinsic fluorescence that can be used to measure the pre-steady-state reactions by stopped-flow spectroscopy.

The majority of kinetic studies to date have focused on the catalytic subfragment of myosin, subfragment 1 (S1),[1] from skeletal muscle [as reviewed by Taylor (1979) and Eisenberg and Hill (1985)]. Cardiac myosin is of interest in that the protein is present as a mixture of isozymes having functional differences that can be correlated with the contractile performance of the heart (Morkin, 1979; Morkin et al., 1983). The data obtained with myosin from normal cardiac tissue (where one isozyme predominates) are useful, then, not only for comparison with skeletal myosin but also for comparison with cardiac isozymes expressed in diseased or damaged tissue.

The first transient kinetic studies of the cardiac protein showed that the combined rate constant for ATP binding (K_0k_{12}) is an order of magnitude lower than that for skeletal S1 (Marston & Taylor, 1980; Taylor & Weeds, 1976). This was confirmed in subsequent studies with improved cardiac S1 preparations (Flamig & Cusanovich, 1983; Smith & Cusanovich, 1984). Transient kinetic analysis of the cardiac protein has since been extended to include a fluorescent ATP derivative (Smith & White, 1985). The individual kinetic constants K_0, k_{12}, k_{23}, and k_{32} and the fluorescence enhancement for M_1 and M_{12} relative to that for M_0 have also been determined for ATP hydrolysis by cardiac S1 by using computer-modeling techniques (Hazzard & Cusanovich, 1986). With this background data for the cardiac S1 reaction, it is now possible to measure the effects of changes in substrate structure on the rate constants for binding (K_0), isomerization (k_{12}), and hydrolysis (k_{23} and k_{32}). In this manner the structural requirements for effective hydrolysis of the nucleotide substrate may be determined.

The hydrolysis of nucleotides having structures analogous to ATP has been previously investigated for skeletal muscle myosin by a variety of techniques [as reviewed in Werber et al. (1972), Seidel (1975), and Eccleston and Trentham (1977)] and for cardiac myosin steady-state hydrolysis (Balint et al., 1978). The results with skeletal myosin indicate the importance of the amino group at position 6 to a maximal fluorescence enhancement upon hydrolysis (Werber et al., 1972; Bagshaw et al., 1974; Seidel, 1975) and to maximal binding of nucleotide measured as $1/K_m$ (Blum, 1960; Kielley et al., 1956). In this work the binding and hydrolysis of a series

† This research was supported by a grant from the Arizona Disease Control Commission.

[1] Abbreviations: S1, myosin subfragment 1; BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane; MES, 2-(*N*-morpholino)ethanesulfonic acid.

0006-2960/89/0428-0807$01.50/0

of nucleotides are measured as rate constants for the individual steps of binding (K_0), isomerization (k_{12}), and hydrolysis (k_{23} and k_{32}) by fluorescence stopped flow. Rate constants and relative fluorescence enhancements for these nucleotides are then compared to those previously measured for ATP (Hazzard & Cusanovich, 1986).

Experimental Procedures

Bovine cardiac myosin S1 was prepared by chymotryptic digestion of myosin (Smith & Cusanovich, 1984): the resulting S1 was free of myosin, HMM, and actin as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Taylor & Weeds, 1976). Enzyme activity was assessed by measurement of rate constants using transient kinetics of ATP hydrolysis at pH 7.0 and 15 °C (Hazzard & Cusanovich, 1986). The S1 protein was frozen in liquid nitrogen in 0.3-mL aliquots and stored in cryotubes at −80 °C until used. Concentrations in milligrams per milliliter were determined as follows: myosin, A_{280} = 0.53; S1, A_{280} = 0.64 with a light-scattering correction of 1.5 A_{320}. Ammonium sulfate absolute grade from Research Plus Labs, ATP grade II from Sigma, and chymotrypsin type 1-S from Sigma were used in the S1 preparation. All other chemicals used in protein preparation were of reagent grade.

Stopped-Flow Measurements and Analysis. Prior to kinetic analysis, the protein was allowed to defrost on ice and then diluted to 8 μM with the standard buffer used in these stopped-flow studies: 50 mM BTP, 100 mM KCl, 0.1 mM DTT, and 0.02% NaN_3, pH 7.0 at 15 °C. This method of S1 storage and preparation for the stopped-flow experiment was found to minimize the degradation that can occur when the cardiac protein is handled (Hazzard & Cusanovich, 1986). The buffer and 5–10 mM nucleotide solutions were prepared daily and stored on ice until used. Buffers and salts were of reagent grade. Nucleotides from Sigma (TTP, UTP, GTP, CTP) and Boehringer-Mannheim (GTP) were used without further purification; however, the nucleotides were used within several days of shipment (on dry ice) and the contents of a bottle were discarded if not used within approximately 24 h after opening. Use of very fresh nucleotide and nucleotide solutions was necessary to eliminate a slow phase observed in the transient kinetics.

