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Magnesium Ion Dependent Adenosine Triphosphatase Activity of Heavy Meromyosin as a Function of Temperature between +20 and −15 °C†

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ABSTRACT: The hydrolysis of Mg^{2+}–adenosine 5′-triphosphate (ATP) by heavy meromyosin has been studied between +20 and −15 °C, especially in the low-temperature range, in a medium containing 30% (v/v) ethylene glycol by fluorometric, spectrophotometric, and potentiometric measurements. The time course of the fluorescence changes of the enzyme during the reaction depends markedly on the temperature in consequence of large differences between the activation energies of the various steps. The observed kinetics have been analyzed according to the simplified scheme of Bagshaw & Trentham [Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331–349]. The following results have been obtained. (1) The rate-limiting step of the reaction changes in this temperature range; at 20 °C M**·ADP·P_i is the predominant steady-state complex, and M*·ADP predominates at −15 °C, with a half-life of ~10 min. (2) As expected, on the basis that it is the dissociation of the M*·ADP complex which becomes rate limiting at low temperature, one observes, in the pre-steady-state below 0 °C, both a proton burst and a lag phase in ADP release. (3) At low temperature, the equilibrium M*·ATP ⇌ M**·ADP·P_i is displaced to the left All the kinetic data obtained in this study are compatible with a simple pathway for the Mg^{2+}–ATP hydrolysis by myosin and with sequential release of the reaction products.

$$M + ATP \rightarrow M^*\cdot ATP \rightleftharpoons M^{**}\cdot ADP\cdot P_i \rightarrow M^*\cdot ADP \rightleftharpoons M + ADP + P_i + H^+$$

Myosin is one of the main protein components of muscle, and its interaction with actin in a cyclic process is responsible for muscular contraction. The hydrolysis of ATP[1] supplies the energy required to drive this cycle, and an active site for this reaction resides on each globular head of the myosin molecule.

The mechanism of ATP hydrolysis by myosin is still the object of debate (Taylor, 1977a; Tonomura & Inoue, 1977). Bagshaw & Trentham (1974), in particular, have proposed a seven-step mechanism for myosin Mg^{2+}-dependent ATP hydrolysis and have drawn attention to the strong temperature dependence of some of the steps. Thus, the rate-limiting step was found to change with temperature between +20 and 0 °C; the magnitude of this change, which is still a matter of uncertainty (Inoue et al., 1977), was small, however, and investigations over a wider temperature range are required to establish it with more certainty and also to better separate the different steps of the reaction.

Douzou (1973, 1977) and Fink (1976) have developed kinetic techniques applicable to studies at subzero temperatures and have emphasized the interest of such studies for the temporal resolution of complex enzymatic reactions into their elementary steps. We have accordingly embarked on a study of the hydrolysis of Mg^{2+}–ATP by heavy meromyosin, a proteolytic subfragment of myosin, between +20 and −15 °C, in a medium containing 30% (v/v) ethylene glycol. In the first place, the effect of ethylene glycol on the conformation and the activity of heavy meromyosin was examined. The fluorescence changes of the enzyme during the hydrolysis reaction were then measured under different experimental

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMPPNP, β,γ-imidoadenosine 5′-triphosphate; AMPCPP, α,β-methyleneadenosine 5′-triphosphate; ATPase, adenosine triphosphatase; HMM, heavy meromyosin; β-NADH, β-nicotinamide adenine dinucleotide; P_i, inorganic phosphate.

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conditions and at various temperatures. Finally, the kinetics of release of the reaction products, ADP, protons (resulting from the phosphate ionization), and also orthophosphate (P_i), have been followed, especially at low temperatures. The results obtained are most easily interpreted by assuming a simple pathway for Mg^{2+}–ATP hydrolysis by myosin, with sequential release of the reaction products. The rate-limiting step of the reaction is found to vary with temperature, and the identity of the long-lived intermediate complexes becomes more explicit at low temperature.[2]

Materials and Methods

Proteins. Pyruvate kinase and lactate dehydrogenase from rabbit muscle were purchased from Boehringer Mannheim (Germany). They were dialyzed against 0.025 M sodium cacodylate–HCl buffer and 0.1 M KCl (pH 6.9) and kept frozen.

Myosin was prepared from rabbit back and hind leg muscles by the method of Perry (1955) with a further step at ionic strength 0.3 M to remove actomyosin.

Heavy meromyosin was prepared by digestion of myosin with chymotrypsin, in the presence of 1 mM $MgCl_2$, according to Weeds & Taylor (1975) and Weeds & Pope (1977). The yield was ~40–50%. The protein was dialyzed in the final phase of the preparation against 0.05 M sodium cacodylate–HCl buffer and 0.2 M KCl, pH 6.9, and kept at 4 °C.

The concentrations of myosin and its subfragments were determined by measuring the absorbance at 280 nm with extinction coefficients of $E_{280nm}^{1\%} = 5.6$ cm^{-1} for myosin and 6.47 cm^{-1} for heavy meromyosin (Weeds & Pope, 1977). In each case, an appropriate correction for light scattering was made. The molecular weights were assumed to be 470 000 for myosin and 340 000 for heavy meromyosin.

Preparations of heavy meromyosin were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The patterns obtained were similar to those reported by Weeds & Pope (1977), showing a very intense heavy-chain and three light-chain bands; minor bands with mobilities slightly greater than the heavy-chain band and some loss of the DTNB light chain were usually observed.

Analytical ultracentrifugation of heavy meromyosin gave a single symmetrical boundary, with a sedimentation coefficient of 7.2 S [cf. 7.2 ± 0.1 S given by Lowey et al. (1969)].

The enzymatic activity of heavy meromyosin after each preparation was routinely controlled with naphthyl triphosphate or Mg^{2+}–ATP. It was almost constant from one preparation to another and agreed with literature values. The enzymatic activity of heavy meromyosin kept at 4 °C generally remained constant for ~8 days; most kinetic experiments in this work were nevertheless done on material not more than 2 or 3 days old.

Nucleotides and Chemicals. Sodium salts of ATP and ADP as well as lithium salts of β,γ-imidoadenosine 5′-triphosphate (AMPPNP) and α,β-methyleneadenosine 5′-triphosphate (AMPCPP) were purchased from Sigma Chemical Co. and used without further purification. Their solutions at neutral pH were stored frozen. The purity of ATP and ADP was checked by polyethylenimine–cellulose chromatography in 0.4 M KH_2PO_4 (pH 3.4) according to Cashel et al. (1969). Under a UV lamp, ATP appeared as a single dark spot on a fluorescent background while ADP showed some trace amounts of impurities coincident with AMP and ATP.

[γ-^{32}P]ATP was obtained from The Radiochemical Centre, Amersham (U.K.).

β-Naphthyl triphosphate was prepared according to Kagawa et al. (1974) and Kuwajima & Asai (1975); it was characterized in terms of its absorption spectrum and that of its enzymatic hydrolysis product (Kuwajima et al., 1975).

Phosphoenolpyruvate and β-NADH were obtained from Sigma Chemical Co. Sodium cacodylate was a Merck product, and ethylene glycol was from Carlo Erba. Other reagents were purchased from Prolabo. These compounds were analytical grade and used without further purification.

Measurements at Subzero Temperatures. Precautions advocated by Douzou (1977) were followed in making the measurements at subzero temperatures. For the preparation of organic solvent–water mixtures containing heavy meromysin, the stock aqueous solution of protein at 4 °C was added slowly with magnetic stirring to the cold ethylene glycol–water mixture. For spectroscopic or potentiometric measurements at low temperatures, ethylene glycol and water were first degassed; the rapid mixing of an aliquot of ATP or other nucleotide with the protein solution was achieved by combined mechanical and magnetic stirring, and the kinetic runs were recorded after 5–10 s, with continuous magnetic stirring.

