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SYNTHESIS OF 2-(DANSYLAMINO)ETHYL TRIPHOSPHATE AND ITS PROPERTIES AS A FLUORESCENT SUBSTRATE OF HEAVY MEROMYOSIN-ATPase

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SUMMARY

2-(Dansylamino)ethanol, which was obtained after the reaction of dansyl chloride with ethanolamine, was phosphorylated by using tetrachloropyrophosphate, and the resulting 2-(dansylamino)ethyl monophosphate was further phosphorylated by using P_i and *N,N'*-dicyclohexylcarbodiimide to obtain 2-(dansylamino)ethyl triphosphate (DTP).

DTP was hydrolyzed by heavy meromyosin-ATPase at a rate similar to the hydrolysis of ATP in the presence of Ca^{2+}. In the presence of 10 mM Mg^{2+}, the K_m of the DTP hydrolysis by heavy meromyosin was $1.9 \cdot 10^{-5}$ M, the hydrolysis apparently being abolished by the addition of ATP.

Marked increases in the intensity of the maxima of the excitation and emission spectra of DTP were observed after the addition of heavy meromyosin in the presence of 83.4 mM Mg^{2+} at 10°. The peak of the emission spectrum shifted from 540 to 530 nm accompanied by an increase in intensity, thus suggesting that the polarity around DTP became rather hydrophobic. Energy transfer from tryptophan and/or tyrosine to the dansyl group of DTP was assumed.

INTRODUCTION

The structure of the active site of myosin-ATPase (EC 3.6.1.3) has been investigated by using substrate analogs, all of which contained purine or pyrimidine bases or their derivatives. TONOMURA *et al.*[1] concluded from their study that the N-6 or O-6 of purine and pyrimidine bases is necessary for the interaction of the substrate analogs with the active site, and that the appropriate distance between the triphosphate and the base should be maintained for a desirable fit of the substrate analog with myosin.

On the other hand, it has been known since the observations of SINGER AND BARRON[2] and of KIELLY AND BRADLEY[3] that the sulfhydryl groups of myosin are

Abbreviations: DTP, 2-(dansylamino)ethyl triphosphate; DDP, 2-(dansylamino)ethyl diphosphate; DMP, 2-(dansylamino)ethyl monophosphate.

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essential for its ATPase activity. Recently, MURPHY AND MORALES[4] have shown by affinity labelling to the –SH group using 6-mercapto-9-β-D-ribofuranosylpurine 5′-triphosphate as the substrate analog that low-molecular-weight components[5] included in the myosin molecule may be indispensable for its enzymic activity.

As an approach to clarify the structure of the enzyme's active site, fluorescent substrate analogs have sometimes been used with success[6,7]. It is now expected that fluorescent reagents will be most useful in the study of the enzyme's active site.

In this paper the synthesis of a new fluorescent substrate, 2-(dansylamino)ethyl triphosphate (DTP), of myosin-ATPase is presented. It was hydrolyzed by heavy meromyosin-ATPase at a comparable rate to the hydrolysis of ATP. The qualitative nature of DTP hydrolysis resembles more that of ITP than that of ATP. It was also shown that the environment around the bound DTP was rather hydrophobic. Some of the results have been presented elsewhere[8,9].

MATERIALS AND METHODS

Heavy meromyosin was prepared from rabbit skeletal muscle by the method similar to that described by SZENT-GYÖRGYI[10].

The dephosphorylation reaction was stopped by addition of trichloroacetic acid at a final concentration of 5 %, and the P_i liberated was determined by the methods of FISKE AND SUBBAROW[11] or MARTIN AND DOTY[12]. The enzymatic activity of heavy meromyosin-ATPase measured at 25° was expressed in units, which were defined as μmoles P_i liberated per min per mg of protein. The concentration of heavy meromyosin was determined by using the absorbance at 280 nm of 0.63 for 1 mg protein per ml solution.

Paper electrophoresis was performed at pH 7–8 (50 mM triethylammonium bicarbonate buffer) and at 30 V/cm for 1–2 h using the Mitsumi electrophoresis instrument. Paper chromatography was performed by using Toyo Roshi 3 A paper (16 cm × 60 cm), following the descending technique. The spots were detected under ultraviolet light from a Manasul lamp.

The carbonate form of DEAE-cellulose was soaked in 0.5 M ammonium carbonate for 1–2 h with stirring and then washed with water in a column (2 cm × 60 cm) until the wash water became neutral. The height of DEAE-cellulose(bicarbonate form) in the column was 47 cm.

The P_i content in 2-(dansylamino)ethyl phosphates was analyzed by the method of ALLEN[13].

Absorption spectra were measured with a Hitachi 124 spectrophotometer.

Fluorescence measurements were carried out with a Hitachi 2PF-3A spectrofluorimeter and the temperature of the cell holder was regulated by circulating water. The fluorescence spectra shown in this paper were not corrected for the spectral response of the photomultiplier and the Xenon lamp. Excitation bandwidths were 3 or 4 nm and the emission band width was 12 nm.