A fluorescence enhancement for GTP hydrolysis by skeletal myosin was reported by Seidel (1975); however, Eccleston et al. (1979) reported no observable increase in fluorescence with the same reaction. In this study, GTP binding to S1 does produce a fluorescence enhancement. In the event that the increase in fluorescence observed here was due to a slight ATP contamination of the GTP sample, the GTP was analyzed by mass spectrometry using a Varian MAT 311A instrument modified for fast atom bombardment operation using an Ion Tech FAB 11N saddle-field gun operating at 8 kV and 1 mA in the negative ion mode. Argon was used as the bombarding gas. GTP samples from Sigma and Boehringer-Mannheim were dissolved in glycerol, and the mass range m/z 100–1000 was scanned at 11.5 s/decade. The data were acquired and averaged by using an SS300 data system. Addition of crown ether (Fujii et al., 1985) resulted in a 2-fold enhancement of the molecular weight related ions. Analysis of the resulting mass spectrum indicated that any ATP, if present, would comprise less than 5% of the GTP sample. No ions were observed at m/z 506, 528, or 550 for ATP or its sodium adduct. In a separate study using high-pressure liquid chromatography, Buitrago et al. (1988) have shown GTP from commercial sources such as Sigma and Boehringer-Mannheim to be 98.8–100% GTP or GTP derivatives. Thus, ATP contamination of GTP samples from commercial sources has not been demonstrated, and the fluorescence observed in the GTP reaction can therefore only be attributed to the myosin–GTP interaction.

The transient kinetic apparatus and methods of data collection and analysis used in this work have been thoroughly described (Hazzard & Cusanovich, 1986). In the case of CTP, TTP, and GTP, k_{obsd} was determined by fitting the fluorescence increase over the first four half-lives to a single-exponential nonlinear least-squares curve. The y intercept from this fit (i.e., the amplitude) was used to calculate the percent fluorescence enhancement, %ΔFl. For UTP, the data exhibit a slight lag phase not observed with the other nucleotides. The k_{obsd} was determined with the same fitting routine, as the lag was not great enough to affect the analysis. The computer-modeling method used to determine the kinetic parameters was based on the explicit solution of a two-step mechanism for nucleotide binding and hydrolysis preceded by a rapid equilibrium [as shown in eq 1 and described in Hazzard and Cusanovich (1986)].

Determination of Additional Kinetic Parameters. The rate constant for the steady-state hydrolysis of nucleotide was determined by stopped-flow single-turnover experiments in which 4 μM S1 was reacted with 0.5–4.0 μM nucleotide. The decrease in fluorescence that follows the transient phase was monitored as it returned to its original base-line level over a period of 1200 s for TTP and CTP, 400 s for GTP, and 1000 s for UTP. The rate constants was calculated by fitting the observed fluorescence decrease to a single exponential after the first 10 data points that included the transient kinetics were discarded. The reaction with GTP exhibited a slow phase comprising approximately 2% of the total fluorescence change. As 98% of the fluorescence decrease could be best described by a single exponential, the rate constant reported here reflects the major phase of the reaction.

Correction of Fluorescence Values for Inner Filter Effect. In addition to the observed rate constant, the stopped-flow trace also yields the magnitude of the fluorescence enhancement. TTP, CTP, UTP, and GTP have a significantly greater absorption at 295 nm (the excitation light used in these experiments) than does ATP, which results in a decreased fluorescence enhancement. The effect of this inner filter on the determination of fluorescence enhancement is as follows. The fluorescence changes that are observed during the course of the reaction are illustrated in Figure 1A. The fluorescence increases to a maximum level (V_{max}), where a plateau is observed, marking the end of the transient kinetic phase of the reaction. ΔFl is reported as ΔV, which is calculated as the voltage at time t, V_t, minus the maximum voltage, V_{max}. The intrinsic fluorescence of the S1 in the absence of substrate corresponds to the voltage at time zero, V_0, which is not directly observed in the reaction itself. Nucleotide binding and the subsequent isomerization of the S1 occur very rapidly, resulting in a portion of the fluorescence enhancement being "lost" in the dead time. Extrapolation to time zero will not yield an intercept equal to the value of V_0 as the observed rate constant is a mixture of both the binding and the much slower hydrolysis steps. The y intercept observed at the dead time, V_{dt}, will therefore be dependent on nucleotide concentration, as has been previously demonstrated for skeletal (Johnson & Taylor, 1978) and cardiac S1 (Hazzard & Cusanovich, 1986). The fluorescence maximum (V_{max}) does not vary with nucleotide concentration. Thus, the true fluorescence enhancement, $\Delta Fl_{tot} = V_{max} - V_0$, is not actually observed in the experiment, rather, it is the observed fluorescence enhancement, ΔFl_{obsd}, that re-

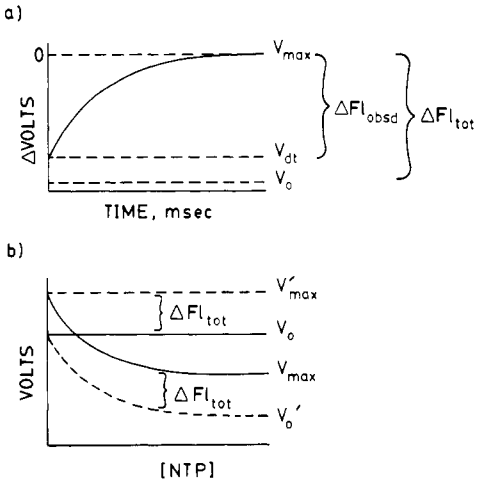

FIGURE 1: (a) Nomenclature for the fluorescence change observed upon mixing myosin S1 with nucleotide: V_0, myosin S1 in buffer, before addition of substrate; V_{dt}, fluorescence at 3 ms after mixing; V_{max}, maximum fluorescence. (b) Correction of V_0 for primary absorption of excitation light. The difference between the observed V_{max} at zero [NTP], V_{max}', and V_0 is taken as the total fluorescence enhancement or ΔFl_{tot}. ΔFl_{tot} is subtracted from V_{max} at all [NTP] to yield V_0'.

