Transient Phase of Adenosine Triphosphate Hydrolysis by Myosin, Heavy Meromyosin, and Subfragment 1†

Edwin W. Taylor‡

ABSTRACT: The transient phase of adenosine triphosphate (ATP) hydrolysis (early burst) was investigated for myosin, heavy meromyosin (HMM), and subfragment 1 (S-1) over a range of temperatures and pH's. The burst size at pH 8, 20 °C, is 0.8–0.85, based on steady-state and transient measurements. The equilibrium constant for the enzyme-substrate to enzyme-product transition is 0.85 ± 0.05. It is concluded that both myosin heads undergo the rapid hydrolysis step and that there are no significant differences for S-1 vs. HMM or myosin. The transient data are fitted reasonably well by a single rate process, but available evidence is consistent with some heterogeneity and a range of rate constants differing by a factor of two. At pH 6.9 and 3 °C, the burst size is 0.5 and the hydrolysis is slower than the configuration change measured by fluorescence. The results are consistent with the kinetic scheme

$$M \overset{(1)}{\rightleftharpoons} M\cdot ATP \overset{(2)}{\rightleftharpoons} M\cdot ATP^{**} \overset{(3)}{\rightleftharpoons} M\cdot Pr^{**} \overset{(4)}{\underset{\downarrow P_i}{\rightleftharpoons}} M\cdot ADP^{*} \overset{(5)}{\rightleftharpoons} M\cdot ADP \overset{(6)}{\rightleftharpoons} M + ADP$$

The lower burst at low temperature and pH can be partly explained by a reduction in the equilibrium constant, K_3, and ATP can be synthesized on the enzyme by a pH–temperature jump.

A characteristic property of myosin ATPase[1] is the hydrolysis of approximately one molecule of substrate per active site at a rate which greatly exceeds the steady-state rate. This effect termed the early burst was studied extensively by Tonomura who postulated an elaborate mechanism to account for the burst as well as other properties of the enzyme (Kinoshita et al., 1969; Tonomura and Inoue, 1974). Lymn and Taylor (1970,1971) proposed a simple scheme for the early burst (Lymn and Taylor, 1970; Taylor et al., 1970):

$$M + ATP \overset{(1)}{\rightleftharpoons} M\cdot ATP \overset{(2)}{\rightleftharpoons} MPr \overset{(3)}{\rightleftharpoons} M + Pr$$

where MPr refers to a myosin–product intermediate. This mechanism was based on direct measurements of the three steps, the apparent rate of substrate binding k_1[a], the hydrolytic step k_2, and the release of products k_3. The first two rate constants were obtained from transient-state measurements of phosphate formation and the third from rapid chromatography. Although the scheme is too simple, it accounted for the majority of the available evidence pertaining to phosphate hydrolysis. Further studies have required an expansion of the scheme by the inclusion of other intermediate states which can be distinguished experimentally. A reaction sequence has been proposed by Trentham and collaborators (Bagshaw and Trentham, 1974) for S-1 and independently for myosin and S-1 by Koretz and Taylor (1975)

$$M + ATP \overset{(1)}{\rightleftharpoons} M\cdot ATP \overset{(2)}{\rightleftharpoons} M\cdot ATP^{**} \overset{(3)}{\rightleftharpoons} (MPr)^{**} \overset{(4)}{\underset{H^+\downarrow P_i}{\rightleftharpoons}} M\cdot ADP^{*} \overset{(5)}{\rightleftharpoons} M\cdot ADP \overset{(6)}{\rightleftharpoons} M + ADP$$

where the asterisks refer to the degree of fluorescence enhancement. The scheme accounts for the kinetic properties of HMM and S-1 for a range of conditions of temperature and pH (Sleep and Taylor, submitted) based on fluorescence and proton measurements. The hydrolysis step is assumed to occur in sequence in the pathway, since the apparent second-order rate constant determined from phosphate measurements agrees with the value obtained from proton or fluorescence measurements (Koretz and Taylor, 1975). The maximum measured rate of hydrolysis is comparable to k_2 at pH 8 and 20 °C; consequently, $k_3 \gtrsim k_2$ and the actual rate has not been measured. The scheme will be referred to as the Bagshaw–Trentham mechanism.

The scheme based on spectroscopic evidence is consistent with an identical independent site mechanism but agreement has not been reached on whether the hydrolysis step is the same for both heads. Lymn and Taylor obtained a burst size of only 0.7–0.8 mol per site and the variation with concentration did not appear to fit a single dissociation constant. The experiments were designed to measure rate constants rather than burst size and the data were not analyzed statistically. A possible reason for a burst of less than one is the finding by Bagshaw and Trentham (1973) that the equilibrium constant, K_3, is only 9, which could reduce the burst to 0.9 mol per site. Other laboratories have reported bursts of 0.6–0.8, but the experiments were generally incidental to other studies (Seidel, 1975).

Tonomura and collaborators have made the only other extensive study of the burst size and they report values of 0.5 mol per site (Inoue et al., 1972), and together with other evidence they have proposed a mechanism in which the two myosin heads and the two classes of S-1 molecules derived from them have different hydrolysis pathways (Tonomura and Inoue, 1974).

The studies reported here were undertaken to try to determine whether or not the heads of myosin are identical and independent in the catalytic properties, and to extend the measurements to low temperatures in order to distinguish steps two and three.

† From the MRC Cell Biophysics Unit, London WC-2, England. *Received February 4, 1976.*

‡ Present address: Department of Biophysics and Theoretical Biology, The University of Chicago, Chicago, Ill. 60637.

[1] Abbreviations used are: ATP and ADP, adenosine tri- and diphosphates; ATPase, adenosine triphosphatase; S-1, subfragment 1; HMM, heavy meromyosin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; Mes, 4-morpholineethanesulfonic acid.

Materials and Methods

Preparation of Proteins. Myosin was prepared from rabbit back and leg muscles following the procedure of Perry (1955), as modified by Starr and Offer (1971). For the preparation of S-1, aggregated myosin in 0.1 M KCl was digested with soluble papain and the reaction was terminated by adding iodoacetic acid to a final concentration of 1 mM at pH 7. According to Weeds and Lowey (personal communication), the method does not produce measurable modification of the SH-1 sulfhydryl group. HMM was prepared by the method of Lowey et al. (1969). Myosin purified by precipitation contains impurities, mostly C protein, as determined by sodium dodecyl sulfate gel electrophoresis (Starr and Offer, 1971). The examination of several preparations showed the content of impurities to be essentially constant, ranging from 11 to 14%. Further purification by chromatography on DEAE-cellulose 52 removed most of the impurities and column-purified material was used in some experiments. The S-1 was also purified for some experiments by chromatography on Sephadex G-200 (Lowey et al., 1969), or DEAE-52.

In the later stages of this work, measurements were made on HMM and S-1 prepared by chymotryptic digestion. Two preparations were further purified by chromatography on DEAE-cellulose 52 for measurement of maximum burst but the two species of S-1 molecules were not separated. We thank Dr. Weeds for providing information on digestion conditions prior to publication (Weeds and Taylor, 1975). On sodium dodecyl sulfate gels, the preparations show fewer bands and the "nonessential" light chain is preserved in the HMM preparation and absent from the S-1 preparation. The magnitude of the burst was slightly larger than for corresponding proteins prepared by trypsin and papain digestions. Consequently, there is no correlation between burst size and presence of the nonessential light chain.