The experimental medium contained 0.025 M cacodylate buffer, 0.1 M KCl, and varying proportions of ethylene glycol. The cacodylate buffer was chosen because of its solubility at low temperatures and the very low-temperature dependence of its protonic activity. According to Douzou et al. (1976) and Larroque et al. (1976), the pH (or pa_H) of a cacodylate solution, which was made up to pH 6.9 in water, was 7.1 in 30% (v/v) ethylene glycol and 7.4 in 50% (v/v) ethylene glycol at any temperature between +20 and −15 °C. These values were checked experimentally.

In general, no correction was made for the volume contraction resulting from the mixing of ethylene glycol and the aqueous solution. From data of Douzou et al. (1976) for the density of ethylene glycol–water mixtures at different temperatures, the correction factor was estimated to be ≤2%, depending on the ethylene glycol percent and the temperature. It was experimentally verified that the volume decrease resulting from the addition of 1 volume of ethylene glycol to 1 volume of 0.05 M cacodylate buffer (0.2 M KCl, pH 7.0) was nearly 2% at 4 °C.

Spectrophotometric Measurements. Spectrophotometric measurements were made with a Cary 118 spectrophotometer, the cell holders and sample compartment of which had been modified in this laboratory. These elements were thermostated by circulation of ethanol from a constant temperature bath or by circulation of temperature-regulated gaseous nitrogen. In the latter case, the equipment was analogous to that described by Maurel et al. (1974) for experiments at subzero temperatures. The temperature in the sample and reference cells was measured with chromel–alumel thermocouples.

In the ADP-linked assay, the reaction mixture was similar to that used by Taylor & Weeds (1976). It contained 0.035–0.17 mg/mL pyruvate kinase, 0.05–0.25 mg/mL lactate dehydrogenase, 0.11 mM β-NADH, 0.45–1.2 mM phosphoenol pyruvate, and 0.2–2 mg/mL heavy meromyosin in a volume of 1.2 or 2.4 mL, depending on the temperature. The buffer contained 0.1 M KCl, 4.3 mM $MgCl_2$, 0 or 30% (v/v) ethylene glycol, and 0.025 M sodium cacodylate–HCl (pH 6.9 or pa_H 7.1). The reaction was initiated by the addition of 5 μL of 0.02 M ATP in aqueous solution or in 30% (v/v) ethylene glycol, and the decrease in absorbance at 340 nm was measured. A molar extinction coefficient of 6220 M^{-1} cm^{-1}

[2] A preliminary account of this work has been presented at the 7th European Conference on Muscle and Motility, Warsaw, 1978.

was used for NADH, at 20 °C, in aqueous solution (Horecker & Kornberg, 1948); in 30% (v/v) ethylene glycol, it was found to be equal to 6500 M^{-1} cm^{-1}. These extinction coefficients increased with decreasing temperature at ~1%/10 °C.

The hydrolysis of naphthyl triphosphate by heavy meromyosin was followed at 322 nm, according to Kuwajima et al. (1975). At this wavelength $E_{product} - E_{substrate}$ was estimated to be 160 M^{-1} cm^{-1} in aqueous solution; it was not significantly different in 50% (v/v) ethylene glycol. The experimental conditions were 0.1 M KCl, 2.8 mM $CaCl_2$, 0.024 M sodium cacodylate–HCl buffer, 0–50% (v/v) ethylene glycol, 0.19 mM naphthyl triphosphate, and 0.2–0.4 mg/mL heavy meromyosin in a volume of 1.1 mL. The pH (or pa_H) of the reaction medium was 6.9 in aqueous solution and 7.4 in 50% (v/v) ethylene glycol. It was verified that, at 5 °C, in aqueous solution the reaction rate increased by only a factor of 1.1 between pH 6.9 and 7.4; no correction was made for the pH change with the proportion of ethylene glycol.

Fluorometric Measurements. Fluorescence kinetic measurements were made on a single-beam spectrofluorometer built by Rodier (1977) in this laboratory. The light from an XBO 900-W xenon arc lamp with a current-stabilized power supply (Fontaine, France) was passed through a Bausch and Lomb UV monochromator into the measurement cell. Emitted light was collected at 90° into a second Bausch and Lomb UV monochromator and detected by a photomultiplier 150 UVP (from Radiotechnique, France). The response was amplified and the resulting voltage applied to a Type EPL 1 potentiometric recorder (from Tacussel Electronique) equipped with a differential voltage recording plug in, Type TD 11G. An opposed reference voltage supplied by a precision power source Model LR-613-DM (from Lambda Electronic Corp.) was applied to the recorder. Thus, a 15% increase in the enzyme fluorescence intensity after the addition of ATP induced a 10-cm displacement on the recorder; the noise was ~5% of the signal. The wavelengths of excitation and of emission were 300 and 338 nm, respectively.

The apparatus was equipped with a hollow cell jacket through which ethanol from a constant temperature bath was circulated. Nitrogen was flushed over the measurement cell to prevent condensation at low temperatures. The magnetically stirred enzyme solution contained 0.025 M sodium cacodylate–HCl buffer, 0.1 M KCl, 5 mM $MgCl_2$, and 0 or 30% (v/v) ethylene glycol. The concentration of protein was 1.85–2.1 mg/mL, and 5 μL of ATP (or analogue) solution was added to the 2-mL protein solution at zero time.

The effect of temperature on the fluorescence intensity of heavy meromyosin at the wavelength of maximal emission (λ = 338 nm) was studied under comparable experimental conditions [enzyme concentration of 1.85 mg/mL, 0 or 5 mM $MgCl_2$, 0.1 M KCl, 0.025 M sodium cacodylate–HCl buffer, 30% (v/v) ethylene glycol, pa_H 7.1]. It was checked that no shift of the fluorescence emission spectrum occurred in the relevant temperature range between +20 and −15 °C.

Fluorescence spectra of heavy meromyosin and acetyltryptophan ethyl ester, in different ethylene glycol–water mixtures, were obtained at 5 °C, with a thermostated absolute differential spectrofluorometer (Fica 55). The wavelength of excitation was 295 nm, and the emission spectrum was recorded between 300 and 450 nm. The protein concentration was 0.15–0.2 mg/mL, and the ester concentration was 0.05 mM. The medium contained 0.025 M sodium cacodylate–HCl buffer, 0.1 M KCl, and a variable percentage of ethylene glycol (experimental conditions of Figure 1).

Polarimetric Measurements. Optical rotatory dispersion spectra of heavy meromyosin [in 0.025 M sodium cacodylate–HCl buffer, 0.1 M KCl, and 0 or 50% (v/v) ethylene glycol] were recorded at 4 °C by means of a Fica spectropolarimeter (series no. 60 000). The protein concentration was 0.8 mg/mL between 600 and 280 nm and 0.06 mg/mL between 300 and 220 nm in a 1-cm path length cell. The values of the reduced mean residue rotation $[m']_{233}$ were calculated according to Fasman (1963) and Adler & Fasman (1968); refractive indexes at 20 °C were used for the calculations.

Potentiometric Measurements. Mg^{2+}- and Ca^{2+}-dependent ATPase activities of heavy meromyosin, in the presence of different percentages of ethylene glycol, were measured potentiometrically with a Radiometer pH stat (Type TTT1) at 5 °C and pH 8 (or pa_H 8). The reaction medium contained 0.1 M KCl, 0.025 M sodium cacodylate, 5 mM $MgCl_2$ or 4 mM $CaCl_2$, 0–50% (v/v) ethylene glycol, and 2.25 mM ATP in a volume of 2.5 mL. The protein concentration was 3.5 mg/mL (Mg^{2+}–ATPase activity) or 0.2 mg/mL (Ca^{2+}–ATPase activity). The titrant was 0.01 N NaOH. The initial rates of the ATPase reaction were given by the slopes of the straight lines obtained during the first minutes of the reaction. In the case of the low Mg^{2+}-dependent ATPase activity, the proton liberation was followed for 20–30 min. It was established that, at 5 °C and pH 8 (or pa_H 8), in a medium identical with that used for kinetic measurements (but without enzyme) the orthophosphate was almost fully dissociated, in aqueous solution and in 50% (v/v) ethylene glycol.