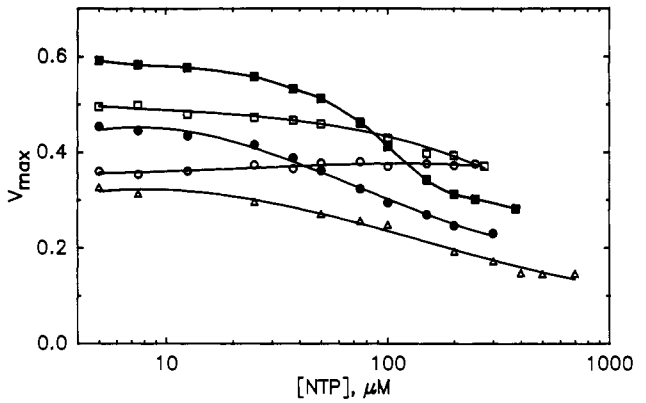

FIGURE 2: Dependence of the apparent maximum fluorescence enhancement, V_{max}, on nucleotide concentration. Myosin S1 concentration is 4 μM. (■) GTP; (□) UTP; (●) TTP; (○) ATP; (△) CTP.

sults. As nucleotide concentration is increased, binding occurs with a greater k_{obsd}, V_{dt} becomes more positive as a larger portion of the fluorescence enhancement is lost in the dead time, and ΔFl_{obsd} decreases. The percent fluorescence enhancement observed is then expressed as

$$\%\Delta Fl = \Delta Fl_{obsd}/V_0 \times 100 \quad (2)$$

and is dependent upon nucleotide concentration.

Regardless of the apparent changes in the voltage at time zero, the fluorescence enhancement returns to the same maximum value, V_{max}, at all ATP concentrations. This occurs because V_{max} is controlled by K_2, the equilibrium between the two fluorescence-enhanced species M_1·T and M_{12}·D·P_i, which is not dependent on substrate concentration. This is manifested experimentally in the myosin–ATP reaction as the independence of V_{max} with respect to [ATP]. However, this is not the case for the nucleotides GTP, TTP, CTP, and UTP: V_{max} is observed to decrease with increasing concentration (Figure 2) due to absorption of the 295-nm excitation light by the nucleotides. This primary absorption is strong enough to result in values of V_{max} that are less than the values for V_0 (which are measured before the addition of nucleotide) at high [NTP].

Although V_{max} is observed to change with [NTP], there is no evidence to suggest that the true value for V_{max} is changing. The effect on the observed V_{max} is therefore taken to be due

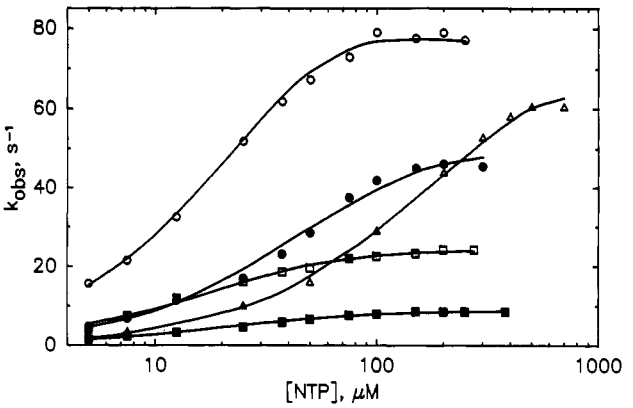

FIGURE 3: Dependence of the observed rate constant of fluorescence enhancement on nucleotide concentration. Symbols are the same as in Figure 2. Lines were generated by computer modeling. Conditions: pH 7.0, 15 °C, BTP–MES–KCl buffer at 200 mM ionic strength.

solely to the inner filter effect. This inner filter effect has a substantial impact on the calculated ΔFl_{obsd}, and a correction must be applied. As shown in eq 2, it is V_0 rather than V_{max} that is used in calculating ΔFl_{obsd}; thus, the correction for the inner filter effect must be applied to V_0. The magnitude of the correction is drawn from the effect of nucleotide concentration on the observed maximum voltage, and this is estimated in the following manner: a value for the maximum voltage in the absence of nucleotide absorption is determined for each nucleotide from the plots in Figure 2 by extrapolation to zero [NTP]. This is referred to as V_{max}'. The total fluorescence enhancement, ΔFl_{tot}, is then $V_{max}' - V_0$. As illustrated in Figure 1b, the ΔFl_{tot} is subtracted from the observed V_{max} at each concentration. This results in the dashed line, which is a corrected V_0 or V_0'. This correction permits ΔFl_{tot} to remain constant regardless of nucleotide concentration, which is the case in the absence of an inner filter effect. The observed fluorescence enhancement at any given [NTP] is then calculated according to

$$\%\Delta Fl = \Delta Fl_{obsd}/V_0' \times 100 \quad (3)$$

Results

Determination of Kinetic Parameters from Stopped-Flow Data. The observed rate constant determined from the monophasic exponential increase in fluorescence with time observed in the stopped flow, k_{obsd}, increases as nucleotide concentration is increased (Figure 3). The absolute value of k_{obsd} and the nature of the concentration dependence for TTP, CTP, UTP, and GTP differ markedly from that for ATP. The observed fluorescence amplitude, expressed as the percent increase in the fluorescence of S1 upon hydrolysis, is dependent upon nucleotide concentration as described under Experimental Procedures. This dependence is observed to be greatest for ATP (Figure 4), whose %ΔFl varies approximately 2-fold over the range of concentrations required to fully saturate the enzyme. The fluorescence enhancement associated with the other nucleotides is not as sensitive to nucleotide concentration. The apparent fluorescence increase for S1 upon reaction with CTP and TTP is greater than that observed for the natural substrate ATP, while the enhancement for S1 with the only other purine studied, GTP, is lower than that for ATP. Fluorescence enhancement in the presence of UTP is closest to the ATP value. The reaction of all nucleotides with S1 results in a decrease in fluorescence enhancement at nucleotide concentrations where binding becomes rate-limiting. The apparent fluorescence maxima decrease in order TTP ~ CTP > ATP > UTP > GTP.