NMR spectra were measured by a JEOL 3H 60 high resolution NMR spectrometer.

Organic solvents were all purified by distillation.

ATP was purchased from Sigma Chemical Company. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) was purchased from Mann Research Laboratories.

RESULTS

Preparation of 2-(dansylamino)ethanol

3.5 g of ethanolamine were added to an equal amount of dansyl chloride dissolved in 300 ml of ethanol, and the mixture was allowed to stand for 2 h at room temperature. The solution was evaporated to 50 ml by heating it to 80–85°. After cooling, a small amount of water was added to the solution and a precipitate of the product appeared. By further addition of water to 2 l, the precipitate was again solubilized and a solution giving green fluorescence was obtained. The solution was filtered through a glass filter, and the filtrate was evaporated to 300 ml at 100°. Yellowish green crystals were obtained from the solution after standing it overnight in the cold room. The precipitate was collected and dried in a desiccator. Yield was 94 %. The molar absorption coefficient of the product at 320 nm was 5410 $mole^{-1} \cdot cm^{-1}$ (reference[14] value: 5310 $mole^{-1} \cdot cm^{-1}$). Only one spot, whose R_F was 0.9, was obtained by paper chromatography developed by ammonium acetate–ethanol (2:7, by vol.). Melting point 100° (reference[13]: 102°). An NMR spectrum measured in dimethyl sulfoxide has shown alcoholic OH at the 4.65 ppm triplet, amide NH at the 7.9 ppm singlet, and naphthalene 6 protons at 7.1–8.6 ppm. The OH and NH signals disappeared by the addition of ^2H_2O.

Preparation of 2-(dansylamino)ethyl monophosphate (DMP)

1 mmole of 2-(dansylamino)ethanol was dissolved in 22 ml of acetonitrile at —5°. 10 mmoles of tetrachloropyrophosphate were added slowly to the solution with stirring. The synthesis of DMP was followed by paper electrophoresis. After reaction for 2 h 2-(dansylamino)ethanol disappeared completely and one large spot moving to the anode was obtained, with small spots containing more anionic compounds. The reaction was stopped by adding 10 vol. of water. Derivatives of 2-(dansylamino)-ethanol in the solution were adsorbed on to 2–3 g of charcoal by shaking vigorously. The charcoal was washed 3 times with 100 ml of a mixture of water–ethanol–benzene (4:8:1, by vol.), with vigorous stirring. 72 % of the material could be desorbed. Benzene and ethanol were removed by evaporation under reduced pressure at 30°. The 2-(dansylamino)ethanol and its derivatives were fractionated by DEAE-cellulose column chromatography under a concentration gradient of triethyl-ammonium bicarbonate buffer (pH 7.0). DMP was obtained as the first main peak at 0.14–0.20 M triethylammonium bicarbonate. The concentration of triethyl-ammonium bicarbonate at the peak was rather variable due to evolution of CO_2 gas during the experiment. The yield from 2-(dansylamino)ethanol was 65 %. Fractions of the first peak were collected and the solvent (triethylammonium bicarbonate) was removed by evaporation under reduced pressure at 30°. The residue was dissolved in a small volume of water. The dissolution–evaporation cycle was repeated until the pH of the aqueous solution became neutral. The final product showed only one spot on paper electrophoresis and also on paper chromatography (R_F, 0.65) developed by ammonium acetate–ethanol (2:7, by vol.). The molar ratio of dansyl group to P_i in the DMP preparation was 1.0.

Preparation of DTP

Synthesis of DTP was performed according to the well-known condensation method of nucleotide monophosphate and P_i using *N,N'*-dicyclohexylcarbodiimide

as a condensing agent[15]. DMP and P_i in final concentrations of 0.1 mM and 1 mM, respectively, were dissolved in 2 ml pyridine containing 2.1 mM tri-*n*-butylamine. Tri-*n*-butylamine was added to dissolve the P_i in the pyridine. The DMP was converted from a triethylammonium salt to a pyridine salt by repeating the dissolution in pyridine–evaporation cycle. Phosphorylation of DMP was started by the addition of 50 mmoles of *N,N'*-dicyclohexylcarbodiimide with stirring. After continuous stirring in the dark at room temperature for 2 days, the light-yellow solution became white and turbid, the turbidity being due to the formation of cyclohexylurea. The precipitate was removed by filtration through a glass filter and was washed with an appropriate amount of water (20–30 ml). An equal volume of ethyl ether was added to the combined filtrate and the water layer was obtained after shaking 2 or 3 times. Any organic solvent in the water layer was removed by evaporation and the aqueous solution was fractionated by DEAE-cellulose column chromatography. As shown in Fig. 1, DTP was separated from DMP and 2-(dansylamino)ethyl diphosphate (DDP). Properties of the components of the first three peaks have not yet been examined. It is presumed that the components are 2-(dansylamino)ethanol and the pyro-type of DMP.