Potentiometric measurements at subzero temperatures were made with a Beckman pH meter, Model 1019, connected to a W + W potentiometric recorder. The glass electrode contained 30% (v/v) ethylene glycol and was supplied by Tacussel Electronique (France). The reference electrode (Tacussel Electronique) was a silver–silver chloride electrode filled with a 0.1 M KCl solution in 30% (v/v) ethylene glycol. The pH meter used as a millivoltmeter was standardized with several buffer systems in 30% (v/v) ethylene glycol of known pa_H values (Larroque et al., 1976). The reaction medium under nitrogen contained 0.1 M KCl, 5 mM $MgCl_2$, 0.025 M sodium cacodylate–HCl buffer, 30% (v/v) ethylene glycol, and 2.5 mg/mL heavy meromyosin in a volume of 5 mL. At zero time, 10 μL of Mg^{2+}–ATP solution in 30% (v/v) ethylene glycol was added to the protein solution, at the same pa_H, and the pa_H changes were recorded as a function of time. A known quantity of alkali was added at different times and was used to convert pa_H changes into moles of protons released. The 100% response time of the electrode (determined after the addition of an aliquot of NaOH to the medium) was no more than ~30 s at −8 °C and ~5 s at 22 °C.

Phosphate Ion Analysis. The quenching technique of Bagshaw & Trentham (1973) was used, with minor modifications, to determinate the relative proportions of $^{32}P_i$ and [γ-^{32}P]ATP during the hydrolysis of 5.5 μM [γ-^{32}P]ATP by heavy meromyosin (16 μM) in excess at −13.5 °C. The reaction medium contained 5 mM $MgCl_2$, 0.1 M KCl, and 0.025 M cacodylate buffer (pa_H 7.1) in 30% (v/v) ethylene glycol. All reagents were also in 30% (v/v) ethylene glycol, and their successive addition at −13.5 °C was carried out manually. After the addition of perchloric acid, the reaction medium became turbid because of the precipitation of protein and salts. Its pH was raised to ~4 with sodium acetate [the concentrated stock solution in 30% (v/v) ethylene glycol being kept at room temperature], and the solution was warmed at 20 °C to allow redissolution of salts. Subsequent manipulations were carried out at 20 °C. After chromatographic separation of substrate

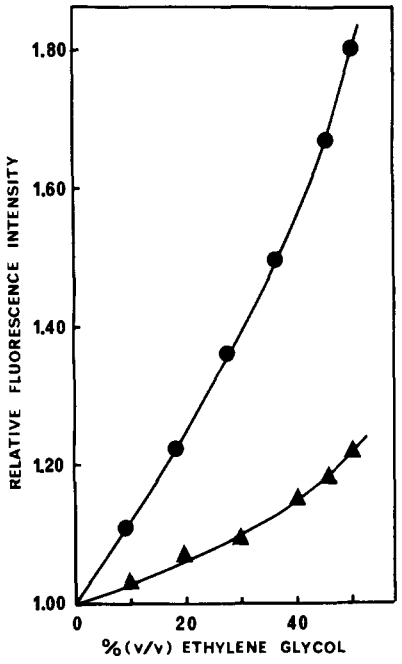

FIGURE 1: Variation with volume fraction of ethylene glycol of the relative fluorescence intensity of heavy meromyosin (▲) and acetyl-L-tryptophan ethyl ester (●) at 5 °C. The wavelength of excitation was 295 nm, and the wavelengths of emission were 338 and 355 nm for heavy meromyosin and acetyl-L-tryptophan ethyl ester, respectively. For other experimental conditions, see Materials and Methods.

and product on polyethylenimine–cellulose (0.75 M KH_2PO_4 buffer, pH 3.4, as developing solvent), the radioactive bands were cut out and eluted with 1.5 mL of 2 M HCl. Eluate aliquots of 0.5 mL were mixed with 10 mL of a water-miscible scintillant and counted for radioactivity in an Intertechnique SL 32 counter. Correction for $^{32}P_i$ contamination was made as indicated in the original paper (Bagshaw & Trentham, 1973).

Results

Effect of Ethylene Glycol on the Conformation of Heavy Meromyosin. Optical rotatory dispersion spectra of heavy meromyosin in aqueous solution and in 50% (v/v) ethylene glycol at 4 °C were nearly identical between 600 and 220 nm. At 233 nm the reduced mean residue rotation $[m']_{233}$ was found to be −8700 ± 150° in aqueous solution while it was −8400 ± 100° in 50% (v/v) ethylene glycol (after correction for the volume contraction). A larger increase of $[m']_{233}$ is expected if the conformation of the protein changes significantly; in 8 M urea, $[m']_{233}$ for myosin was −1670° instead of −8700° for the native enzyme (Simmons et al., 1961).

The fluorescence emission spectrum of heavy meromyosin (excited in the tryptophan band at 295 nm) in 50% (v/v) ethylene glycol shows an increase in intensity relative to that in aqueous solution, with no significant shift in the peak wavelength. As shown in Figure 1, the fluorescence intensity of heavy meromyosin, at the emission maximum, increases smoothly with ethylene glycol concentration, with no evidence of any abrupt change. The effect of glycol is much larger for the model compound acetyl-L-tryptophan ethyl ester, whose chromophore is fully accessible to solvent (Figure 1). According to Steiner et al. (1964), the slope of the change in protein fluorescence relative to that of an appropriate model compound gives a measure of the degree of exposure of the fluorescent side chains to the ambient solvent; it is evident, from Figure 1, that the value of this ratio does not change with the percentage of ethylene glycol. We conclude again that the conformation of the protein is not significantly modified by the addition of the solvent.

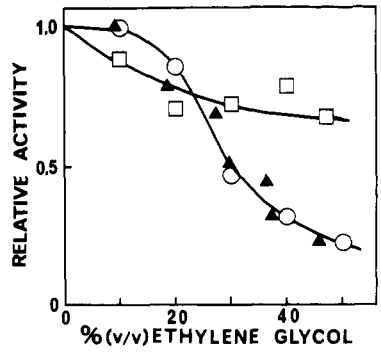

FIGURE 2: Effect of ethylene glycol on the Ca^{2+}-dependent (○) and Mg^{2+}-dependent (□) ATPase activities of heavy meromyosin at 5 °C and pH 8 (or pa_H 8). Effect of ethylene glycol on the naphthyl triphosphatase activity of heavy meromyosin at 5 °C and pa_H 6.9–7.4 (▲). The experimental conditions for the potentiometric (ATPase activities) and spectrophotometric (naphthyl triphosphatase activity) measurements are given under Materials and Methods.

In addition, the effect of temperature on the fluorescence intensity of heavy meromyosin, at the wavelength of maximum emission, was measured between +20 and −15 °C. The medium contained 30% (v/v) ethylene glycol. A linear increase in fluorescence intensity with decreasing temperature was observed, and the change, amounting to 30–35% over this temperature range, was shown to be completely reversible. Thus, no significant structural change of heavy meromyosin occurs in the investigated temperature range.

Effect of Ethylene Glycol on the Enzymatic Activity of Heavy Meromyosin. The effect of ethylene glycol on the enzymatic activity of heavy meromyosin against different substrates is shown in Figure 2. The Ca^{2+}-dependent ATPase and the naphthyl triphosphatase activities decrease markedly with the ethylene glycol concentration above ~20% (v/v) organic cosolvent, while the Mg^{2+}-dependent ATPase activity is hardly affected by the presence of ethylene glycol.