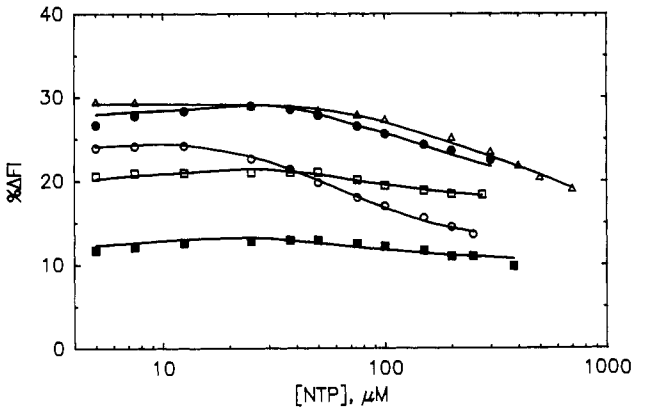

FIGURE 4: Dependence of the observed fluorescence enhancement (%ΔFl from eq 2) on nucleotide concentration. Symbols are the same as in Figure 2. Lines were generated by computer modeling. Conditions the same as for Figure 3.

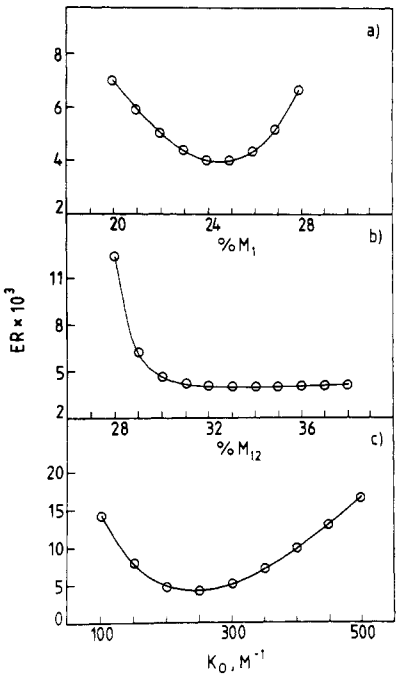

FIGURE 5: Error parameters for the CTP fit shown in Figures 3 and 4. (a) M_{12} and K_0 fixed at 32% and 250 M^{-1}, respectively. (b) M_1 and K_0 fixed at 25% and 250 M^{-1}, respectively. (c) M_1 and M_{12} fixed at 25% and 32%, respectively.

The solid lines of Figures 3 and 4 are drawn through data points that were generated by computer modeling. Typical error wells for these fits are shown in Figure 5 for CTP. Errors are shown only for the relative magnitudes of the two fluorescence enhancements and the value of K_0, as all other parameters were permitted to float. The error well for the variation of $\%M_{12}$ (Figure 5b) is quite broad. As $\%M_{12}$ is increased from 30 to 38, the value for K_2 decreases by 30%; other parameters do not change significantly. This fitting anomaly has been previously observed in the ATP reaction (Hazzard & Cusanovich, 1986). In the absence of a clearly defined error well, the value for $\%M_{12}$ is taken at the beginning of the well (i.e., at 32% for CTP), where the modeling routine is still sensitive to $\%M_{12}$ variations. This is the procedure shown previously to be valid for ATP.

The standard deviation for the fluorescence magnitudes and the kinetic constants was estimated by varying the parameter in question while holding all others constant. The 90% confidence limits of each parameter are set as the variation in the parameter that results in a 50% increase in the total least-squares error (Johnson, 1983). The following standard deviations are average for the four nucleotides: 10% for K_0, 37% for M_1, 4% for M_{12}, 6% for k_{12}, 14% for k_{23}, and 22% for k_{32}. The average point to point error for the fits of k_{obsd} vs [NTP] is ±5.1% for TTP, 4.4% for CTP, 2.9% for GTP, and 5.1% for UTP.

Table I: Normalized Kinetic Parameters for Binding and Isomerization[a]

	K_0 (M^{-1})	k_{12} (s^{-1})	K_0k_{12} (M^{-1} s^{-1})
ATP	1.0 (380)	1.0 (834)	1.0 (3.2 E5)
TTP	1.25	0.24	0.29
CTP	0.66	0.20	0.13
GTP	2.11	0.05	0.10
UTP	1.32	0.28	0.38

[a] Values in parentheses are the measured values for ATP.

Table II: Normalized Kinetic Parameters for Hydrolysis[a]

	k_{23} (s^{-1})	k_{32} (s^{-1})	$k_{23} + k_{32}$ (s^{-1})	K_2
ATP	1.0 (57)	1.0 (17)	1.0 (74)	1.00 (3.5)
TTP	0.65	0.47	0.61	1.40
CTP	0.33	0.77	0.43	0.43
GTP	0.12	0.06	0.11	1.40
UTP	0.37	0.24	0.34	1.49

[a] Values in parentheses are the measured values for ATP.