Concentrations in milligrams/milliliter were obtained from the relations $(A_{280} - 1.5\ A_{320})/0.533$, $A_{280}/0.647$, and $A_{280}/0.78$ for myosin, HMM, and S-1, respectively. As there is some variation in turbidity of myosin preparations, the scattering is not completely compensated by subtraction of absorption at 320 nm and could lead to some error in concentration determinations. The molecular weights were assumed to be 4.7, 3.5, and 1.15×10^5 for myosin, HMM, and S-1. Molecular weights for proteins prepared by chymotryptic digestion were assumed to be the same as the corresponding values for tryptic and papain digestions.

Phosphate Analysis. In all experiments, hydrolysis was measured by use of [γ-^{32}P]ATP obtained from Radiochemical Centre, Amersham. There is no completely satisfactory method for the determination of small quantities of phosphate in the presence of ATP. The method used previously (Lymn and Taylor, 1970) was used in most of the steady-state determinations, namely, precipitation of phosphate as the triethylamine–molybdate complex. ATP was removed by adsorption to charcoal before adding molybdate which is an effective catalyst for ATP hydrolysis in acid solution. Radioactive phosphate was counted as the precipitate using an end window counter (Nuclear Chicago) or redissolved in 2 ml of acteone and counted in Triton–toluene (Packard Tri-Carb). The ATP blank could be reduced to 1% by this method.

A simple assay was developed for Cerenkov counting, since with this method a large aqueous sample can be counted. The reaction mixture in 1 ml of 1 N perchloric acid was added to 4 ml of charcoal (10 mg/ml in 0.1 N HCl) and agitated intermittently for 5 min at 0 °C. The mixture was filtered by suction on a 0.45-nm Millipore filter. Celite was added if necessary as a filter aid, since it does not adsorb phosphate. The filter was washed with 0.1 N HCl and the filtrate in a volume of 10 ml was counted directly. Recovery of phosphate is quantitative and zero blanks are 2.0% but very reproducible. Filtration on a charcoal–Celite column (2 ml of a 1:1 charcoal–Celite mixture) reduced the blank to 1% and this method was used in later experiments.

The radioactive ATP stock solution was repurified by batch elution from DEAE-Sephadex A-50 if the zero blank exceeded 3%.

Assay Conditions. In steady-state experiments, the reaction was initiated by mixing equal volumes of enzyme and substrate in a hand operated "T" mixer. Particularly with myosin, the mixing step can produce aggregation and precipitation of protein; thus, direct mixing in a test tube was avoided. Reaction was terminated with 1–2 volumes of 2 N perchloric acid. Carrier ATP and phosphate were added with the acid (10^{-4} M). For each experimental point, an aliquot of the mixture was counted to determine total radioactivity. A complete hydrolysis sample was included to check recovery unless the ATP concentration was extremely high. The ratio of ATP/sites was maintained around 5 unless the ATP concentration was greater than 5×10^{-5} M. Individual samples were processed immediately and the time for various steps in the analysis was kept constant as far as possible to reduce variations from hydrolysis of ATP during analysis. Five or six measurements were made over the time range from 5 to 60 s. The burst and rate were calculated by a least-square fit of the data to a straight line. For very low ATP concentrations, the first point was taken at 10–15 s to ensure a steady state had been obtained.

The quench-flow apparatus described previously (Lymn and Taylor, 1970) was used in the initial part of this study. The majority of the experiments were performed with a second machine, which will be briefly described. The drive syringes are thermostated by circulating water in an aluminium block and reaction times from 5 ms to several seconds were obtained by operating in direct drive or delay-line modes. The experiments described here were performed over a period of 2 years, during which the machine had undergone a number of modifications. In its present form, the reagents are mixed in an eight-jet Gibson mixer and pass through a lucite cylinder to a second mixer (Berger or offset-T). Reaction times are varied by interchanging lucite cylinders of different lengths and by varying gas pressure to cover a time range up to 100 ms. For longer reaction times, a delay line is used, and drive occurs in two steps with a time interval set by an electronic timer and relay. The reaction is terminated by driving the sample out of the line with buffer from a fourth syringe, which enters via a T connection just below the first mixer. The delay line consisted of a lucite cylinder enclosed in a water jacket. Thick lucite cylinders (2-cm diameter) were used because of their low-heat conduction. Change in sample temperature between mixing and quenching was <0.5 °C for a 2-s reaction.

Kinetic Equations

A main purpose of these studies is to decide whether the kinetics of hydrolysis is consistent with the Bagshaw–Trentham mechanism over a range of pH, temperature, and concentration conditions. A general solution to the rate equations for a six-step mechanism is too complex to be useful, but a knowledge of the rate and equilibrium constants determined previously can be used to obtain an approximate solution. Under the conditions employed, the first step is a rapid equilibrium (Bagshaw et al., 1974; Sleep and Taylor, submitted) and the rate of ATP dissociation (k_{-2}) is small compared to the

TABLE I: Magnitude of Phosphate Early Burst.[a]

Procedure	Burst Size (mol/site)
Steady state, column purified	Myosin 0.79 ± 0.08(6) S-1 0.78 ± 0.06(6)
Steady state, precipitation	Myosin 0.69 ± 0.06(16) S-1 0.70 ± 0.03(12)
Transient, 0.5–1 s	Myosin, HMM, S-1 0.80 ± 0.13(12)
Excess enzyme, 0.5–1 s	Myosin, HMM, S-1 0.85 ± 0.05(6)
Transient-Chase	Myosin, HMM, S-1 0.84 ± 0.03(6)

[a] All measurements at pH 8, 20 °C, 10 mM Tris buffer, 5–10 mM $MgCl_2$. Numbers in parentheses refer to number of measurements. Steady-state measurements are average values for 12 myosin and 10 S-1 preparations. ATP concentration, 10^{-5} M to 3×10^{-4} M, in 0.5 M KCl. Transient refers to extent of hydrolysis at 0.5–1 s, ATP concentration 10^{-5} to 10^{-4} M, 0.5 M KCl (myosin), 0.05 M–0.1 M KCl (HMM and S-1). The value is the average for a variety of experiments, and includes the variation from different purification and digestion methods. Excess enzyme refers to fraction of ATP hydrolyzed at 0.5–1 s by tenfold excess of enzyme sites. In transient-chase experiments, the active-site concentration is determined by final amount of ATP hydrolyzed after mixing with a 100-fold excess of unlabeled ATP at 1 s. Initial ATP to site ratio was 2.5–4 mol/site. Error limits refer to standard deviation of the mean.

steady-state rate at 20 or 0 °C (Mannherz et al., 1974; Wolcott and Boyer, 1974; Arata et al., 1975); consequently, the first two steps can be treated as an essentially irreversible step of rate λ_1. The hydrolysis step (3) is fast compared to the steady-state rate and evidence is presented that the step is reversible; thus, k_3 and k_{-3} are large compared to k_4. For temperatures above 15 °C, the rate-limiting step is k_4; thus, steps 4–6 can be treated as a single process of rate k_4. The K_M is much smaller than the dissociation constants of phosphate or ADP and rate measurements were made with substrate present in four- to fivefold excess over products. Reversal of the dissociation step can be neglected under these conditions.