Fluorometric Measurements on Mg^{2+}–ATP Hydrolysis by Heavy Meromyosin between +20 and −15 °C. During the reaction of heavy meromyosin with Mg^{2+}–ATP the fluorescence of the enzyme changes as a function of time (Werber et al., 1972; Mandelkow & Mandelkow, 1973; Bagshaw & Trentham, 1974). The kinetics of these fluorescence changes in 30% (v/v) ethylene glycol are shown in Figure 3 at a number of temperatures between +21 and −15 °C and at two substrate concentrations (in large and small excess over enzyme). It is evident that the temperature has a pronounced effect on the form of the kinetics. On the other hand, the time course of the fluorescence change of heavy meromyosin between +21 and 0 °C is found to be very similar in aqueous solution and in 30% ethylene glycol.

At temperatures above 10 °C (upper curve of Figure 3), an immediate enhancement of the fluorescence to a level F_1 is observed at the onset of the reaction. The fluorescence then remains at this level until nearly all the substrate has been hydrolyzed; toward the end of the reaction it falls to a final level F_3 which is different from the initial value but identical with that obtained after addition of ADP to the native enzyme under the same experimental conditions. (This was found to hold well at all temperatures.) The readdition of an excess of ATP to the solution containing heavy meromyosin and hydrolyzed ATP produces the same kind of fluorescence changes. The catalytic constant of the hydrolysis reaction may be calculated from such a kinetic curve by using Chance's equation as described by Morita (1967); i.e., $k_{cat} = S_0/(E_0 t_{1/2})$,

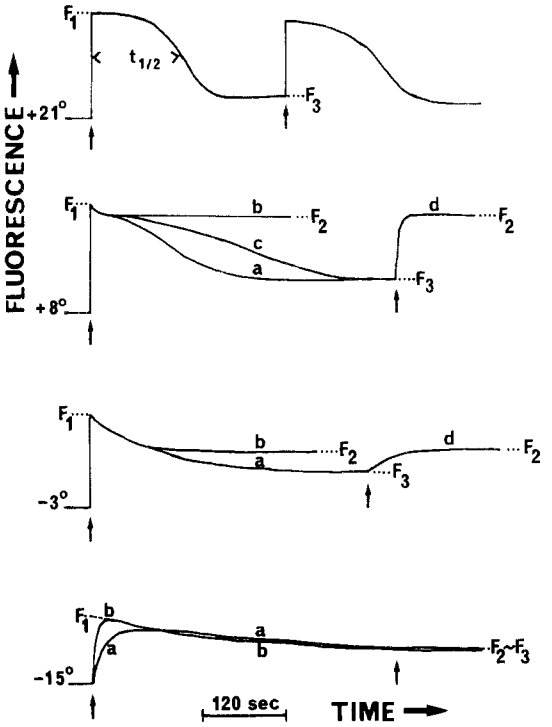

FIGURE 3: Time course of heavy meromyosin fluorescence during the hydrolysis of Mg^{2+}–ATP at selected temperatures between +21 and −15 °C. Experimental conditions: pa_H 7.1; 0.1 M KCl, 5 mM $MgCl_2$, 0.025 M sodium cacodylate–HCl buffer, 30% (v/v) ethylene glycol, and 5.3–6 μM heavy meromyosin in 2 mL total volume. At zero time, 5 μL of ATP was added, as indicated by the arrows. The final concentrations of ATP are the following: at +21 °C, 93 μM; at +8 °C, 25.3 μM (curve a), 186 μM (curve b), 50.6 μM (curve c); at −3 °C and −15 °C, 11.6 μM (curve a) and 186 μM (curve b). Readdition of ATP (185 μM in final concentration) was done at all temperatures, except at 21 °C (93 μM ATP). The rate curves obtained in the presence of a large excess of ATP (such as curves b and d) are recorded only for a few minutes and are not followed to their conclusion. The significance of the different fluorescent levels F_1, F_2, and F_3 is discussed in the text.

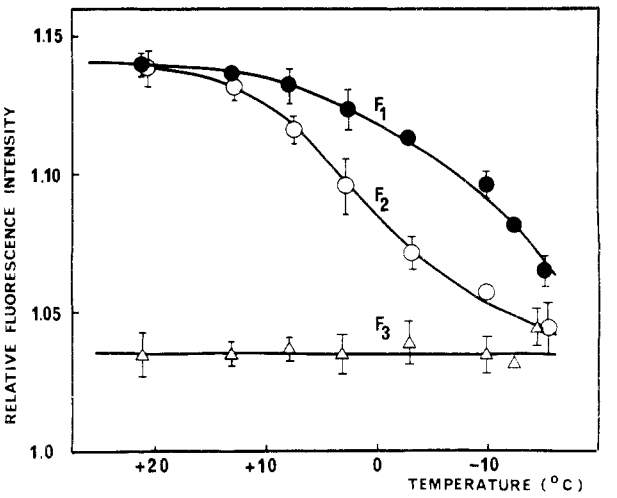

FIGURE 4: Effect of temperature on the amplitudes of the different enzyme fluorescence levels, F_1 (●), F_2 (○), and F_3 (△), observed during the reaction of heavy meromyosin with Mg^{2+}–ATP in 30% ethylene glycol. Experimental conditions are as described in Figure 3. The level F_2 is also determined after readdition of ATP to the reaction medium containing the enzyme and hydrolyzed ATP, and only the mean value of both measurements is given here. The reported values are the arithmetic means of at least five measurements. It may be noted that the addition of ADP (12 or 24 μM) to the free enzyme (5.5 μM) induces an enzyme fluorescence increase to the level F_3 at all temperatures.

where S_0 is the initial substrate concentration, E_0 is the enzyme concentration,[3] and $t_{1/2}$ is the time required for the fluorescence to drop to a point midway between its maximal and final levels. At 21 °C, the value of k_{cat}, measured in this manner, is 0.115 ± 0.005 s^{-1} in 30% (v/v) ethylene glycol and 0.085 ± 0.005 s^{-1} in aqueous solution; these values agree well with the spectrophotometric measurements (vide infra).

At temperatures between +10 and −10 °C (middle curves in Figure 3), a rapid initial fluorescence increase to the level F_1 is also observed, but the fluorescence does not stay at this level and rapidly falls according to a first-order rate constant k_0 to a level F_2, which corresponds to the steady-state fluorescence. It remains at this level for a greater or lesser period of time depending on the initial substrate concentration before it decays slowly to the final level F_3. This slow decay from F_2 to F_3 (as in curve c of Figure 3 at +8 °C) is due partly to the low value of k_{cat} [the rate constant of this decay is proportional to k_{cat}, as shown by Mandelkow & Mandelkow (1973)] and partly to some inhibition of the reaction by the product ADP (which can also be brought about by adding ADP at the beginning of the reaction). By working at two extreme substrate concentrations relative to that of the enzyme, we can precisely determine these two levels F_2 and F_3, but because of the complexity of the kinetics the application of Chance's equation to the calculation of k_{cat} is unreliable. The readdition of an excess of ATP to the solution containing heavy meromyosin and hydrolyzed ATP does not lead to the same kinetic time course as the first addition; instead, a fluorescence increase, with first-order rate constant k_0', to a level similar to F_2 is observed. At a given temperature, k_0 and k_0' have closely similar values.

At temperatures lower than −10 °C (lower curve in Figure 3), the initial enhancement of the fluorescence (to F_1) has a low amplitude and is no longer instantaneous; it becomes time dependent in the range 5–60 s, depending on the substrate concentration. For a low substrate concentration (11.6 μM), the fluorescence transient appears monophasic (curve a at −15 °C in Figure 3) with a rate of increase of 0.055 ± 0.005 s^{-1}. The subsequent diminution of the fluorescent intensity to the level F_2 (high substrate concentration) or F_3 (low substrate concentration) is slow, and the kinetics at two different substrate concentrations are nearly superimposable. The readdition of ATP to the medium containing the enzyme and hydrolyzed ATP (or to a new medium containing enzyme and ADP) does not induce any measurable fluorescence change.