Table III: Normalized Values for Fluorescence Enhancement[a]

	M_1·T	M_{12}·D·P_i	total %ΔFl	% M_1·T
ATP	1.00 (0.10)	1.0 (0.17)	1.0 (0.27)	1.0 (37)
TTP	1.40	0.94	1.11	1.27
CTP	2.50	0.41	1.19	2.11
GTP	0.40	0.53	0.48	0.84
UTP	0.10	1.18	0.78	0.14

[a] Error is ±10%. Values in parentheses are the measured values for ATP.

The kinetic parameters resulting from computer modeling are listed in Tables I and II. Nucleotide binding by cardiac S1, measured as the equilibrium constant K_0 for the formation of the collision complex M_0·T, varies with nucleotide structure over a range of 3 kJ/mol. Only CTP has a lower K_0 than ATP; for the other nucleotides the equilibrium is shifted to the right. The forward rate constant for isomerization, k_{12}, has been reduced by approximately 4-fold for all nucleotides except GTP, where the reduction is 20-fold. The estimated k_1, however, ranges over only 5 kJ/mol due to the very small value for the reverse rate constant. Within this group of nucleotides, GTP invokes the greatest change in both K_0 and k_{12} relative to values for ATP. Due to the lower k_{12} constants for all nucleotides other than ATP, the combined binding rate constant K_0k_{12} is decreased by as much as an order of magnitude from the value for ATP in the order ATP > UTP ~ TTP > CTP ~ GTP. For the hydrolysis step both forward and reverse rate constants contribute significantly for all nucleotides. The equilibrium constant K_2 ranges from 1 to 5, and the combined hydrolysis rate constant, $k_{23} + k_{32}$, decreases by an order of magnitude in the order ATP > TTP > CTP > UTP > GTP.

The relative fluorescence enhancements of M_1·T and M_{12}·D·P_i resulting from computer modeling are listed in Table III. The average point to point error for the fits of %ΔFl vs [NTP] is ±1.4% for TTP, 1.1% for CTP, 3.0% for GTP, and 0.9% for UTP. Total fluorescence enhancement decreases in the order CTP ~ TTP ~ ATP > UTP > GTP. The low fluorescence for UTP is due to the lack of fluorescence enhancement upon isomerization, as the enhancement upon hydrolysis is similar to that for ATP. The low total fluores-

Table IV: Single-Turnover Rate Constants for Cardiac S1

nucleotide	k_{34} (s^{-1})	nucleotide	k_{34} (s^{-1})
TTP	0.009 ± 0.001	UTP	0.013 ± 0.001
CTP	0.005 ± 0.001	ATP	0.009 ± 0.001
GTP	0.050 ± 0.005		

cence for GTP cannot be ascribed to a loss in fluorescence of M_1·GTP or M_{12}·GDP·P_i as the %M_1·GTP is very similar to that for ATP. Although none of the M_{12}·NDP·P_i complexes exhibit a fluorescence greater than the M_{12}·ADP·P_i complex (the UTP value is within experimental error), the M_1·TTP and M_1·CTP complexes exhibit fluorescence enhancements greater than that for ATP. In fact, in the case of CTP the fluorescence of M_1·T comprises well over half of the total fluorescence enhancement. The only nucleotide in which the total fluorescence enhancement is distributed between M_1·T and M_{12}·D·P_i similarly to ATP is the other purine, GTP.

Single-Turnover Hydrolysis of Nucleotide. The single-turnover experiment measures the rate constant for the overall rate-limiting step of myosin nucleotide hydrolysis, which is the release of inorganic phosphate or k_{34} according to eq 4. The

$$M_0 + T \underset{}{\overset{K_0 k_{12}}{\rightleftharpoons}} M_1{\cdot}T \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} M_{12}{\cdot}D{\cdot}P_i \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} M_1{\cdot}D + P_i \qquad (4)$$

values for k_{34} determined here are given in Table IV. The rate constant is considered to be similar for ATP, TTP, and UTP, low for CTP, and significantly higher than the norm for GTP.

Discussion

The binding and hydrolysis of ATP by cardiac myosin S1 have been described (Hazzard & Cusanovich, 1986) in kinetic terms as a three-step process that begins with the formation of an electrostatically stabilized collision complex M_0·T, which is in rapid equilibrium with the reactants. This is followed by an essentially irreversible isomerization to M_1·T, resulting in an increase in the protein fluorescence of about 10%. In this conformation the γ-phosphate bond of the bound nucleotide is hydrolyzed to form M_{12}·D·P_i, which can readily reform the high-energy bond in a back reaction to M_1·T. The hydrolysis step in the reaction with ATP results in a further increase in fluorescence of 17% to yield a total fluorescence enhancement of 27% (relative to the fluorescence of M_0) upon hydrolysis.

Upon initial inspection it would appear unlikely that structural characteristics of the nucleotide ring would impact significantly on the kinetic parameters describing binding or hydrolysis for the following reasons. In the case of binding, the equilibrium constant for the formation of M_0·T is controlled to a significant extent by electrostatic factors—the binding is enhanced by the presence of a protonated species carrying a positive charge at the active site that would presumably interact with the nucleotide phosphates. With respect to hydrolysis, the distance of the phosphates from the nucleotide rings precludes any inductive effects that might enhance the hydrolysis of the γ-phosphate bond if it is assumed that the nucleotide is binding in an extended conformation, as in the homologous adenylate kinase (Fry et al., 1985, 1986). It is reasonable, therefore, to conclude that the interactions between myosin and the nucleotide are dominated by the nucleotide phosphate esters. However, interactions of active-site residues with the purine or pyrimidine ring could serve to properly position the phosphate bonds for hydrolysis and in this regard could impact on the processes of binding, isomerization, and hydrolysis.