The yield of DTP from DMP was 78 %. When the amount of *N,N'*-dicyclohexylcarbodiimide used was less then 50 mmoles, the yield of DTP was less. Only one spot was detected with the DTP preparation by paper electrophoresis and by paper chromatography. The molar ratio of the dansyl group to P_i was 1:3.3. DTP was used as the triethylammonium salt. Aqueous DTP solution was rather stable, *i.e.* after 3 months in the deep freezer at −20°, more than 97 % of the DTP remained unhydrolyzed.

Absorption and fluorescence spectra

Fig. 2 shows the absorption spectrum of 2-(dansylamino)ethanol. Absorption maxima were obtained at 215, 245, and 328 nm in 20 mM Tris–HCl buffer (pH 8.0).

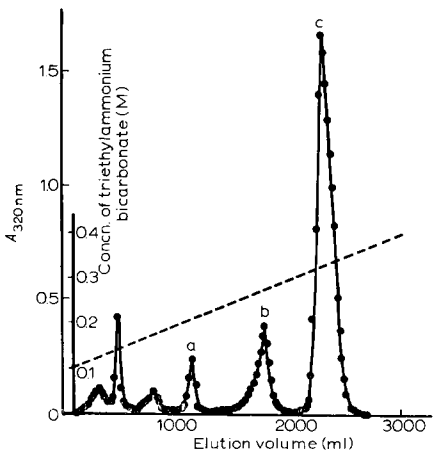

Fig. 1. DEAE-cellulose column chromatography of 2-(dansylamino)ethyl phosphates. Aqueous solution of a mixture of 2-(dansylamino)ethyl phosphates was applied to the column (COOH form, 2 cm × 47 cm). Elution was performed under a linear concentration gradient of triethylammonium bicarbonate from 0.1 to 0.4 M (– – – –). DMP(a), DDP(b), and DTP(c) were eluted at 0.20, 0.27, and 0.32 M triethylammonium bicarbonate, respectively. Fractions of 20 ml were collected. It took 36 h to complete this chromatographic fractionation. Three peaks eluted in front of DMP were not examined; see text.

The absorption spectra of phosphorylated derivatives of 2-(dansylamino)ethanol were indistinguishable from that of 2-(dansylamino)ethanol in the range 230 to 400 nm. Since the molar absorption coefficients of the phosphorylated derivatives at 328 nm were apparently identical with that of 2-(dansylamino)ethanol, absorbance at 328 nm of these compounds was adjusted to 0.03 and the fluorescence intensities, which were excited at 328 nm and measured at 540 nm, were compared. The fluorescence intensities of DMP, DDP and DTP were only 4, 2 and 1 % higher, respectively, than that of 2-(dansylamino)ethanol.

Increase in the concentration of ethanol up to 70 % at neutral pH increased 9-fold the intensity of the fluorescence of 2-(dansylamino)ethanol, which was measured in the range 440 to 620 nm (Fig. 3). At the same time, the peak of the fluorescence emission spectrum shifted from 540 to 523 nm. The fluorescence intensity of 2-(dansylamino) ethanol, wich was excited at 328 nm and measured at 485 nm, was increased by the addition of bovine serum albumin in 40 mM Tris buffer (pH 7.8) at 25°. As shown in Fig. 4, the intensity at 485 nm increased 82-fold by the addition of the protein in a final concentration of 5 mg/ml. The fluorescence emission spectrum maximum was at 485 nm in the presence of 5 mg/ml of the protein, but it shifted to a longer wavelength with a decrease in protein concentration being at 540 nm in the absence of the protein.

A small decrease in the fluorescence intensity was observed by adding KCl or $MgCl_2$ to 2-(dansylamino)ethanol and its phosphorylated derivatives. When $MgCl_2$ was added in a final concentration of 0.2, 0.5 or 1.0 M, the extent of quenching of phosphorylated derivatives was 9, 20 or 28 %, respectively, but when the same