The values of the fluorescence levels F_1, F_2, and F_3 observed during the hydrolysis of Mg^{2+}–ATP by heavy meromyosin in 30% (v/v) ethylene glycol and in aqueous solution are shown as a function of temperature in Figures 4 and 5, respectively. For these determinations, the substrate was present in sufficient excess over the enzyme, especially at low temperatures. At any given temperature, the values of F_1, F_2, and F_3 are lower in 30% (v/v) ethylene glycol than in water, but the decrease of F_1 and F_2 with temperature is more marked in aqueous solution.

The values of k_{cat}, k_0 (or k_0') and k_b in the temperature ranges where measurements are possible are shown in Figure 6 in the form of an Arrhenius plot. Here k_b represents the

[3] The heavy meromyosin molecule has two ATPase sites, but the question of the relation between the two active sites was not considered in this work. The kinetic data were analyzed by considering only the activity of the total protein and were not related to the activity of one site. In the presence of an ATP regenerating system we have measured that at 20 °C 1.6–1.8 ATP molecules/heavy meromyosin molecule were required to give the maximal fluorescence signal F_1. At this temperature, it is generally assumed that both active sites are identical and independent.

Table I: Thermodynamic Parameters of the Overall Reaction of Mg^{2+}-ATP Hydrolysis by Heavy Meromyosin and the Dissociation of the Heavy Meromyosin-ADP Complex in Aqueous Solution and in 30% (v/v) Ethylene Glycol[a]

thermodynamic parameter[b]	k_{cat} in aq soln at $T > +5$ °C		k_{cat} in 30% (v/v) ethylene glycol at $T > +5$ °C		k_{cat} in 30% (v/v) ethylene glycol at $T < -5$ °C	k_b in 30% (v/v) ethylene glycol
	c	d	c	d	c	d
$\Delta G^{\ddagger\ddagger}$ (kJ/mol)	+78.5	+78.5	+77.5	+77.5		+70.5
$\Delta H^{\ddagger\ddagger}$ (kJ/mol)	+41	+41.5	+61.5	+55.5	+116	+122.5
$\Delta S^{\ddagger\ddagger}$ [J/(mol deg)]	−126	−123.5	−53.5	−73	+146.5	+176

[a] Other conditions: 5 mM $MgCl_2$; 0.1 M KCl; 0.025 M cacodylate buffer; pH 7 (or pa_H 7). [b] A linear regression program was used in these calculations with a Hewlett-Packard Model 9830 A calculator. [c] Spectrophotometric measurements. [d] Fluorometric measurements.

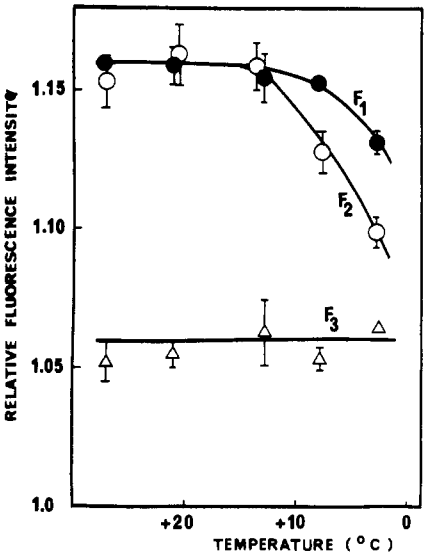

FIGURE 5: Effect of temperature on the amplitudes of the different fluorescence intensities, F_1 (●), F_2 (○), and F_3 (△), observed during the reaction of heavy meromyosin with Mg^{2+}-ATP in aqueous solution. Experimental conditions are as described in Figure 3, except that the organic cosolvent is absent. The level F_2 is also determined after readdition of ATP to the reaction medium containing the enzyme and hydrolyzed ATP, and only the mean value of both measurements is given here. The reported values are the arithmetic means of at least five measurements.

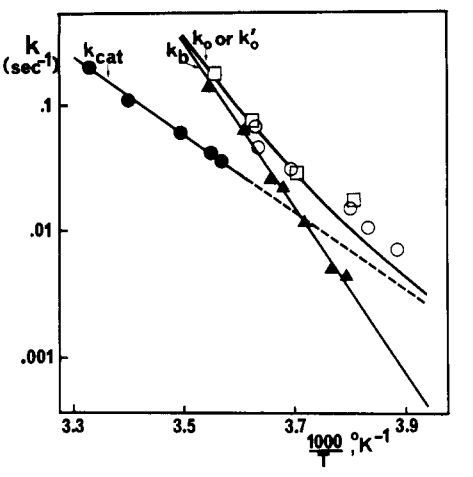

FIGURE 6: Arrhenius plots of the rate constants k_{cat} (●), k_0 (○) or k_0' (□), and k_b (▲). Experimental conditions are as described in Figure 3. The theoretical curve, calculated from the sum of values of k_a' and k_b at a given temperature, passed through the experimental values of k_0 (or k_0'). As explained under Discussion, k_a' is taken to be equal to k_{cat} at the high temperatures and to the extrapolated values on the broken line at the low temperatures.

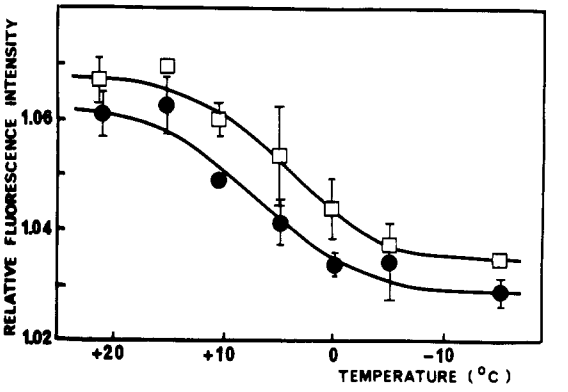

FIGURE 7: Effect of temperature on the amplitude of the enzyme fluorescence increase observed during the reaction of heavy meromyosin with α,β-methyleneadenosine 5′-triphosphate (●) and β,γ-imidoadenosine 5′-triphosphate (□). Experimental conditions: pa_H 7.2; 0.1 M KCl; 5 mM $MgCl_2$; 0.025 M sodium cacodylate–HCl buffer; 30% (v/v) ethylene glycol; enzyme concentration = 5.5 μM; ATP analogue concentration = 25 or 50 μM.

rate constant for dissociation of the heavy meromyosin–ADP complex. According to Arata et al. (1974), it is obtained by adding a sufficient concentration of the displacing agent pyrophosphate [only 0.25 mM in this work because of the limited solubility of pyrophosphate in 30% (v/v) ethylene glycol] to the complex resulting from the addition of ADP (12 μM) to heavy meromyosin (5 μM) and recording the rate of the ensuing fluorescence decrease. Thermodynamic parameters for k_{cat} and k_b are reported in Table I.

Finally, and for comparison, the fluorescence changes of heavy meromyosin induced by the addition of the ATP analogues AMPCPP or AMPPNP in the presence of Mg^{2+} and 30% (v/v) ethylene glycol have been studied. The form of the kinetics of hydrolysis of AMPCPP is the same at all temperatures between +21 and 0 °C (the lowest temperature at which the reaction may be followed to completion) and is analogous to that observed during the hydrolysis of Mg^{2+}-ATP at $T > 10$ °C. The values at different temperatures of the amplitude of the initial fluorescence rise induced by the addition of AMPCPP to HMM and of the fluorescence enhancement due to the addition of AMPPNP to HMM are shown in Figure 7. (Under our experimental conditions, these fluorescence increases are instantaneous only at temperatures above 0 °C.) The fluorescence intensities associated with the complexes HMM–AMPPNP and HMM–AMPCPP rapidly decrease with temperature, with a transition midpoint at about +5 °C.