The kinetic scheme can be treated as a three-step process

$$\mathrm{M + ATP} \overset{\lambda_1}{\rightleftharpoons} \mathrm{M{\cdot}ATP^{**}} \overset{\lambda_2}{\rightleftharpoons} \mathrm{M{\cdot}Pr^{**}} \overset{k_4}{\rightleftharpoons} \mathrm{M + ADP + P_i}$$

The solution of the rate equations for MPr** and the formation of free phosphate $(P_i)_f$ subject to the conditions on the rate constants outlined above is

$$\mathrm{MPr^{**}} = \left[1 - \frac{\lambda_1}{\lambda_1 - \lambda_2}\exp(-\lambda_2 t) + \frac{\lambda_2}{\lambda_1 - \lambda_2}\exp(-\lambda_1 t)\right] \times \left(\frac{K_3}{K_3 + 1}\right)\left(\frac{S}{S + K_m}\right)$$

$$(P_i)_f = \int_0^t k_4(\mathrm{MPr^{**}})dt$$

where S refers to substrate concentration, $\lambda_2 = k_3 + k_{-3}$, and MPr** and $(P_i)_f$ are expressed relative to myosin site concentration, two per myosin molecule. For moderate ATP concentrations such that $K_1k_2S \gg k_4$, $\lambda_1 = K_1k_2S/(K_1S + 1)$. The quantity K_1k_2 is the apparent second-order rate constant for ATP binding determined from fluorescence measurements. The condition is satisfied for concentrations greater than 0.5 μM. If $\lambda_2 > \lambda_1$, the time dependence of MPr** formation (the early burst) will fit a single exponential $[1 - \exp(-\lambda_1 t)]$, which appears to be the case at 20 °C. The steady-state behavior is obtained from the transient solution for large t. The small value of K_1 (circa 10^4 M^{-1}) compared to the measured quantity K_m^{-1} leads to a fairly simple expression for the Michaelis constant. The burst, r, is defined as the steady-state value of MPr** and the expressions for r and the steady-state rate, V, are:

$$r = \left(\frac{K_3}{K_3 + 1}\right)\left(\frac{S}{S + K_M}\right) \qquad V = \frac{V_M S}{S + K_M}$$

where

$$K_M = \frac{k_4}{K_1k_2}\left(\frac{K_3}{K_3 + 1}\right) \qquad V_M = k_4\left(\frac{K_3}{K_3 + 1}\right)$$

The quantity measured experimentally is total phosphate which is equal to MPr** plus $(P_i)_f$. The burst is determined by linear extrapolation of the steady-state rate to $t = 0$. This procedure defines the apparent burst $r^a = r + \int_0^t k_4(\mathrm{MPr^{**}})dt - k_4rt$, where t is large. The apparent burst is equal to r if the time lag in reaching the steady-state rate of free phosphate formation is small compared to k_4^{-1}. The condition is satisfied at moderate ATP concentrations, but in order to compare the concentration dependence of r and V it is necessary to choose ATP concentrations such that K_1k_2S is comparable to k_4. In this range, $\lambda_1 = K_1k_2S + K_3k_4/(K_3 + 1)$, and $\lambda_2 \gg \lambda_1$. Substitution of these values and solution of the integral give

$$r^a = r\left(1 - \frac{k_4}{K_1k_2S + K_3k_4/(K_3 + 1)}\right)$$

Under the experimental conditions employed, r is underestimated by linear extrapolation for ATP concentration less than 3×10^{-7} M. At low temperatures, k_4 and k_5 in the complete scheme are comparable (Bagshaw and Trentham, 1974; Sleep and Taylor, submitted). The step k_4 in the approximate scheme has to be replaced by two steps

$$\mathrm{M{\cdot}Pr^{**}} \overset{k_4}{\rightleftharpoons} \mathrm{M{\cdot}ADP^{*}} \overset{k_5}{\rightleftharpoons} \mathrm{M + ADP}$$

where again the two steps can be treated as irreversible for the conditions employed. The values of rate constants measured at low temperature by Sleep and Taylor and phosphate measurements reported here still satisfy the assumptions used in solving the rate equation. The relations given above are preserved with k_4 replaced by the quantity $k_4k_5/(k_4 + k_5)$.

Results

Size of the Early Phosphate Burst. The early burst was first compared for myosin and S-1 under the same conditions (0.5 M KCl, pH 8, 20 °C) by extrapolation of the steady-state rate to zero time. Twelve myosin and ten S-1 preparations were used to obtain a representative sample for statistical analysis. Myosin purified by standard precipitation procedures contains 11–14% of other components, as determined by densitometry of sodium dodecyl sulfate gels stained with Coomassie blue. Column purification removes these contaminants, mostly C protein, and the burst is slightly increased (Table I). The standard error in a single determination of the burst estimated from the coefficient of the least-squares fit is typically ±10%. The comparison shows that C protein itself has no direct effect on the burst. For S-1, column purification removes about 10% of the total protein and there is a small increase in burst size.

The burst measured within 1 to 2 days after preparation of myosin by the precipitation method was larger than the average of all determinations. Storage of myosin for 3–6 days at 4 °C led to a decrease of 10–15% in the burst. A problem with col-

umn purification is a probable loss of activity during the procedure and a better estimate of the myosin burst is provided by measurements on fresh preparations, corrected for the impurities which averaged 12%. On this basis, the burst is 0.85–0.89 mol per site. A further error arises in estimating the S-1 burst, since some inactive material is probably produced by proteolytic digestion. Chymotryptic S-1 gave slightly larger values than papain S-1, ranging from 0.80 to 0.85 for column-purified material. Within experimental error, the burst per S-1 or myosin site was essentially the same when measured under the same conditions.

Transient experiments provide a measure of the burst from the extent of hydrolysis in 0.5–1.0 s at moderately high ATP concentrations. The average from a variety of experiments with myosin, HMM, and S-1 was 0.8 ± 0.13. The figure given is the average from a variety of experiments on column-purified and standard preparations. The largest values were obtained with chymotryptic HMM and S-1. An example of a transient experiment is given in Figure 2.

The standard deviation of the mean of 20 steady-state measurements on myosin was ±10%; thus, the error in the mean is three times larger than expected from random errors in the phosphate measurements. The precision of the measurement is therefore limited by the variation in content of impurities and inactive enzyme. Accepting the argument of Bagshaw and Trentham (1973), the deviation from unity arises from the value of the equilibrium constant K_3, which determines the amount of M·ATP** present in the steady state. Conversely, the fraction of ATP rapidly hydrolyzed for enzyme sites present in large excess over substrate is an alternate measure of the size of the early burst which is independent of impurities. The extent of hydrolysis at 0.5–1.0 s for ten to one site excess was measured for myosin, HMM, and S-1 and gave a value of 0.85 ± 0.05 mol per site (Table I).

A further method, which is independent of impurities or inactive enzyme, is provided by the finding that ATP is essentially irreversibly bound (Mannherz et al., 1974). The burst was measured at 0.5–1.0 s and a large excess of unlabeled ATP was mixed with the sample using the quenched-flow apparatus. The reaction was stopped with acid after a time equal to five times the lifetime of the product complex. The total ATP hydrolyzed measures the content of active ATP binding sites and was 5–20% less than the measured protein concentration, depending on the method of purification. The corrected burst obtained by this method was 0.84 ± 0.03.

It is concluded that the early phosphate burst is 0.8–0.85 mol per site for myosin, HMM, or S-1 at pH 8 and 20 °C. The value agrees with the result expected for an equilibrium between M·ATP** and M·Pr** determined by chase experiments. Both myosin heads and all S-1 molecules must contain a site at which ATP is tightly bound and rapidly hydrolyzed.

The size of the burst was measured for a range of conditions of ionic strength, pH, and temperature. The magnitude was essentially independent of ionic strength up to 0.5 M in KCl and decreased slightly with decreasing pH or temperature. At 3 °C and pH 6.9, the value is 0.5 ± 0.05. Modification of the SH-1 sulfhydryl group did not affect the burst.