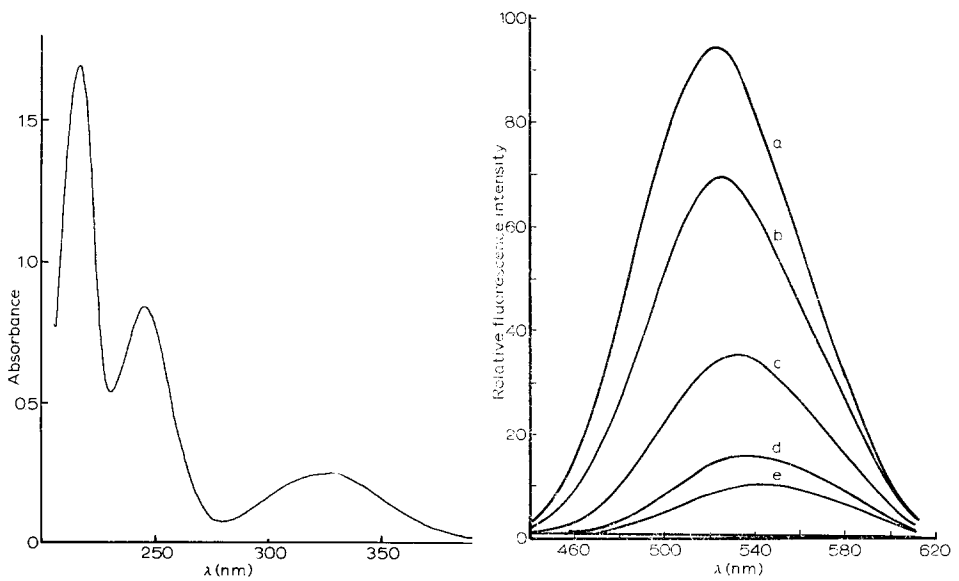

Fig. 2. Absorption spectrum of 2-(dansylamino)ethanol. Concentration of 2-(dansylamino)ethanol was $5.2 \cdot 10^{-5}$ M. Solvent: 20 mM Tris–HCl buffer (pH 8.0).

Fig. 3. Effect of ethanol on the fluorescence emission spectrum of 2-(dansylamino)ethanol. Excitation at 328 nm. Concentration of ethanol in percentage (v/v): a, 70; b, 50; c, 30; d, 10; e, none. Concentration of 2-(dansylamino)ethanol was $6.4 \cdot 10^{-6}$ M.

concentrations were added to 2-(dansylamino)ethanol itself, quenching was 4, 13 or 22 %, respectively. No difference in the effect of KCl was observed between 2-(dansylamino)ethanol and the phosphorylated derivatives, and the quenching with 2 M KCl was less than 15 %. In the presence of 0.5 M KCl and 100 mM $MgCl_2$, the quenching of DTP was 5–10 %.

Fig. 5 shows the effect of pH on the fluorescence intensity of DTP, which was excited at 328 nm and measured at 540 nm. The quenching of dansyl group fluorescence by decreasing the pH to the acid region has already been reported by FÖRSTER[16]. It was explained by the protonation of the dimethylamino group. Quenching of the fluorescence intensity of DTP was clearly observed below pH 6, while no fluorescence was observed below pH 3. The absorption at 328 nm was also decreased by decreasing the pH.

Hydrolysis of DTP by heavy meromyosin

Hydrolysis of DTP by heavy meromyosin was measured in a reaction mixture containing 0.1 mg/ml heavy meromyosin, 0.3 mM DTP, 4.85 mM $CaCl_2$, 0.5 M KCl and 50 mM Tris–maleate buffer (pH 7.0), at 25°. As shown in Fig. 6, P_i was liberated from DTP at the same rate as from ATP. P_i liberation from DDP under the same conditions was negligible. P_i liberation from DTP was hardly observed when heavy meromyosin which was inactivated by preincubation in 0.5 M KCl (pH 7.0) at 80°

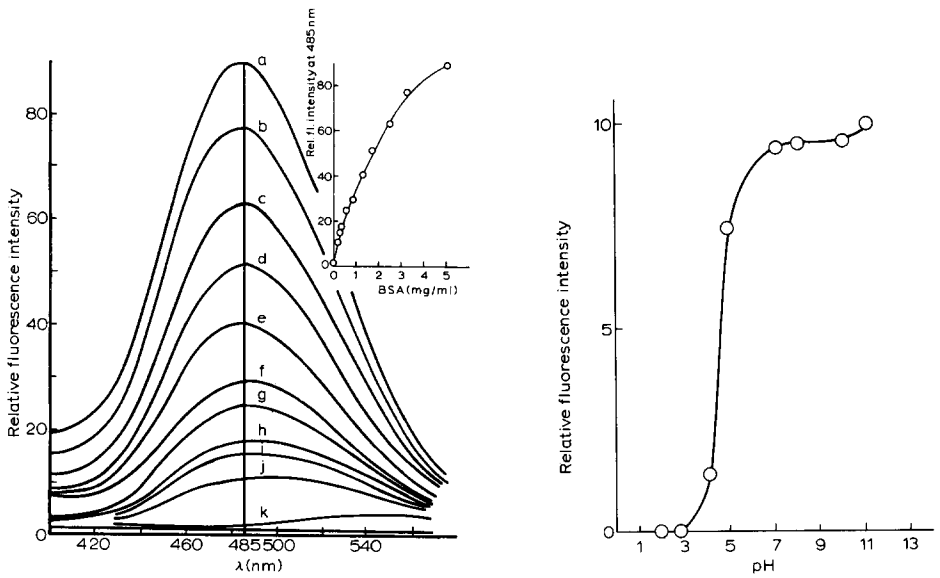

Fig. 4. Effect of bovine serum albumin on fluorescence emission spectrum of 2-(dansylamino)-ethanol. Concentration of 2-(dansylamino)ethanol was $1.7 \cdot 10^{-6}$ M. Concentration of bovine serum albumin in mg/ml: a, 5.0; b, 3.3; c, 2.5; d, 1.7; e, 1.25; f, 0.84; g, 0.63; h, 0.42; i, 0.31; j, 0.21; k, none. Inset shows the relation between the fluorescence intensity at 485 nm and the concentration of bovine serum albumin (BSA).