Spectrophotometric Measurements of the Mg^{2+}-ATP Hydrolysis by Heavy Meromyosin between +20 and −15 °C. The rate v of ADP release during the enzymatic hydrolysis of ATP (at the concentration 0.1 mM) was followed spectrophotometrically under steady-state conditions, between +20 and −15 °C, by means of a linked assay system using pyruvate kinase and lactate dehydrogenase (Imamura et al., 1966;

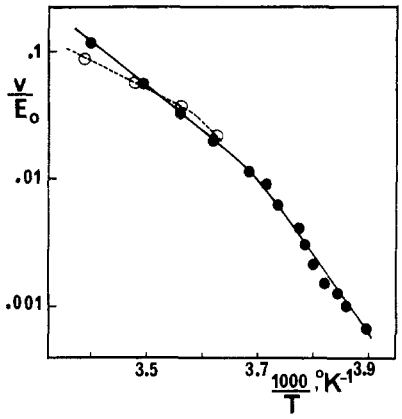

FIGURE 8: Arrhenius plot of the ratio of the reaction velocity to the enzyme concentration, v/E_0 (equivalent to the catalytic rate constant k_{cat}), for the hydrolysis of Mg^{2+}–ATP (0.1 mM) by heavy meromyosin in 30% (v/v) ethylene glycol (●) or in aqueous solution (○). The experimental conditions are given under Materials and Methods. The points at temperatures higher than 0 °C are the mean of four measurements.

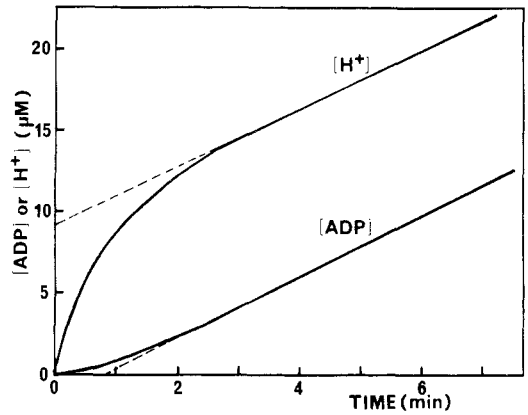

FIGURE 9: Time course of ADP and proton release at low temperature. Experimental conditions for ADP release experiments: pa_H 7.1; T = −6 °C; 4 mM $MgCl_2$; 0.1 M KCl; 27.5% (v/v) ethylene glycol; 0.022 M cacodylate buffer; 44 μM ATP; 5.1 μM heavy meromyosin; 0.1 mM NADH; 1 mM phosphoenolpyruvate; pyruvate kinase = 0.17 mg/mL; lactate dehydrogenase = 0.25 mg/mL. Extrapolation of the steady-state region of the progress curve to zero gives the value of the relaxation time, τ. For direct comparison with the potentiometric measurements it was verified that the time course of ADP release at pa_H 8.4 was indistinguishable from that at pa_H 7.1. Experimental conditions for the measurement of proton release after addition of Mg^{2+}–ATP to heavy meromyosin: pa_H 8.4; T = −7.5 °C; 5 mM $MgCl_2$; 0.1 M KCl; 30% (v/v) ethylene glycol; 0.025 M cacodylate buffer; 45 μM ATP; 7.25 μM heavy meromyosin. Extrapolation of the steady-state rate region of the progress curve to zero gives the size of the proton burst, π.

Trentham et al., 1972). The corresponding Arrhenius plot (v/E_0 vs. $1/T$) is shown in Figure 8; it is characterized by a discontinuity at ~0 °C. At temperatures above 0 °C the activation energy of the reaction is +65 kJ/mol, whereas below −5 °C it is +120 kJ/mol. The activation energy of the hydrolysis reaction in aqueous solution is lower (+45 kJ/mol); a change in the slope of the Arrhenius plot below +5 °C may be anticipated (Figure 8). The thermodynamic parameters of the hydrolysis reaction in water and in 30% (v/v) ethylene glycol are given in Table I ($k_{cat} = v/E_0$; it was shown that v was practically identical for [ATP] = 0.1 and 0.02 mM in the temperature range investigated).

Moreover, in the time course of ADP release at low temperature (T < 0 °C), a lag phase was apparent in the first moments of the reaction (Figure 9). This lag phase remained practically the same when the concentrations of both coupled

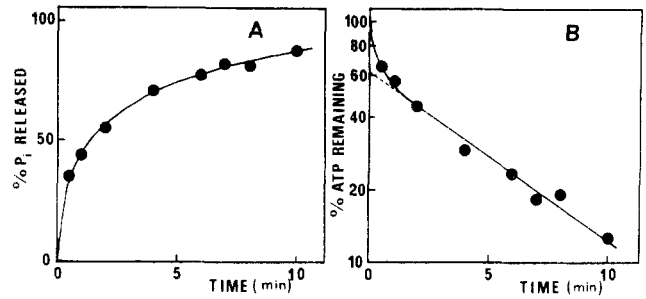

FIGURE 10: Kinetics of ATP cleavage and P_i liberation during a single turnover of heavy meromyosin. Experimental conditions: pa_H 7.1; T = −13.5 °C; 5 mM $MgCl_2$; 0.1 M KCl; 30% (v/v) ethylene glycol; 0.025 M cacodylate buffer; 5.5 μM [γ-^{32}P]ATP; 16 μM heavy meromyosin. (A) Release of $^{32}P_i$; (B) semilogarithmic plot of [γ-^{32}P]ATP remaining unhydrolyzed.

enzymes were simultaneously doubled or halved. The relaxation time τ, defined as the time required to reach $1/e$ of the steady-state signal, was estimated to be 55 s at −6 °C and was much longer than the time required for the normal conversion of ADP by the coupled enzyme assay at this temperature; 95% of extra ADP (at a low concentration on the order of 10 μM) added to the medium was converted within 10 s. Therefore, this lag phase characterizes the initial, slow hydrolysis reaction catalyzed by heavy meromyosin and not the fast reactions catalyzed by pyruvate kinase and lactic dehydrogenase.

Potentiometric Measurements of Mg^{2+}–ATP Hydrolysis by Heavy Meromyosin at Subzero Temperature. Since a lag phase in ADP release is observed in the pre-steady-state of the Mg^{2+}–ATP hydrolysis reaction by heavy meromyosin at low temperatures, it is of interest to determine also the time course of formation of the second hydrolysis product, P_i. Green & Mommaerts (1953) have shown that the hydrolysis of ATP by myosin liberated one orthophosphate ion and one proton per molecule of ATP at around pH 8. We have therefore carried out potentiometric measurements of proton release at −7.5 °C and at pa_H 8.4 with the conditions described under Materials and Methods.

A proton burst followed by a steady-state proton release is observed at this temperature during the first 2 min of the reaction (Figure 9). Under the experimental conditions of this study, ~1.25 ± 0.1 mol of H^+/mol of enzyme (mean of seven experiments) appears at the beginning of the reaction, as estimated by extrapolation to zero time of the linear part of the curve.

For comparison, the proton release associated with the binding of ADP to the enzyme was also measured under the same experimental conditions. About 0.7 mol of H^+/mol of enzyme became rapidly detectable at a rate limited by the response time of the electrode.

Phosphate Ion Liberation during a Single Turnover of Heavy Meromyosin at Subzero Temperature. With the purpose of characterizing the main intermediate complex present at the beginning of the reaction at low temperatures (see Discussion), the quenching technique of Bagshaw & Trentham (1973) was used. Under conditions in which the enzyme concentration is higher than that of the substrate, the fractional release of phosphate ion or the survival of ATP as a function of time is shown in Figure 10.

The plots are biphasic; after a rapid initial cleavage of 30–40% ATP, the remaining substrate is hydrolyzed with a first-order rate constant of 2.7×10^{-3} s^{-1} at −13.5 °C (Figure 10B). Extrapolation of the linear plot to zero time gives the proportions of ATP (62%) and ADP + P_i (38%) bound to heavy meromyosin in the equilibrium mixture [see Taylor &

Weeds (1977) for such an analysis at +25 °C].