Concentration Dependence of the Burst and Steady-State Rate. Myosin and S-1 were compared in 0.5 M KCl for ATP concentrations ranging from 10^{-7} to 3×10^{-4} M (pH 8, 20 °C). The variation in burst and steady-state rate normalized to the same maximum value is illustrated in Figure 2 in which the data are plotted vs. log [ATP]. The error bars refer to the total range generated by the standard deviations of the bursts

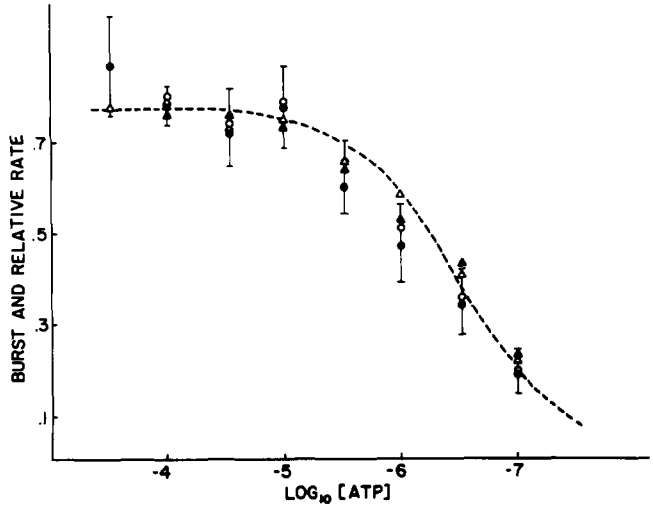

FIGURE 1: Burst size and relative steady-state rate vs. log (ATP) for myosin and subfragment 1 in 0.5 M KCl, pH 8.0, 20 °C. (●) Myosin burst; (▲) myosin rate; (○) S-1 burst; (△) S-1 rate. The dashed curve gives the concentration dependence for a single class of site $K_m = 3 \times 10^{-7}$ M. The burst sizes for the two lowest ATP concentrations have been corrected for time lag as described in the text.

for myosin and S-1. To obtain reasonable statistics, data was combined from 81 bursts and rate measurements on a dozen preparations; thus, the errors include the variation among the preparations. Myosin values were corrected for 12% impurities for samples which were not purified by chromatography. Some uncertainty is introduced in comparing the concentration dependence of the burst and rate, since a correction is necessary for the time lag in reaching the steady state for ATP concentrations of 3×10^{-7} and 10^{-7} M. The correction factors are 1.1 and 1.35, respectively.

The conclusions that can be drawn are limited by the difficulty in obtaining high precision in phosphate-burst measurements. Within a ±10% error, myosin and S-1 show the same concentration dependence of the early burst. Steady-state rate measurements also exhibited a similar concentration dependence. The dashed curve is the expected variation for a single class of site of dissociation constant 0.3 μM. The data points lie within one standard deviation of the curve but the fit is poor, particularly for the burst data. Scatchard plots of the burst measurements also give a poor fit to a straight line. The K_M values obtained by least-square fit to a straight line were in the range 0.25 ± 0.05 μM for burst and rate measurements.

The kinetic scheme requires the burst and rate to have the same concentration dependence. While the condition was approximately satisfied, there appears to be a measurable deviation from a single Michaelis constant for both myosin and S-1. The deviation could indicate a fourfold difference between rate constants for the two classes of myosin heads, which have recently been separated by Weeds and Taylor (1975), or some heterogeneity in the myosin population, which is obtained from mixed back and leg muscles. In the former case, roughly equal amounts of the two classes are present and a three- or fourfold difference in rates should have been detected in transient measurements by fluorescence on the mixture or steady-state measurements on separated S-1 species (Weeds and Taylor, 1975). A mixture of several myosin classes whose K_M values covered a twofold range would account for the data shown in Figure 1 and would not have been detected in transient and steady-state experiments.

The kinetic scheme predicts $K_M = [K_3/(K_3 + 1)]k_4/K_1k_2$.

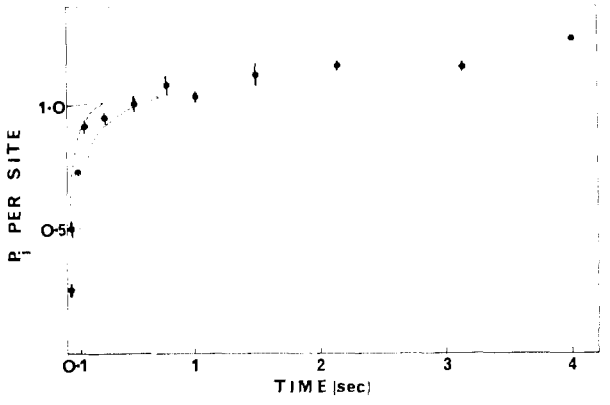

FIGURE 2: The transient and steady-state phases of ATP hydrolysis for HMM. Data are expressed as P_i per site, site molecular weight 178 000. Chymotryptic HMM, 0.05 M KCl, pH 7, 20 °C, 1.2×10^{-5} M ATP, ATP/site ratio equal to 5. Bars indicate range of duplicate measurements. In all figures, the concentrations are the values after mixing. Dashed curves are calculated for rate constants of 8 and 20 s^{-1}.

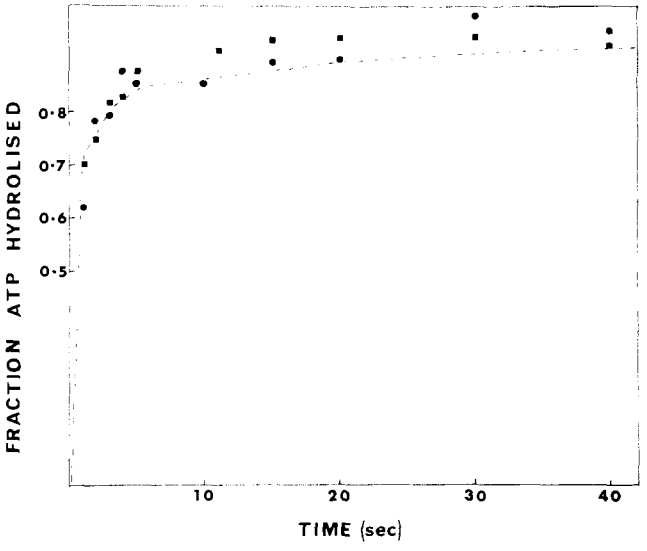

FIGURE 3: Hydrolysis of ATP in a stoichiometric reaction with myosin. Myosin site concentration, 10^{-5} M, 0.5 M KCl, pH 8, 20 °C, (●) ATP/site equals one; (■) ATP/site equals 0.86. Dashed curve calculated for a second-order reaction, second-order rate constant 5×10^5 M^{-1} sec^{-1}, maximum burst 0.85, steady-state rate 0.02 s^{-1}.