Fig. 5. Effect of pH on the fluorescence intensity of DTP. The pH's above 10 were adjusted by KOH and pH 2 was adjusted by HCl. 20 mM citrate buffer was used for the pH in the range from 2.8 to 6.0, and 20 mM Tris–HCl buffer was used from pH 7.1 to 8.1. The absorbance of DTP ($6.4 \cdot 10^{-6}$ M) at 328 nm was 0.03 in the solution at pH 7.6.

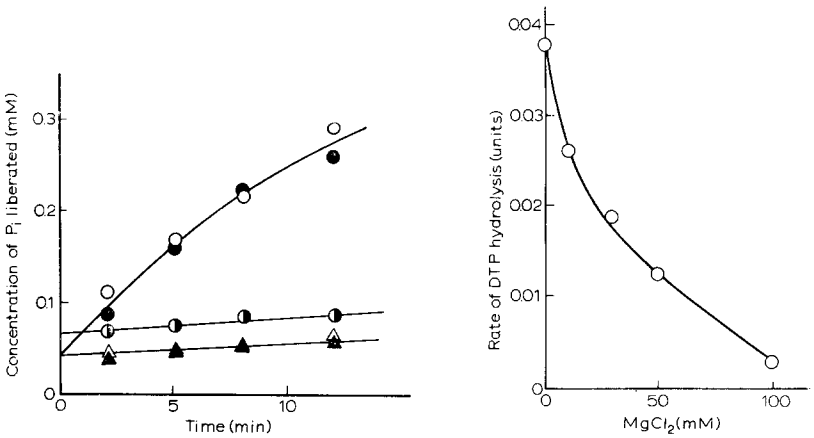

Fig. 6. DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.1 mg/ml heavy meromyosin, 0.3 mM DTP (or ATP, DDP), 0.5 M KCl, 4.85 mM $CaCl_2$, and 50 mM Tris–maleate buffer (pH 7.0). Temperature, 25°. ●, ▲; DTP. ○, △; ATP. ◑; DDP. ▲, △; heat-denatured heavy meromyosin was used.

Fig. 7. Effect of Mg^{2+} inhibition on DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.1 mg/ml heavy meromyosin, 0.3 mM DTP, 0.5 M KCl, 20 mM Tris–HCl buffer (pH 7.6) and various concentrations of $MgCl_2$. Temperature, 9.8°.

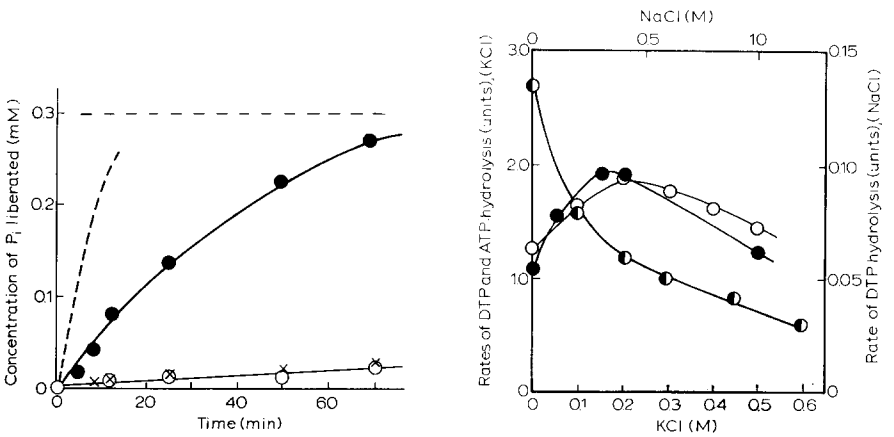

Fig. 8. Inhibitory effect of ATP on DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.02 mg/ml heavy meromyosin, 0.3 mM DTP and/or ATP, 0.5 M KCl, 10 mM $MgCl_2$, and 50 mM Tris–maleate buffer (pH 7.0). Temperature, 25°. Substrate: ●, DTP; ○, ATP; ×, 0.3 mM DTP and 0.3 mM ATP. Broken line, time course of P_i liberation from ATP in the presence of Ca^{2+} in place of Mg^{2+}. The vertical broken line shows the level of DTP concentration added.

Fig. 9. Effect of NaCl or KCl concentration on DTP hydrolysis by heavy meromyosin. Reaction mixture used for the experiment with NaCl consisted of 0.05 mg/ml heavy meromyosin, 0.58 mM DTP, 10 mM $MgCl_2$, 20 mM Tris–HCl buffer (pH 7.6) and various concentrations of NaCl (●). Temperature, 9°. Reaction mixture used for the experiment with KCl consisted of 0.025 mg/ml heavy meromyosin, 0.93 mM DTP, 4.85 mM $CaCl_2$, 20 mM Tris–HCl buffer (pH 7.6) and various concentrations of KCl (○). Temperature, 25°. In order to save the DTP its concentration was kept as low as possible. Reaction mixture used for ATP hydrolysis in the presence of KCl consisted of 0.025 mg/ml heavy meromyosin, 2 mM ATP, 5 mM $CaCl_2$, 50 mM Tris–maleate buffer (pH 7.0) and various concentrations of KCl (◑). Temperature, 25°.