In light of these considerations, it is not unexpected that the binding equilibrium constant K_0 is observed to vary over a relatively narrow range of 3 kJ/mol. This demonstrates not only that the same type of binding process is being observed for all nucleotides but also that binding is in fact relatively insensitive to nucleotide ring structure. Binding is therefore influenced primarily by electrostatic interactions (via the nucleotide phosphate groups) and to a lesser extent by non-electrostatic interactions, such as hydrogen bonding, with the nucleotide rings. The order of binding, in terms of decreasing K_0, is GTP > UTP ~ TTP > ATP > CTP. The nucleotides with a K_0 greater than that for ATP differ from ATP and CTP at the C6 position: GTP, UTP, and TTP have a C6 carbonyl, a hydrogen-bond acceptor, whereas ATP and CTP have C6 amino groups, hydrogen-bond donors. Also, when a carbonyl is present at C6, the N1 has a hydrogen available for hydrogen bonding. Thus, the presence of a C6 carbonyl appears to promote the formation of a stabilized collision complex.

The order of isomerization, in terms of decreasing k_{12}, is ATP > UTP ~ TTP ~ CTP > GTP. This order, unlike that for binding, does not correlate with the substituent at position C6, rather, the group at C2 is important. ATP carries only a proton at this position, whereas UTP, TTP, and CTP have a C2 carbonyl function and GTP has an amino group. This suggests that the presence of a bulky group at C2 restricts the conformational change from M_0·T to M_1·T. A bulky group at this position has been postulated to restrict binding (or some aspect of the binding process as reflected in the K_m) in adenylate kinase (O'Sullivan & Noda, 1968; Fry et al., 1985). The kinetic barrier to isomerization could be due to the collision complex M_0·T having a more tightly bound nucleotide than in the case of ATP. For GTP, then, the increased K_0 and decreased k_{12} may reflect an increased stability of the M_0·T complex relative to either the free reactants or the M_1·T complex.

Those nucleotides with a bulky group at C2 may encounter restrictions not only to isomerization but also to the conformational change that occurs upon hydrolysis. This is observed as a similar order of decrease of k_{12} to that of k_{23} and k_{32}: k_{12}, ATP > UTP ~ TTP ~ CTP > GTP; k_{23}, ATP > TTP > CTP ~ UTP > GTP; k_{32}, ATP > CTP > TTP > UTP > GTP. The change in rate constants for isomerization and hydrolysis is the same in magnitude as well, decreasing a total of 90 ± 5% in going from ATP to GTP. Thus, hydrolysis and isomerization share at least two important characteristics. Both events result in an enhancement of tryptophan fluorescence, and rate constants for both are affected similarly by alterations in nucleotide structure.

The amplitudes of the fluorescence increases in isomerization and hydrolysis are also sensitive to nucleotide structure, but are more difficult to interpret. The fluorescence enhancements reported here for the kinetic intermediates M_1·T and M_{12}·D·P_i reflect not only extinction coefficients but also concentration and therefore can be dependent upon relative values of the rate constants for isomerization and hydrolysis. (Fluorescence amplitudes are not otherwise expected to correlate with rate constants, as the fluorescence reflects the structure of the product complex rather than the structure of the transition state.) If the extinction coefficients for M_1·T and M_{12}·D·P_i were unaffected by nucleotide structure, then correlations encompassing all nucleotides could be drawn with k_{12} and K_2. The absence of such correlation indicates that extinction coefficients are different for some of the nucleotides. In this sense, the fluorescence enhancement of ATP should be greater than all other nucleotides, as the concentrations of M_1·T and

M_{12}·D·P_i will be greater at any given point in time during the reaction. As this is not the case, it is deduced that the extinction coefficients for M_1·T and M_{12}·D·P_i for the ATP complex are lower than those for TTP and CTP, which have comparable enhancements to that of ATP.

The amino group at C6 has been postulated to be necessary for maximal fluorescence enhancement in skeletal myosin, as the order in decreasing total enhancement is ATP ~ CTP > UTP > GTP (Seidel, 1975; Weber et al., 1972). The total fluorescence enhancement measured in this work is in agreement with this order. However, TTP is shown to exhibit a fluorescence increase equal to that of ATP and CTP, and this nucleotide has a carbonyl at C6. Closer inspection of the relative contributions of M_1·T and M_{12}·D·P_i to the total fluorescence enhancement shows that the nature of the fluorescence increase of S1 in the presence of ATP is quite different from that of CTP. With ATP, the majority of the fluorescence increase is due to hydrolysis, whereas in CTP, the majority is due to isomerization. The distribution of fluorescence enhancement in the case of TTP is intermediate between ATP and CTP, where the same degree of fluorescence increase occurs in isomerization as in hydrolysis.