In 0.5 M KCl, the calculated value is 0.03–0.04 μM. The steady-state measurements, while showing some deviation from a single K_M, would still require values in the range from 0.2 to 0.4 μM. The main difference in experimental conditions is the very low ATP concentration (circa 10^{-7} M) necessary to determine K_M, while transient measurements are made at ATP concentrations of at least 5 μM. Steady-state measurements were made in 0.5 M KCl in order to compare myosin with S-1. A possible explanation of the high K_M is competitive inhibition by other ion species present, particularly chloride ion since an association constant of 10 M^{-1} would be sufficient to account for the discrepancy. The variation in the steady-state rate of S-1 in pH 7, Tris–Mes buffer in the absence of KCl gave a K_M of 0.1–0.2 μM in somewhat better agreement, but a discrepancy remains. A possible explanation is the presence of a class of binding sites with a lower affinity for ATP (circa 10^6 M^{-1}) which were not distinguished in previous transient measurements of fluorescence enhancement. The relative magnitude of the fluorescence charge was measured for ATP concentrations from 0.7 to 2.0 μM, the latter value corresponding to maximum fluorescence change. The lowest S1 concentration that can be used in the stopped-flow apparatus is 0.1 μM, which gives a fluorescence signal about three times the background signal produced by buffer alone. The ratio of ATP to S1 was 2:1 or 1:1, which gives a transient increase in fluorescence lasting for at least a few seconds. The free ATP concentration at the maximum value of the transient was corrected for the amount bound to S1 using the magnitude of the transient relative to the maximum value at high ATP concentrations. The measurements yielded an apparent dissociation constant K_m of 0.05–0.08 μM (S1, pH 8, 0.1 M KCl, 10 mM $MgCl_2$). The measurements rule out the possibility of a class of weaker ATP binding sites and the K_m agrees within a factor of two with the value calculated from the kinetic constants.

Transient Measurements. The evidence requires that both myosin sites undergo the rapid hydrolysis step, although there may be small differences between sites or heterogeneity in the population. The extent of such differences was investigated by examining the complete time course of the phosphate transient at high ATP concentrations and at stoichiometric ATP concentrations.

An experiment at a moderate ATP concentration is shown in Figure 2 (1.2×10^{-5} M ATP, 0.05 M KCl, pH 7, 20 °C, chymotryptic HMM). The apparent rate for the fluorescence enhancement is 12–18 s^{-1} for these conditions. Examination of the time range from 0.1 to 1 s showed that the phosphate burst is complete in 0.5 s and is (fortuitously) one per site in this particular experiment. The dashed curves calculated for rate constants of 8 and 20 s^{-1} (i.e., second-order rate constants of 7×10^5 M^{-1} s^{-1} and 1.6×10^6 M^{-1} s^{-1}) bracket the experimental points. The error in duplicate analyses averages 3% and it cannot be proven that the data fits a single exponential over the entire range but it is evident that a variation of a factor of two in rates is a reasonable estimate of the extent of heterogeneity. Similar experiments with HMM and myosin over a tenfold ATP concentration range yielded the same general result, both sites per molecule gave an early burst and kinetic heterogeneity, if present, was not more than a factor of two in the rate constants.

A second test of homogeneity is provided by examining the complete hydrolysis of ATP at a site concentration equal to or greater than the ATP concentration. The reaction was studied at ATP/site ratios of 0.1, 0.25, 0.5, and 1.0. The results at the lowest ratio were similar to those of Bagshaw and Trentham (1973) and will not be described in detail (Table I). In Figure 3, the hydrolysis reaction is shown for an ATP/site ratio of 1.0 (myosin 10^{-5} M sites, 0.5 M KCl, pH 8, 20 °C). If the preparation were to contain 10% inactive enzyme, the substrate would be slightly in excess and lead to a rather longer tail than expected for a homogeneous population. The experiment was also carried out at 0.86 ATP/site to circumvent this problem. The apparent rate constant determined by proton or phosphate measurements is $5–6 \times 10^5$ M^{-1} s^{-1} (Koretz and Taylor, 1975). The dashed curve was calculated for a second-order reaction with a rate constant of 5×10^5 M^{-1} s^{-1}, a burst of 0.85 per site and a steady-state rate of 0.02 s^{-1}. The agreement is reasonably good considering that the parameters were not fitted to the data but derived from other experiments. Heterogeneity in the population, if present, is again no more than a factor of two.

The possibility has been considered that the two heads of myosin have different kinetic parameters and that one head may either not hydrolyze ATP or at least do so at a very different rate than the other head. The experiments described

TABLE II: Dependence of Phosphate Early Burst of Myosin, HMM, and S-1 on Experimental Conditions.[a]

Protein	Parameter	Burst Size
S-1	0.5 M KCl	0.74 ± 0.03 (2)
	0.1 M KCl	0.83 ± 0.11 (3)
HMM, S-1, myosin	20 °C, pH 8.0	0.74 ± 0.05 (7)
	0 °C, pH 8.0	0.68 ± 0.06 (6)
	20 °C, pH 6.5	0.63 ± 0.05 (7)
	0 °C, pH 6.5	0.51 ± 0.04 (7)
S-1	IAA modified	0.91 ± 0.12 (3)

[a] Experiments at pH 8, 20 °C, in 10 mM Tris buffer, 3×10^{-5} M ATP, 5–10 mM $MgCl_2$, 0.1 M KCl, unless indicated. Myosin experiments are in 0.5 M KCl in all cases. Comparisons of burst size at different ionic strengths, pHs, or temperatures were made on paired samples from the same preparation. The values quoted for S-1 and myosin in 0.5 M KCl, pH 8, 20 °C, are from a different series of experiments than Table I.

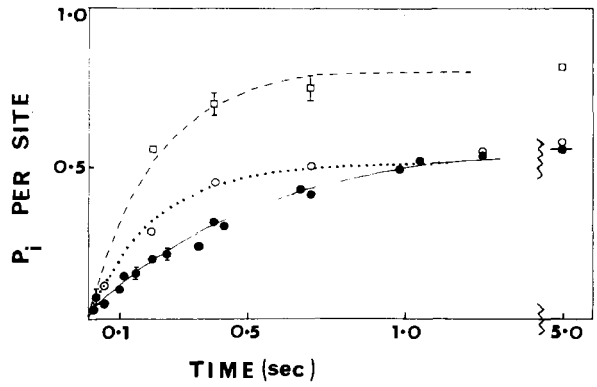

FIGURE 4: Phosphate burst for HMM at pH 6.9 and 3 °C in 0.05 M KCl. Site concentration 1.25×10^{-5} M, [γ-^{32}P]ATP/site ratio equal to 4. (●) P_i/site, reaction stopped in acid at indicated times; (○) reaction quenched with 100-fold excess of ATP at times corresponding to position of points along the time axis. Reaction stopped in acid by hand (2–3 s delay); (···) fluorescence transient normalized to final value of burst (0.49); (□) reaction quenched with ATP at times corresponding to position of points and stopped with acid at 200 s; (- - -) fluorescence transient normalized to final P_i value (0.82).

above would easily have distinguished two classes of sites present in equal amounts if the rate constants differed by a factor of four or five. A "tail" was observed in some experiments but the range in rate constants cannot be more than a factor of two or three.

Transient measurements were made at low pH and temperature in order to determine whether the conformation change and hydrolysis steps could be resolved. Also, as recorded in Table II, the burst is only 0.5 mol/site and it is necessary to ask whether only one head per myosin gave a burst under these conditions.

In Figure 4, the phosphate burst is shown for HMM at 5×10^{-5} M ATP, pH 6.9, 3 °C (solid circles). The apparent rate constant is only 3 s^{-1}, while the fluorescence transient for the same preparation was 5.5 s^{-1} (dotted curve). According to the kinetic scheme, the time dependence should fit two exponential terms but the accuracy of phosphate measurements is not sufficient to separate the two rate constants. The apparent rate increased with ATP concentration but the plateau could not be accurately measured because of the large errors in the zero-time blank for ATP concentrations >0.2 mM. The concentration dependence is shown in Sleep and Taylor (in press). The maximum rate appeared to be in the range 5–10 s^{-1}.