for 60 min was used in place of native heavy meromyosin. As shown in Fig. 7, DTP hydrolysis was inhibited by the addition of $MgCl_2$ in 0.5 M KCl and 20 mM Tris–HCl buffer (pH 7.6) at 9.8°. 70 % of the original activity was preserved after the addition of $MgCl_2$ in a final concentration of 10 mM, but only 7 % or less was observed in the presence of 100 mM $MgCl_2$. The rate of ATP hydrolysis by heavy meromyosin in 0.35 M KCl at pH 8.2 decreased to less than 30 % of the original activity with the addition of $MgCl_2$ in a final concentration of 10 mM (ref. 17). As shown in Fig. 8, ATP hydrolysis was inhibited nearly completely in the presence of 10 mM $MgCl_2$, while under the same conditions DTP hydrolysis was clearly shown. When both DTP and ATP were added at an equal concentration of 0.3 mM, the rate of P_i liberation was the same as that obtained in the presence of ATP as the only substrate. The result strongly suggests that ATP and DTP compete for the same active site and that the affinity of ATP for the site is stronger than that of DTP. This conclusion was confirmed by the measurement of K_m's for ATP and DTP as described below.

Heavy meromyosin-ATPase activity in the presence of Ca^{2+} is simply decreased by increasing the concentration of KCl. When DTP was used as the substrate in place of ATP, the level of P_i liberation was nearly one-half that of ATP hydrolysis in the absence of KCl. As shown in Fig. 9, this level increased with increase in the concentration of KCl and, after the maximum activity was attained at 0.2 M KCl, it gradually decreased. Graphs of the decrease in the rate of ATP hydrolysis and of the increase in DTP hydrolysis intersected at 0.1 M KCl. The rate of DTP hydrolysis was also examined in the presence of 10 mM $MgCl_2$ at various concentrations of NaCl (Fig. 9). An effect similar to that of KCl was obtained and the maximum activity was attained at 0.3–0.4 M NaCl.

DTP was not hydrolyzed by heavy meromyosin in the presence of 10 mM EDTA and 0.5 M KCl at pH 7, 25°, while ATP hydrolysis was activated under the same conditions.

The Michaelis constant (K_m) and the maximum velocity (v_{max}) of DTP hydrolysis by heavy meromyosin were measured in the presence of Mg^{2+} or Ca^{2+} at pH 7.0 and 25°. In the presence of 10 mM $MgCl_2$ and 0.5 M KCl, the K_m was $1.9 \cdot 10^{-5}$ M and v_{max} was 0.24 unit. In the presence of 4.85 mM $CaCl_2$ and 0.5 M KCl, the K_m was found to be $1.8 \cdot 10^{-4}$ M and v_{max} was 1.56 units. In the case of ATP hydrolysis by heavy meromyosin, K_m and v_{max} with 2 mM Mg^{2+} were less than 10^{-6} M and 0.02 unit, respectively[17,18], and those with 5 mM Ca^{2+} were of the order of magnitude of 10^{-5} M and 0.5–0.6 unit, respectively (T. NAKATA, unpublished observation).

Fig. 10 shows the pH–activity relationship of DTP hydrolysis by heavy meromyosin with that of ATP hydrolysis as a reference. The pH dependency of DTP hydrolysis was similar to that of ATP hydrolysis, *i.e.* a maximum at around pH 6–7 and a minimum at around pH 8–9 were observed with DTP.

Dependence of the v_{max} of DTP hydrolysis on temperature was measured in the presence of 4.85 mM $CaCl_2$ and 0.5 M KCl at pH 7.6. As shown in Fig. 11, the temperature dependence gave a biphasic plot curving sharply near 14°. The activation energies were obtained as 12 and 34 kcal/mole above and below 14°, respectively.

Fluorescence of the DTP–heavy meromyosin system

Fluorescence excitation and emission spectra were measured before and after the addition of heavy meromyosin in a final concentration of 0.39 mg/ml to the