Discussion

Measurements at subzero temperature generally require the introduction of an organic cosolvent, with a consequent possibility of effects on the conformation and the activity of enzymes. Ethylene glycol may be considered as an inert solvent for proteins (Tanford et al., 1962); Kay & Brahms (1963) have shown by optical rotatory dispersion experiments that the conformation of heavy meromyosin, at 25 °C, was not appreciably modified when the medium contained up to 67% (v/v) ethylene glycol. Their observation is confirmed in the present work by polarimetric and fluorometric measurements at a lower temperature, viz., 4–5 °C. Moreover, a change from +20 to −15 °C induces no significant structural modification of heavy meromyosin in 30% (v/v) ethylene glycol.

The enzymatic activity of heavy meromyosin under steady-state conditions is, however, in various ways affected by the presence of ethylene glycol. A strong inhibitory effect on the Ca^{2+}-dependent ATPase and naphthyl triphosphatase activities is observed at 5 °C, whereas the Mg^{2+}-dependent ATPase activity is only slightly diminished in 30% or even 50% organic cosolvent. Spectrophotometric and fluorometric measurements of the Mg^{2+}–ATP hydrolysis by heavy meromyosin, at a series of temperatures between 0 and 20 °C, show that the activation energy of the reaction is increased in 30% (v/v) ethylene glycol relative to that in water (Table I); thus, depending on the temperature, either activation or inhibition of the reaction by ethylene glycol can occur. [This last result probably applies equally to the Ca^{2+}-dependent ATPase activity of heavy meromyosin because at 25 °C (Kay & Brahms, 1963) or at 37 °C (Kaldor, 1968) the effect of ethylene glycol on this activity is small.]

The mechanism of the Mg^{2+}–ATP hydrolysis by heavy meromyosin is similar in aqueous solution and in 30% (v/v) ethylene glycol as indicated by the similarity of the rate curves in both media at temperatures above 0 °C. The temperature effect on the shape of the fluorescence kinetics of ATP hydrolysis is, however, somewhat more noticeable in aqueous solution than in 30% (v/v) ethylene glycol (compare Figures 4 and 5); this is probably due to differences in activation energies for the kinetic steps implicated in these fluorescence changes.

This limited effect of ethylene glycol on the enzymatic activity of heavy meromyosin may therefore result from a minor conformational change of the active site of the enzyme or a slightly modified reactivity of the entities implicated in the hydrolysis reaction, the gross conformation of the protein being unaffected.

The kinetics of the fluorescence changes of heavy meromyosin during the reaction with Mg^{2+}–ATP have been analyzed, as a first approach, according to the scheme of Bagshaw & Trentham (1974)

$$\begin{aligned} M + ATP \rightleftharpoons M{\cdot}ATP \rightleftharpoons M^*{\cdot}ATP \rightleftharpoons M^{**}{\cdot}ADP{\cdot}P_i \rightleftharpoons \\ (M^*{\cdot}ADP{\cdot}P_i \rightleftharpoons)\ M^*{\cdot}ADP \rightleftharpoons M{\cdot}ADP \rightleftharpoons M + ADP \\ + P_i + H^+ \end{aligned}$$

in which M is heavy meromyosin and asterisked intermediates are species with enhanced protein fluorescence relative to the free enzyme. The complex $M^*{\cdot}ADP{\cdot}P_i$, the existence of which is questionable (Goody et al., 1977), is placed in parentheses.

The fast initial enhancement[4] of fluorescence to the level F_1 corresponds to the formation of the most highly fluorescent complex $M^{**}{\cdot}ADP{\cdot}P_i$, which is in rapid equilibrium with $M^*{\cdot}ATP$. The subsequent drop in fluorescence to the level F_2 (apparent at temperatures lower than +10 °C) is linked to the establishment of the steady state of the reaction; the amplitude of F_2 depends on the relative concentrations of the two components differing in fluorescence, i.e., $M^{**}{\cdot}ADP{\cdot}P_i$ (in rapid equilibrium with $M^*{\cdot}ATP$) and $M^*{\cdot}ADP$, in the steady state. The final fluorescence level F_3 corresponds to the fluorescence of the $M^*{\cdot}ADP$ complex.

We consider first the significance of the changes in amplitude of F_1 and F_2 with temperature.

The amplitude of F_1 decreases slowly with temperature. One possible explanation for this phenomenon is that the equilibrium

$$M^*{\cdot}ATP \overset{K_3}{\rightleftharpoons} M^{**}{\cdot}ADP{\cdot}P_i$$

is displaced to the left, in favor of the less fluorescent complex $M^*{\cdot}ATP$, when the temperature decreases. There is some evidence from the literature (Taylor, 1977b; Inoue et al., 1977) that the equilibrium constant K_3 is lower at low temperature. We find (Figure 10) that, at −13.5 °C, the complex $M^*{\cdot}ATP$ is in twofold excess over $M^{**}{\cdot}ADP{\cdot}P_i$; the equilibrium constant K_3 is therefore ~0.6 at −13.5 °C, while under closed experimental conditions its value ranges from 4 to 9 at 20–25 °C (Taylor & Weeds, 1977; Taylor, 1977b; Bagshaw & Trentham, 1973).

On the other hand, the relative fluorescent intensity of the complex $M^*{\cdot}ATP$ is probably temperature dependent while that of $M^{**}{\cdot}ADP{\cdot}P_i$ must be little or not at all affected by a temperature decrease. Indeed, as shown in Figure 7, the relative fluorescence intensities of the complexes HMM–AMPCPP and HMM–AMPPNP decrease rapidly with temperature [see also Morita (1977)]; at low temperatures the relative fluorescence intensities reach the constant value of the fluorescence of the $M^*{\cdot}ADP$ complex. Now, in the hydrolysis of AMPCPP the predominant steady-state intermediate is the complex $M^*{\cdot}AMPCPP$ (Mannherz et al., 1973) and the identity of this intermediate does not change with temperature (at least between +21 and 0 °C). Thus, the complex of heavy meromyosin with AMPCPP may be considered a good model for the complex $M^*{\cdot}ATP$.

The gradual decrease with temperature of the amplitude of F_1 is therefore most probably due to a shift of the equilibrium $M^*{\cdot}ATP \rightleftharpoons M^{**}{\cdot}ADP{\cdot}P_i$ to the left, in favor of $M^*{\cdot}ATP$, the fluorescence of which is temperature dependent.

The amplitude of F_2 decreases rapidly with temperature. As previously suggested by Bagshaw & Trentham (1974), such a decrease is probably a consequence of a change in the identity of the rate-limiting step. For purposes of discussion, the preceding scheme may be written in the simplified form

[4] Johnson & Taylor (1978) have shown by stopped-flow measurements that this fluorescence increase is biphasic at +5 °C since the rate of conversion of $M{\cdot}ATP$ into $M^*{\cdot}ATP$ is faster than that of $M^*{\cdot}ATP$ into $M^{**}{\cdot}ADP{\cdot}P_i$. At −15 °C, where this fluorescence increase is no longer instantaneous, the dead time of the apparatus precludes observation of the initial stages of the reaction, and the kinetics of the fluorescence change appear monophasic. It may be noted that this transient phase seems to have a large dependence on temperature; at 20 °C its apparent second-order rate constant (in aqueous solution) is $2 \times 10^6\ M^{-1}\ s^{-1}$ (Johnson & Taylor, 1978), while at −15 °C [in 30% (v/v) ethylene glycol] it is $\sim 5 \times 10^3\ M^{-1}\ s^{-1}$.

$$M + ATP \xrightarrow{fast} M^*\cdot ATP \rightleftharpoons M^{**}\cdot ADP\cdot P_i \xrightarrow{k_a} M^*\cdot ADP \xrightarrow{k_b} M + ADP + P_i + H^+$$