An additional constraint on $k_3 + k_{-3}$ is imposed by chase experiments, which also strengthen the conclusion that the mechanism is sequential. At various times during the transient, [^{32}P]ATP binding was blocked by mixing with a 100-fold excess of unlabeled ATP and the reaction was then stopped within 2–3 s by adding acid. The extent of hydrolysis is shown by the open circles in Figure 4. Since the substrate is tightly bound in the MATP** complex and is converted to MPr** at a rate of at least 3 s^{-1}, any [^{32}P]ATP** would come to equilibrium with MPr** by the time the reaction is stopped in acid. A small correction of 0.02 was made for the steady-state flux out of the MPr** state. The data points fit the shape of the fluorescence transient, which is shown by the dotted line in the figure.

If $k_3 + k_{-3}$ was much larger than the rate of the fluorescence step, M·ATP** and MPr** would be in equilibrium during the transient and there would be no increase in P_i in a 3-s chase. The increase thus depends on the relative rates of the two steps and from the measured value of the rate of the fluorescence step the increase can be calculated for trial values of $k_3 + k_{-3}$. The best fit was obtained for a rate of 6–8 s^{-1}. A further estimate is obtained from rate measurements of acto-HMM or acto-S-1 ATPase (Sleep and Taylor, in press). Interposing the dissociation step effectively raises the apparent rate constant, a plateau is reached at a lower ATP concentration, and the rate process fits a single exponential. The value of the maximum rate was 7 s^{-1}. It is concluded that $k_3 + k_{-3}$ is 6–8 s^{-1}, which is about one-half the value for the conformation change k_2.

The size of the burst at pH 6.9 and 3 °C is approximately 0.5. In various experiments, the results ranged from 0.45 to 0.60. If we accept the explanation of Bagshaw and Trentham (1973), the low value could result from a reduction of the equilibrium constant, K_3, to a value of 1–1.5. Alternatively, it might be supposed that only one head per myosin gives a burst under these conditions.

A second type of chase experiment is also shown in Figure 4. After mixing with excess unlabeled ATP at various times, the reaction was allowed to proceed for 200 s (5 halflives) and was then stopped in acid. There is a large increase (open squares) compared to the results of quenching immediately, and after the reaction has reached a steady state the maximum hydrolysis was 0.82 mol/site. The dashed curve is the fluorescence transient normalized to the final P_i value. The fluorescence increase counts M·ATP** plus MPr** and by allowing the reaction to reach completion both intermediates should be measured as P_i. Comparison with the fluorescence change provides an internal check on the validity of the phosphate measurements. The experiment shows a maximum increase of 65% in the amount of hydrolysis after the end of the transient, while at 20 °C the increase is at most 20%. The increase corresponds to an equilibrium constant of 1.6. The final hydrolysis value is slightly lower than the average value obtained in quenching experiments at 20 °C. Some dissociation at ATP from MATP** is possible according to the rate constant of Arata et al. (1975) (0.002 s^{-1} at 0 °C and 0.5 M KCl), which would slightly reduce the final value.

A further test of the hypothesis that the low burst size results from a shift in equilibrium constant was made by combining the ATP chase with a temperature and pH jump at the end of the transient phase. If the burst size is determined by an equilibrium between MATP** and MPr**, then a jump from pH 6.9, 3 °C to pH 8, 20 °C and vice versa should lead to immediate hydrolysis or synthesis of ATP in an amount equal to the difference in burst size for the respective conditions.

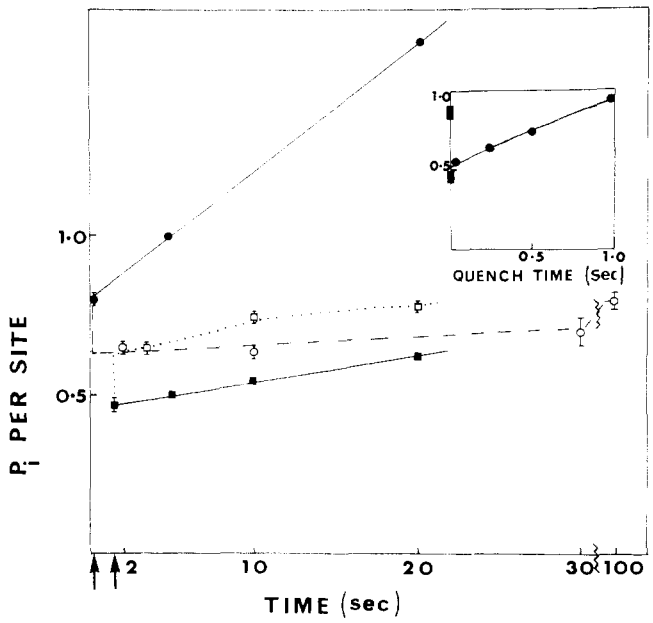

FIGURE 5: Effect of pH–temperature jump on the enzyme-substrate to enzyme–product equilibrium of subfragment 1. $^{32}P_i$ formation, pH 8, 20 °C (●), at 100 ms. (First arrow) reaction was quenched with a 200-fold excess of ATP in the quench-flow apparatus and fired into 5 ml of buffer to give pH 6.9 and 3 °C; reaction was stopped in acid at times indicated (○); $^{32}P_i$ formation at pH 6.9 and 3 °C (■); at 1.5 s. (Second arrow) reaction was quenched with ATP and fired into buffer to give pH 8 and 20 °C; reaction was stopped in acid at times indicated (□). Radioactive ATP concentration 6×10^{-5} M, ATP/site ratio equals 2.5, all solutions were 0.05 M in KCl. Inset: reaction was started at pH 9 and 20 °C, quenched with ATP at times indicated, fired into buffer to give pH 6.0 and 3 °C, and stopped immediately (2–3 s). Bars indicate range in burst size for the two pH–temperature conditions. The decrease relative to the upper bar is the amount of ATP synthesis per site.

The results of this type of experiment for S-1 are shown in Figure 5 at an ATP concentration of 6×10^{-5} M and ATP/site ratio of 2.5. The sample was mixed with a large excess of unlabeled ATP and fired into 4–5 volumes of buffer to give the appropriate temperature and pH. Reaction was stopped with acid in 2 s or allowed to proceed for various times. The lower curve (solid squares) shows P_i formation at pH 6.9 and 3 °C. The sample was quenched at 1.5 s when the transient was essentially complete and jumped to pH 8 and 20 °C. The P_i burst increased from 0.45 to 0.65 within 1 to 2 s and then slowly increased to a final value of 0.75. The reaction at pH 8 and 20 °C reached a steady state in less than 20 ms. The jump was applied at 100 ms and the burst (open circles) decreased from 0.8 to 0.65 (i.e., ATP was synthesized). Hydrolysis continued slowly at 3 °C and reached a final value of approximately 0.8. The final values are <1 P_i/site but are not much below the average found in counting sites by quenching in a typical S-1 preparation. However, the change produced by the jump was only half the expected value. Some adverse effect of mixing may have reduced the observed change but this cannot be a complete explanation.