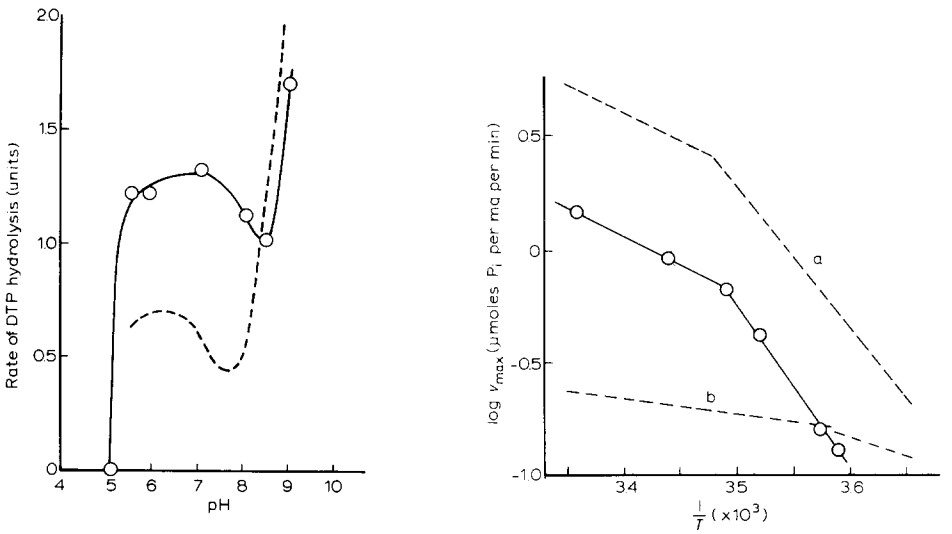

Fig. 10. pH–activity relationship of DTP hydrolysis by heavy meromyosin. Reaction mixture consisted of 0.1 mg/ml heavy meromyosin, 0.3 mM DTP, 0.5 M KCl, 4.85 mM $CaCl_2$, and 20 mM Tris–maleate buffer (pH 5.1–7.3) or 20 mM Tris–HCl buffer (pH 7.9–9.1). Temperature, 25°. The broken line shows the pH–activity curve of ATP hydrolysis as a reference under the same conditions.

Fig. 11. Temperature dependency of the v_{max} of DTP hydrolysis by heavy meromyosin. The v_{max} was measured using a reaction mixture of 0.05 mg/ml heavy meromyosin, 0.5 M KCl, 4.85 mM $CaCl_2$, 20 mM Tris–HCl buffer (pH 7.6) and various concentrations of DTP, at temperatures from 6 to 25°. Data of ITP hydrolysis (a) and ATP hydrolysis (b) were taken from the results of AZUMA AND TONOMURA[22] obtained in 0.6 M KCl, 7 mM $CaCl_2$, and 25 mM Tris–HCl buffer (pH 7.05).

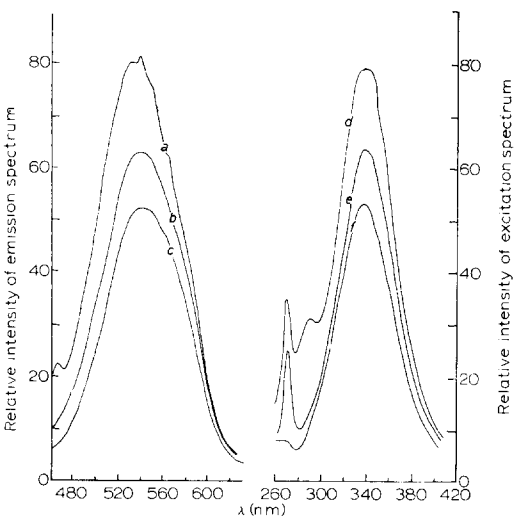

Fig. 12. Fluorescence excitation and emission spectra of DTP obtained after addition of heavy meromyosin. a, b, c, emission spectra, excitation at 340 nm. d, e, f, excitation spectra, emission at 537 nm. Reaction mixture consisted of 0.39 mg/ml heavy meromyosin, 7.95 μM DTP, 83.4 mM $MgCl_2$, and 33.3 mM Tris–HCl buffer (pH 7.6). Temperature, 10°. Time elapsed until the measurement of the maximum of each spectrum after addition of heavy meromyosin: a, 20 sec; b, 90 sec; c, 160 sec; d, 33 sec; e, 89 sec; f, 159 sec.

reaction mixture containing 2-(dansylamino)ethanol, DDP or DTP in the presence of 83.4 mM $MgCl_2$ and 33.3 mM Tris–HCl buffer (pH 7.6), at 10°. The molar ratio of the added 2-(dansylamino)ethanol, DDP or DTP, to heavy meromyosin was nearly 7. The fluorescence excitation spectrum was obtained by the measurement of fluorescence at 537 nm, and the fluorescence emission spectrum was obtained by excitation at 340 nm.

The excitation and emission intensities increased a little with the addition of heavy meromyosin to 2-(dansylamino)ethanol (7.1 μM) or DDP (6.8 μM). Fig. 12 shows the fluorescence excitation and emission spectra obtained by the addition of heavy meromyosin to DTP (8.0 μM). In contrast to the above experiments using 2-(dansylamino)ethanol or DDP, an enhancement of about 60 % in these intensities was observed immediately after the addition of heavy meromyosin to DTP, followed by a rapid decrease to the level obtained in the presence of DDP. The peak of the emission spectrum shifted from 540 to about 530 nm along with the increase in intensity.

The fluorescence excitation spectrum measured 33 sec after the addition of heavy meromyosin to DTP, clearly showed a peak at 290 nm which was not observed at 89 sec. When DDP (6.8 μM) or 2-(dansylamino)ethanol (7.1 μM) was present in place of DTP, the peak at 290 nm was not observed. The intensity at 290 nm was measured continuously after addition of heavy meromyosin in a final concentration of 0.39 mg/ml to various concentrations of DTP in the presence of 83.4 mM $MgCl_2$ and 0.5 M KCl at 16°. The first measurement was made 3–5 sec after the addition of heavy meromyosin. When the concentration of DTP was more than 19.8 μM, a plateau of intensity at 290 nm was observed which gradually decreased to a certain low level. The half-life of the decrease in intensity was obtained from a series of experiments as 26, 48, 62, or 102 sec with 8.0, 19.8, 31.8, or 39.8 μM DTP, respectively.