The fluorescence change due to isomerization for the M_1·CTP complex is significantly higher than that of M_1·ATP: this does not correlate with the isomerization rate constant, which, while low, is no different than k_{12} for TTP or UTP. What is distinctive about CTP is the low value for K_2, indicating that the concentration of M_1·CTP is greater than that of M_1·ATP; thus, the CTP complex contributes more to the total fluorescence enhancement than does the corresponding ATP complex. For GTP and UTP the fluorescence of M_1·T is significantly lower than that of ATP. For GTP, the k_{12} is very low, which will result in a lower concentration of the M_1·T complex and therefore a lower fluorescence enhancement. In the case of UTP, the kinetic constants k_{12}, k_{23}, k_{32}, and K_2 are very similar to those for the TTP reaction, which results in fluorescence enhancement comparable to that of the ATP reaction, so the concentration of M_1·T will be similar for both UTP and TTP. Thus, the only rationale for the very low fluorescence enhancement of the UTP complex is a reduced extinction coefficient.

The fluorescence enhancement upon hydrolysis differs significantly from that for ATP only in the case of CTP and GTP. The low values for CTP and GTP result from the relatively low concentrations of M_{12}·D·P_i relative to M_1·T as explained above.

Thus, differences in the concentrations of the kinetic intermediates can provide reasonable explanations for the variations in fluorescence enhancement observed among TTP, CTP, and GTP. In the case of TTP and CTP an increase in extinction coefficients relative to the ATP intermediates has been postulated. For GTP there is no evidence for an increase in extinction coefficients: they may well be similar to the ATP values. In the UTP complex formed upon isomerization there must be a decrease in the inherent fluorescence. This M_1·UTP complex still hydrolyzes the γ-phosphate bond, producing a fluorescence enhancement comparable to that for ATP.

Of all the parameters measured in this work, the effect of nucleotide ring structure is most pronounced in the rate constants for isomerization and hydrolysis, where a bulky group at the C2 position may restrict conformational changes that occur during both events. The similar sensitivity of both isomerization and hydrolysis to specific structural characteristics of the nucleotide ring suggests that these two events involve comparable structural changes in the myosin protein. The similarities noted here for isomerization and hydrolysis must be carefully considered in light of the very different thermodynamic properties that have been measured and/or estimated for these two processes. Isomerization proceeds with a large change in free energy and a moderate loss in entropy, while hydrolysis results in only a small change in free energy but a large increase in entropy (Hazzard & Cusanovich, 1986). The large entropy gain in hydrolysis may be due to a breakdown of ordered water structure, occurring as exposed hydrophobic groups (such as tryptophans) become more buried (Kodama & Woledge, 1979). Heat capacity measurements support this interpretation (Kodama, 1981). The decrease in entropy in the overall binding process (which includes isomerization) may be related to a hydrophobic effect as well (Kodama, 1981). Thus, isomerization and hydrolysis may act in tandem to permit the myosin molecule to accept and store the free energy of hydrolysis of the bound nucleotide.

To summarize, the binding of nucleotide to cardiac myosin S1 is enhanced, but only slightly, by the presence of a carbonyl at the C6 position of the nucleotide. The isomerization of the S1–nucleotide complex and the ensuing nucleotide hydrolysis are inhibited by a bulky group at the C2 position. The magnitude of the fluorescence enhancement appears to be controlled largely by the relative values of the rate and equilibrium constants.

Registry No. ATP, 56-65-5; TTP, 365-08-2; CTP, 65-47-4; UTP, 63-39-8; GTP, 86-01-1; ATPase, 9000-83-3.

References

Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331–349.

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., & Trentham, D. R. (1974) *Biochem. J. 141*, 351–364.

Balint, M., Wolf, L., Tarcs-Falvi, A., Gergely, J., & Sreter, F. A. (1978) *Arch. Biochem. Biophys. 190*, 793–799.

Blum, J. J. (1960) *Arch. Biochem. Biophys. 81*, 104–119.

Buitrago, F., Canales, J., & Sillero, A. (1988) *Anal. Biochem. 171*, 389–392.

Eccleston, J. F., & Trentham, D. R. (1977) *Biochem. J. 163*, 15–29.

Eccleston, J. F., & Trentham, D. R. (1979) *Biochemistry 18*, 2896–2904.

Eisenberg, E., & Hill, T. L. (1985) *Science (Washington, D.C.) 227*, 999–1006.

Flamig, D. P., & Cusanovich, M. A. (1983) *J. Biol. Chem. 258*, 977–983.

Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1985) *Biochemistry 24*, 4680–4694.

Fry, D. C., Kuby, S. A., & Mildvan, A. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 907–911.

Fujii, I., Isobe, R., & Kanematsu, K. (1985) *J. Chem. Soc., Chem. Commun.*, 405–408.

Hazzard, J. H., & Cusanovich, M. A. (1986) *Biochemistry 25*, 8141–8149.

Johnson, K. A. (1983) *J. Biol. Chem. 258*, 13825–13832.

Johnson, K. A., & Taylor, E. W. (1978) *Biochemistry 17*, 3432–3442.

Kielley, W. W., Kalckar, M., & Bradley, L. B. (1956) *J. Biol. Chem. 219*, 95–110.

Kodama, T. (1981) *J. Biol. Chem. 254*, 6382–6386.

Kodama, T., & Woledge, R. C. (1979) *J. Biol. Chem. 254*, 6382–6386.

Marston, S. B., & Taylor, E. W. (1980) *J. Mol. Biol. 139*, 573–600.

Morkin, E. (1979) *Circ. Res. 44*, 1–7.