To obtain a somewhat larger change, jump experiments were performed between pH 9, 20 °C and pH 6, 3 °C. The shift to low pH and temperature took place at various times between 30 ms and 1 s. The values immediately after the jump are plotted in the insert of Figure 5. The bars on the axis give the range of values of the burst for the two conditions. Extrapolated to zero time the jump gives nearly the expected amount of ATP synthesis but the extent of reversal of the reaction decreases with time with a halflife of about 0.5 s. In several experiments with S-1 and HMM, a jump to higher pH and temperature gave up to three-fourths of expected hydrolysis when applied in the first 1–2 s, but no synthesis of ATP was obtained at times greater than 1 s.

The fast synthesis or hydrolysis of bound ATP is strong evidence for a change in K_3 with pH and temperature, although the reaction appears to be more complex than predicted from the kinetic scheme. Inoue et al. (1974) have also described ATP synthesis on the enzyme by an ionic-strength jump.

The low-temperature results confirm the finding by Tonomura that the burst size is smaller (Kanazawa and Tonomura, 1965) and that the rate of P_i formation is approximately half the rate of proton release (Tonomura et al., 1969).

Conclusions

Steady-state and transient measurements give a value of the phosphate early burst of 0.8–0.85 mol per site for myosin, HMM, and S-1 at pH 8 and 20 °C. Bagshaw and Trentham have suggested that the maximum burst is determined by K_3, the equilibrium constant of the MATP** to M·Pr** step. The burst calculated from K_3 is 0.85 ± 0.05 in good agreement with the direct measurements.

The presence of 12–14% impurities in myosin prepared by standard precipitation procedures and the slow decrease in burst with time of storage at 4 °C may account for the low values obtained in earlier experiments. The present evidence requires that ATP is essentially irreversibly bound and rapidly hydrolyzed by both heads of myosin. With some reservations, the evidence is consistent with the general kinetic scheme proposed by Bagshaw and Trentham (1974) for S1 and extended to an identical independent site model for myosin by Sleep and Taylor (submitted). The essential part of the scheme is repeated here for convenience:

$$M \overset{(1)}{\rightleftharpoons} M\cdot ATP \overset{(2)}{\rightleftharpoons} M\cdot ATP^{**} \overset{(3)}{\rightleftharpoons} MPr^{**} \overset{(4)}{\rightleftharpoons} M + ADP + P_i$$

The hydrolysis step for excess or stoichiometric ATP concentrations is fitted reasonably well by a single rate constant at 20 °C; at worst the variation is a factor of two. The rate is approximately equal to the value obtained from fluorescence or proton measurements; thus, the two sites per myosin must be very similar in rate behavior, if not identical.

In spite of the large number of measurements which were made it is difficult to obtain high precision in the determinations of the burst. The data are sufficient to show, within an error of ±10–15%, that the variation in burst or rate is the same for myosin or S-1, as required by the kinetic scheme, but there appears to be a significant deviation from a single K_m which may be explained by some heterogeneity in the population of myosin prepared from back and leg muscles (Barany, 1967). The deviation is such as to produce Scatchard plots which are concave downward. Positive cooperativity is difficult to rule out in transient experiments, except by very precise measurements of rate constants. The effect of positive cooperativity would be to give a Scatchard plot which is concave upwards and which passes through a maximum value. Deviations from linearity are in the opposite direction and are very similar for myosin and S-1. The steady-state burst and rate measurements provide another piece of evidence against positive cooperativity between myosin sites.

Further evidence for a sequential mechanism was obtained from phosphate-rate measurements at 3 °C. In contrast to

previous studies at 20 °C, the apparent rate of hydrolysis was less than the rate of the conformation change at moderate ATP concentrations. Accurate determination of $k_3 + k_{-3}$, the actual rate of hydrolysis step, is difficult, since the time dependence predicted by a sequential model requires a fit to two exponentials of similar magnitude which can not be made accurately even with very precise data. Also, there is some variation introduced by the large-temperature dependence of the rates of the two processes and measurements have to be compared which were made in the stopped-flow and quenched-flow machines.

Chase experiments indicated the presence of M·ATP** in amounts in excess of the equilibrium value, which is consistent with a sequential process and sets an upper limit to $k_3 + k_{-3}$ of 8–9 s^{-1}. Thus, the rate of the hydrolysis step has not been accurately measured, but it does appear to be slower than step 2. The low-burst size at low temperature is in part a consequence of a decrease in K_3. Chase experiments gave $K_3 = 1.6$ at pH 6.9 and 3 °C, while the same experimental procedure gives $K_3 = 6$–7 at pH 8.0 and 20 °C. Both values are consistent with the burst measured directly.

In two respects, the evidence is not explained by the Bagshaw–Trentham scheme. First, the K_m value predicted from the kinetic scheme is considerably smaller than the value obtained from the concentration dependence of the burst or steady-state rate. The discrepancy is smaller at low-ionic strength but is still a factor of four or five. Since both heads contribute to the fluorescence signal and phosphate burst, there is no simple explanation for the discrepancy.

Second, the magnitude of the phosphate burst has been explained as an equilibrium between substrate and products on the enzyme (Bagshaw and Trentham, 1973) but the pH–temperature jump experiments suggest that this interpretation is incomplete. The previous evidence from cold chase experiments shows only that some tightly bound substrate is present in the steady state which is converted to products at the steady-state rate. It was also found at 20 °C that even with substrate present at a 2:1 ratio over sites, a cold chase at 2–5 s is still followed by a 10–20% hydrolysis per enzyme site. This experiment removes two objectives to the original study, with enzyme in large excess, that the equilibrium applies only to a fraction of the enzyme sites and that it is not a property of the steady state. However, the results could still be interpreted as evidence for a branched pathway, as proposed by Tonomura (Tonomura and Inoue, 1974), rather than an equilibrium. Stronger evidence for reversal of step 3 is given by the jump experiment at very short times and by exchange of 75% of the three terminal oxygens of enzyme-bound ATP in 2 s (Bagshaw et al., 1975). The evidence for reversal of the reaction, the magnitude of the burst, and the values of the rate constants at 20 °C require an equilibrium at step 3 which is established within 30 ms. The jump experiments could indicate the presence of a second myosin product state as proposed by Tonomura (Nakamura and Tonomura, 1968) but it is preferable to leave open the question of whether the kinetic scheme is complete, since the evidence is not conclusive. The conclusions drawn here are in partial disagreement with those of Tonomura (recently reviewed by Tonomura and Inoue, 1974) who proposed two reaction pathways which "probably take place in different heads" (Arata et al., 1975). Possibly because of the complexity of the original scheme, the contributions of Tonomura have not been properly recognized. The pathway for one of the heads is essentially the same as the Bagshaw–Trentham mechanism, except for the inclusion of a final step, a configuration change of free myosin. As their studies were prior to those of Bagshaw and Trentham, the mechanism could be attributed to both groups. The major disagreement is the postulation of a second pathway for the other head, which does not include a rapid hydrolysis step and therefore requires the average phosphate burst to be only 0.5 mol/site or 0.43 mol/site if the equilibrium at step 3 is included. Earlier studies by Tonomura (for example, Tonomura et al., 1962; Kinoshita et al., 1969) gave 0.6 mol/site (recalculated for the currently accepted molecular weight) on preparations which were not column purified and his result is consistent with our figure of 0.69 ± 0.06. However, more recent measurements on column-purified myosin gave a lower value (Inoue et al., 1972), while we have obtained 0.85 ± 0.05. The size of the early burst is the strongest evidence for Tonomura's second pathway and, in view of the disagreement, we do not consider that there is sufficient positive evidence to warrant the inclusion of a second hydrolysis mechanism.

Acknowledgments

I thank R. Butt and A. Maggs for technical assistance.