Depending on the temperature, the species M*·ATP, M**·ADP·P_i, and M*·ADP are present in the reaction steady state in different ratios, with $k_{cat} = (k_a'k_b)/(k_a' + k_b)$ with $k_a' = (k_aK_3)/(1 + K_3)$. At 20 °C (Trentham et al., 1976), M**·ADP·P_i is the predominant steady-state complex; in which case, $F_2 \simeq F_1$ and $k_{cat} \simeq k_a' \simeq k_a$ since $k_b > k_a'$ and $K_3 > 1$. At temperatures lower than −10 °C, M*·ADP becomes predominant because of the strong temperature dependence of k_b ($\Delta H^{\ddagger} = 122.5$ kJ/mol) compared to that of k_a ($\Delta H^{\ddagger} \simeq$ 55–60 kJ/mol); in this case, $F_2 \simeq F_3$ and $k_{cat} \simeq k_b$ since $k_b < k_a, k_a'$. This last result is apparent from a comparison of Figures 8 and 6 for the changes of k_{cat} (spectrophotometric measurements) and k_b (fluorometric measurements) with temperature; at −13.5 °C, the values of k_{cat} (1.05×10^{-3} s^{-1}) and k_b (1.55×10^{-3} s^{-1}, extrapolated value) are of similar magnitude, while the calculated value of k_a', viz., to 3.05×10^{-3} s^{-1}, is somewhat higher. (The experimental value of k_a' is 2.7×10^{-3} s^{-1}.)

The decay of the initial fluorescence F_1 to F_2, corresponding to the steady state, is an exponential process occurring at a rate $k_0 = k_a' + k_b$; likewise, addition of ATP to the final reaction mixture induces a fluorescence change which follows an exponential course at a rate characterized by $k_0' = k_a' + k_b$ (Bagshaw & Trentham, 1974). As shown in Figure 6, the experimental values of k_0 (or k_0') as a function of $1/T$ fit rather well with the calculated curve of $k_a' + k_b$. Here, as a first approximation, k_a' is derived by extrapolation from the values of k_{cat} measured at high temperatures (at −13.5 °C, the extrapolated value of k_a' is 4.9×10^{-3} s^{-1} while the experimental value from the data of Figure 10 is 2.7×10^{-3} s^{-1}). Deviations from this theoretical curve are observed at low temperatures (≤−10 °C), but k_0 values may be overestimated under these conditions due to the small and slow changes of the signal.

The validity of such an interpretation of the fluorometric data is substantiated by a change in the slope of the Arrhenius plot of k_{cat} (Figure 8). Such a change has in fact been observed in ATP hydrolysis by myosin (Watterson et al., 1975, and references therein), subfragment-1 (F. Travers, personal communication), and even heavy meromyosin (Ishigami & Morita, 1977). It is usually interpreted as reflecting a change in the nature of the rate-limiting step of the reaction with temperature.

A more direct proof of the validity of the scheme of Bagshaw & Trentham would be afforded by a demonstration of sequential release of the two products of the reaction. This cannot be observed at 20 °C since the rate-limiting step of the reaction precedes release of the products. At temperatures below 0 °C, by contrast, both a proton burst and a lag phase in the release of ADP are observed during the pre-steady-state of Mg^{2+}–ATP hydrolysis by heavy meromyosin.

The release of ADP is measured indirectly by means of the coupled enzyme assay. The lag phase (Figure 9) reflects the slow reaction catalyzed by heavy meromyosin and not a delayed response of the coupled enzymes as can sometimes happen in this kind of assay (Easterby, 1973, and references therein).

The significance of the proton burst observed at −7.5 °C is less certain, however. It may be associated with the liberation of free P_i into the medium and/or with the proton-dependent isomerization of heavy meromyosin in the various intermediate enzyme–substrate and enzyme–product complexes.

In the first case, the size of the proton burst, π, depends on the ratio of the kinetic constants k_a' and k_b. It may indeed be shown that to a first approximation (Bender et al., 1966) $\pi = E_0[[k_a'/(k_a' + k_b)]/(1 + K_m/S)]^2$ (where E_0 is the total enzyme concentration, S is the substrate concentration, and K_m is the Michaelis constant). Under conditions in which $S > K_m$ and $k_b \gg k_a'$, π vanishes and there is no burst; if $k_a' \gg k_b$, $\pi = E_0$. While at 20 °C protons are liberated linearly with time (Tonomura et al., 1962), at low temperatures where k_a' is greater than k_b the size of the proton burst becomes significant.

In the second case, it has been shown that a transient proton release associated with the isomerization of heavy meromyosin occurs in the first steps of the hydrolysis at 20 °C (Koretz & Taylor, 1975; Chock & Eisenberg, 1974). This proton burst (~0.6 mol of H^+/mol of enzyme at pH 8) is also observed at 20 °C or even at −7.5 °C, as shown in the present work, following the binding of various ATP analogues or ADP (Marsh et al., 1977) to the enzyme.

Under our experimental conditions in which $S > E_0$, the protons released in the pre-steady-state of the reaction at −7.5 °C come from the first steps of the reaction up to the steps of the formation of the steady-state intermediates (which are a mixture of M*·ATP, M**·ADP·P_i, and M*·ADP). The proton burst observed is therefore due both to pH-dependent enzyme isomerizations in the intermediate complexes and to the liberation of free phosphate into the medium. For a burst of 1.25 ± 0.1 protons/mol of enzyme at −7.5 °C, it may be estimated that 0.6–0.7 proton comes from enzyme ionizations and ~0.6 proton comes from ionization of liberated phosphate. Preliminary measurements of $^{32}P_i$ release have been performed after acid quenching of the reaction of an excess of [γ-^{32}P]ATP with HMM; a transient P_i liberation is observed in the first 2 min of the reaction before the steady-state P_i release and after an initial immediate P_i burst of low amplitude which corresponds to the formation of M**·ADP·P_i.

All of our kinetic data may thus be accommodated in the scheme of Bagshaw & Trentham. Inoue et al. (1977, and references therein) have proposed a different reaction scheme with two pathways, the first of which is analogous to that of Bagshaw & Trentham but without any sequential release of products. A kinetic argument for the occurrence of a second pathway was the low rate of reappearance of the steady-state complexes after the readdition of ATP to a medium containing heavy meromyosin and hydrolyzed ATP (see Figure 3). This phenomenon is rather well explained by the fact that heavy meromyosin is not free in the presence of hydrolyzed ATP but is present as the complex M*·ADP, the dissociation of which is slow, particularly at low temperature; the rate of return to the steady state is therefore $k_a' + k_b$, as indicated above.

Kinetic studies of enzymatic reactions at subzero temperatures allow the characterization of reaction intermediates which may be isolated. Thus, at −15 °C, the complexes M*·ATP (in rapid equilibrium with M**·ADP·P_i) and M*·ADP, which are identified in the reaction pathway, have half-lives of about 5 and 10 min, respectively [as estimated from the values of k_{cat} (spectrophotometric measurements) and k_b (fluorometric measurements)]. The isolation of these complexes by rapid gel filtration (Taylor et al., 1970) would therefore be possible. The nature and the physicochemical properties of these complexes which are yet poorly characterized might thus be open to analysis.

In conclusion, we note that the results on the kinetic behavior of heavy meromyosin at low temperature may also be relevant to an understanding of the complex temperature-dependent kinetics of actomyosin. Thus, the species M*·ATP and M**·ADP·P_i are transiently formed during Mg^{2+}–ATP hydrolysis by actomyosin (Johnson & Taylor, 1978, and references therein), and Marston (1978) has recently suggested that the step M*·ATP $\rightleftharpoons$ M*·ADP·P_i might become rate limiting at low temperature in the hydrolysis of Mg^{2+}–ATP by actin subfragment-1 ATPase. The application of low-temperature techniques might allow the analysis of this phenomenon.

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