Morkin, E., Flink, I. L., & Goldman, S. (1983) *Prog. Cardiovasc. Dis. 25*, 435–464.
O'Sullivan, W. J., & Noda, L. (1968) *J. Biol. Chem. 243*, 1424–1433.
Seidel, J. C. (1975) *J. Biol. Chem. 250*, 5681–5687.
Smith, S. J., & Cusanovich, M. A. (1984) *J. Biol. Chem. 259*, 9365–9368.
Smith, S. J., & White, H. D. (1985) *J. Biol. Chem. 260*, 15146–15155.
Taylor, E. W. (1979) *CRC Crit. Rev. Biochem. 6*, 103–164.
Taylor, R. S., & Weeds, A. G. (1976) *Biochem. J. 159*, 301–315.
Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2883.

A Differential Scanning Calorimetric Study of the Binding of Sulfate Ion and of Cibacron Blue F3GA to Yeast Phosphoglycerate Kinase†

Cui Qing Hu and Julian M. Sturtevant*
Departments of Chemistry and of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511
Received April 26, 1988; Revised Manuscript Received August 9, 1988

ABSTRACT: In continuation of earlier work [Hu, C. Q., & Sturtevant, J. M. (1987) *Biochemistry 26*, 178–182], differential scanning calorimetry has been employed in a study of the effects on the thermal denaturation of yeast phosphoglycerate kinase of two inhibitors of the enzyme, sulfate ion and the dye Cibacron blue F3GA. Sulfate ion, as is usual with ligands that dissociate during unfolding of the host protein, raises $t_{1/2}$, the temperature of half-completion of the denaturation, has only a modest effect, stemming from the enthalpy of dissociation of the ligand, on the enthalpy of denaturation, and has little or no effect on the heat capacity change resulting from denaturation. In sharp contrast, Cibacron blue F3GA lowers $t_{1/2}$ and drastically decreases both the enthalpy and heat capacity changes due to denaturation. The DSC results with sulfate ion are consistent with previous kinetic data [Scopes, R. K. (1978) *Eur. J. Biochem. 91*, 119–129; Khamis, M. H., & Larsson-Raznikiewicz, M. (1981) *Biochim. Biophys. Acta 657*, 190–194], which indicate two binding sites for sulfate ion at one of which the ligand acts as a competitive inhibitor. The results with Cibacron blue F3GA indicate that the dye induces a major destabilizing structural change in the enzyme in addition to rendering it enzymically inactive.

In earlier work (Hu & Sturtevant, 1978) the thermodynamics of the binding of MgADP, MgATP, and 3-phosphoglycerate (3-pG)[1] to yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) (PGK) and of the thermal unfolding of the enzyme in the absence and presence of these ligands was studied. In this paper we extend our differential scanning calorimetric (DSC) study to include the effects on the thermal denaturation of PGK of two inhibitors of the enzyme, sulfate ion and Cibacron blue F3GA (CB3GA), in the absence and the presence of 3-pG and MgATP. Sulfate ion has been reported to play a dual role, as both an activator at low concentration and a competitive inhibitor at high concentration (Scopes, 1978; Khamis & Larsson-Raznikiewicz, 1981; Mas et al., 1988), with two dissimilar binding sites on the protein. CB3GA also appears to behave as a competitive inhibitor with either one (Thompson & Stellwagen, 1976) or two (Beissner & Rudolph, 1979) binding sites on the protein.

Materials and Methods

Yeast phosphoglycerate kinase was purchased from Sigma Chemical Co. (lot no. 64F-8130) as an ammonium sulfate precipitate. Enzyme solutions were prepared by exhaustive dialysis against a buffer containing 50 mM PIPES and 0.1 mM DTE at pH 7.00. Protein concentrations were determined spectrophotometrically by taking the absorbance of a 1 mg mL^{-1} solution at 280 nm to be 0.57[2] (Blake et al., 1972). Protein solutions were used within 2 days of preparation.

The magnesium salt of ATP and the disodium salt of D-(−)-3-phosphoglyceric acid were products of Sigma Chemical Co. CB3GA was purchased from Fluka. All other chemicals were of reagent grade. Doubly deionized water was used throughout.

The DSC experiments were performed with a DASM-4 instrument (Privalov, 1980) purchased from Mashpriborintorg, Moscow, USSR. All scans were run at 1 K min^{-1}. Base lines were drawn as described by Takahashi and Sturtevant (1981), and enthalpies were evaluated by planimeter integration. van't Hoff enthalpies were calculated by the equation

$$\Delta H_{vH} = 4RT^2_{1/2}C_{ex,1/2}/\Delta H_{cal}$$

where $T_{1/2} = 273.15 + t_{1/2}$, $t_{1/2}$ is the temperature in degrees Celsius of half-completion of the denaturation, $C_{ex,1/2}$ is the

† This work was supported by Grant GM-04725 from the National Institutes of Health and Grant DMB-8421173 from the National Science Foundation.

[1] Abbreviations: CB3GA, Cibacron blue F3GA; DSC, differential scanning calorimetry; DTE, dithioerythritol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); 3-pG, 3-phosphoglycerate; PGK, yeast phosphoglycerate kinase.

[2] This value is listed by Blake et al. (1972) and by Pickover at al. (1979) and was used by us in our earlier paper (Hu & Sturtevant, 1987), whereas Krietsch and Bucher (1970) and Thompson and Stellwagen (1976) gave the value 0.49. All of our values for ΔH_{cal} and ΔC_p^d can be brought into line with any future version of the absorbance by simple multiplication by the appropriate factor. The values for ΔH_{vH} would remain unchanged.

0006-2960/89/0428-0813$01.50/0