DISCUSSION

The molecular structure of DTP is shown in Fig. 13, together with that of ATP. The sizes of the compounds appear to be very close to one another and the similarity implies that DTP could be a substrate analog of ATP.

Like ATP, DTP was actually hydrolyzed easily by heavy meromyosin, and the competition between ATP and DTP in the heavy meromyosin-ATPase reaction indicates that DTP binds to the ATP binding site (Fig. 8). DTP was not hydrolyzed by heavy meromyosin in the presence of 10 mM EDTA and 0.5 M KCl, while ATP hydrolysis was activated by EDTA. The nucleotide polyphosphates tested so far show a marked difference in their rates of hydrolysis depending on the structure of the purine or pyrimidine ring, those possessing an amino group in the 6-position being highly preferable for hydrolysis in the presence of EDTA[19]. The marked inhibition of DTP hydrolysis by EDTA might therefore be due to the lack of any amino group in the naphthalene ring in DTP.

The dansyl group is known as a fluorescent reagent, but it is also useful as an indicator of a hydrophobic environment. The addition of an organic solvent (ethanol) or bovine serum albumin to 2-(dansylamino)ethanol increased the fluorescence intensity and induced the blue shift of the fluorescence emission spectrum. When heavy meromyosin was added to 2-(dansylamino)ethanol, DDP or DTP, increase

in intensity and the blue shift in the fluorescence spectrum of the dansyl group were also observed. The increment of fluorescence intensity obtained with DTP was much higher than that obtained with other compounds, and the fluorescence intensity once increased with DTP decreased to the level of a mixture of DDP and heavy meromyosin after the hydrolysis of DTP to DDP. Since it was indicated that DTP binds to the ATP binding site, DTP is expected to be a hydrophobic probe of the active site of heavy meromyosin.

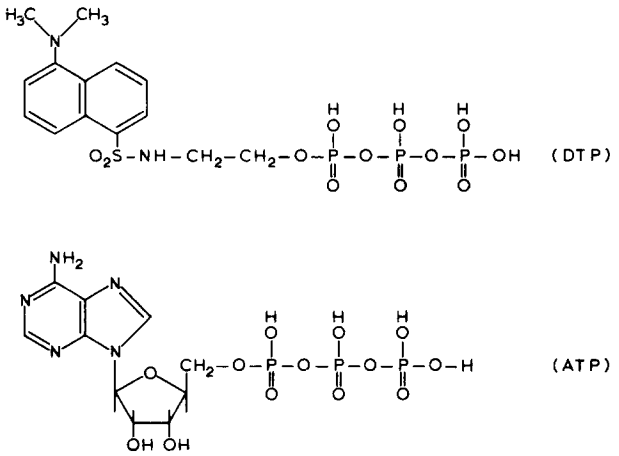

Fig. 13. Molecular structures of DTP and ATP.

CHEUNG AND MORALES[20] have shown that the maximum binding of 8-aniline-1-naphthalene sulfonate to myosin was obtained as 2 (mole/mole) and that the binding sites were located towards the head region of the myosin molecule. The results suggest that the hydrophobic region of the myosin molecule is very restricted and that the noncovalent binding of dansyl compounds to heavy meromyosin would also be limited.

The maxima of absorption and fluorescence emission spectra of DTP were at 328 and 534 nm, respectively. The absorption of DTP overlaps with the fluorescence of tyrosine and tryptophan. Energy transfer from the protein chromophore (tyrosine and/or tryptophan) to the dansyl group of DTP can therefore be assumed to occur. When heavy meromyosin was added to DTP, a particular excitation spectrum that rapidly disappeared was observed around 290 nm. The result shows that the excitation spectrum around 290 nm may be related directly to the energy transfer from the protein chromophore to DTP and that DDP is no longer able to participate in energy transfer after DTP hydrolysis. The rate constant of the decay of fluorescence intensity at the 290 nm peak was 0.06 unit in the presence of 100 mM $MgCl_2$ and 0.5 M KCl at 16° using CHANCE's[21] formula. This value was similar to the rate constant of DTP hydrolysis by heavy meromyosin under the same conditions.

According to MORITA[17], a red shift of the absorption spectrum around 290 nm is observed accompanied with the formation of Michaelis complex of heavy meromyosin. This shift is decreased by the hydrolysis of ATP to ADP. She explained the phenomenon of red shift by the displacement of tryptophan and tyrosine to the hydrophobic region. Binding of DTP to the active site might cause a displacement

of the tryptophan, which moves by the binding of ATP, and the tryptophan probably participates in the energy transfer to the dansyl group of bound DTP.

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