References

Arata, T., Inoue, A., and Tonomura, Y. (1975), *J. Biochem. (Tokyo) 77,* 895–900.

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

Bagshaw, C. R., and Trentham, D. R. (1973), *Biochem. J. 133,* 323–328.

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

Bagshaw, C. R., Trentham, D. R., Wolcott, R. G., and Boyer, P. D. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2592–2596.

Barany, M. (1967), *J. Gen. Physiol. 50,* 197–218.

Chock, S. P., and Eisenberg, E. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4915–4919.

Inoue, A., Arata, T., and Tonomura, Y. (1974), *J. Biochem. (Tokyo) 76,* 661–666.

Inoue, A., Shibata-Sekiya, K., and Tonomura, Y. (1972), *J. Biochem. (Tokyo) 71,* 115–124.

Inoue, A., and Tonomura, Y. (1974), *J. Biochem. (Tokyo) 76,* 755–764.

Kanazawa, T., and Tonomura, Y. (1965), *J. Biochem. (Tokyo) 57,* 604–615.

Kinoshita, N., Kubo, S., Onishi, H., and Tonomura, Y. (1969), *J. Biochem. (Tokyo) 65,* 285–301.

Koretz, J. F., and Taylor, E. W. (1975), *J. Biol. Chem. 250,* 6344–6370.

Lowey, S., Slayter, M. S., Weeds, A. G., and Baker, H. (1969), *J. Mol. Biol. 42,* 1–29.

Lymn, R. W., and Taylor, E. W. (1970), *Biochemistry 9,* 2975–2983.

Lymn, R. W., and Taylor, E. W. (1971), *Biochemistry 10,* 4617–4624.

Mannherz, H. G., Schenck, H., and Goody, R. S. (1974), *Eur. J. Biochem. 48,* 287–295.

Nakamura, H., Tonomura, Y. (1968), *J. Biochem. (Tokyo) 63,* 279–293.

Perry, S. V. (1955), *Methods Enzymol. 2.*

Seidel, J. C. (1975), *J. Biol. Chem. 250,* 5681–5687.

Starr, R., and Offer, G. (1971), *FEBS Lett. 15,* 40–44.

Taylor, E. W., Lymn, R. W., and Moll, G. (1970), *Biochemistry 9,* 2984–2991.

Tonomura, Y., and Inoue, A. (1974), *Mol. Cell Biochem. 5,*

127-143.
Tonomura, V., Kitagawa, S., and Yoshimura, J. (1962), *J. Biol. Chem. 237*, 3660-3666.
Tonomura, Y., Nakamura, H., Kinoshita, N., Onishi, H., and Shigekawa, M. (1969), *J. Biochem. (Tokyo) 66*, 599-618.
Weeds, A. G., and Taylor, R. S. (1975), *Nature (London) 257*, 54-56.
Wolcott, R. G., and Boyer, P. D. (1974), *Biochem. Biophys. Res. Commun. 57*, 709-716.

Proton Magnetic Resonance Studies of Actinomycin D Complexes with Mixtures of Nucleotides as Models for the Binding of the Drug to DNA†

Thomas R. Krugh,*,‡ Eddie S. Mooberry,§ and Yu-Chih Chen Chiao

ABSTRACT: The proton magnetic resonance spectra of actinomycin solutions with mixtures of deoxynucleotides have been investigated to determine the relative preference for the binding of guanine and adenine nucleotides to the two nucleotide binding sites of actinomycin D. An analysis of the chemical shifts of the actinomycin D resonances shows that adenine and guanine nucleotides competitively bind to the benzenoid portion of the phenoxazone ring of actinomycin D while guanine nucleotides bind stronger than adenine nucleotides to the quinoid portion of the phenoxazone ring. The chemical shift data for the titrations of actinomycin D with pdG-dG, pdC-dC, and an equimolar mixture of these complementary deoxydinucleotides show that: (1) pdG-dG forms a stacked complex much like dGMP; (2) pdC-dC does not bind to actinomycin D under the conditions used in these experiments; (3) in the titration of actinomycin D with the equimolar mixture of pdG-dG + pdC-dC, a miniature intercalated complex is formed.

The binding of actinomycin D to DNA has been studied by a variety of techniques over the past 15 years (e.g., see the reviews by Hollstein, 1974; Meienhofer and Atherton, 1973; and the many references therein). Müller and Crothers (1968) proposed that the phenoxazone ring of actinomycin D (Figure 1) intercalates into the DNA double helix, with the two cyclic pentapeptides located in the minor groove of the helix. The intercalation model for actinomycin D binding to DNA was supported by the data of Waring (1970) and Wang (1971). When actinomycin D binds to DNA, there is a general, but not absolute, requirement for a guanine base at the intercalation site (e.g., see Wells and Larson, 1970). Sobell et al. (1971) determined the three-dimensional structure of an actinomycin D-deoxyguanosine (1:2) crystalline complex, which was used to construct a detailed molecular model for the intercalation of actinomycin D at a dG-dC sequence of DNA (Sobell and Jain, 1972; Sobell, 1973). The complexation of actinomycin D to DNA involves base stacking, hydrogen bonding, and hydrophobic forces and thus a detailed understanding of the complex formation requires a knowledge of the relative contributions of each of these forces to the stabilization of the complex. The question of the origin of the guanine requirement is also of great interest. In previous nuclear magnetic resonance (NMR)[1] and optical titrations (Krugh, 1972; Krugh and Neely, 1973 a,b; Krugh and Chen, 1975), we have established that actinomycin D will bind two guanine containing deoxydinucleotides (see also Schara and Müller, 1972) or two dGMP molecules (see also Arison and Hoogsteen, 1970; Danyluk and Victor, 1970; Patel, 1974). The NMR titrations provide an opportunity of monitoring the binding of the nucleotides to the individual binding sites on the phenoxazone ring of actinomycin D. An actinomycin D titration with dAMP (Krugh and Neely, 1973a; Krugh and Chen, 1975) suggested that dAMP binds almost as strongly as dGMP to the benzenoid portion of the phenoxazone ring and much weaker than dGMP at the quinoid portion of the phenoxazone ring. The present manuscript and the following manuscript (Chiao and Krugh, 1977) explore the preferential binding of the nucleotides to actinomycin D as a model for the binding of the drug to DNA.

Experimental Section

The deoxydinucleotides were purchased from Collaborative Research, Inc., and were used without further purification. A few of the samples were either treated with Chelex-100 (Bio-Rad) or a small amount of EDTA (ethylenediaminetetraacetic acid, sodium form) was added if there was appreciable line broadening observed in the dinucleotide proton magnetic resonance spectra. The dinucleotides were weighed into a sample tube and dissolved in 5 mM D_2O phosphate buffer to yield a concentration in the 50 to 80 mM concentration range. The pD was then adjusted using DCl or NaOD to give a pH meter reading of 7.0. The concentration of the dinucleotides

†From the Department of Chemistry, University of Rochester, Rochester, New York 14627. *Received August 23, 1976.* This investigation was supported by Research Grant No. CA-14103 and Career Development Award No. CA-00257 from the National Cancer Institute, Department of Health, Education and Welfare.

‡Alfred P. Sloan Fellow 1976-78; National Institutes of Health Research Career Development Awardee, 1976-1981.

§Present address: Department of Chemistry, North Carolina Agricultural and Technical State University, Greensboro, North Carolina 27411.

[1]Abbreviations used: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetracetic acid; TSP, sodium 3-trimethylsilylpropionate-*2,2,3,3-d*$_4$.