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$1,N^6$-ETHENO-2-AZA-ADENOSINE TRIPHOSPHATE: ENORMOUS INCREASE IN FLUORESCENCE INTENSITY INDUCED BY ITS BINDING TO HEAVY MEROMYOSIN AND ESTIMATION OF THE KINETIC PARAMETERS

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SUMMARY: The fluorescence spectrum of an ATP analogue, $1,N^6$-etheno-2-aza-adenosine triphosphate, changed greatly upon its binding to heavy meromyosin. The wavelength of the fluorescence maximum excited at 350 nm shifted from 485 nm to 453 nm and the fluorescence intensity at the maximum increased 2.8 times. The time course of the change in the fluorescence intensity measured at 440 nm with excitation at 350 nm was analyzed on the basis of a reaction scheme of Michaelis-Menten type. The analysis gave reasonable kinetic parameters for the hydrolysis of $1,N^6$-etheno-2-aza-adenosine triphosphate by heavy meromyosin.

INTRODUCTION

Various kinetic parameters, such as K_m and K_i values, of the enzymatic hydrolysis of ATP and its analogues have been measured by a number of methods. The most common method involves colorimetric assay of the concentration of inorganic phosphate (P_i) liberated at various time intervals during the enzymatic reaction and at various total concentrations of substrate. However, this method is very laborious and is not accurate if the K_m value is small. The change in ultraviolet absorption of heavy meromyosin (HMM) induced by the binding of ATP has been utilized for the estimation of the K_m value (1). The slight decrease (~15%) in the fluorescence intensity of $1,N^6$-etheno-adenosine triphosphate (e-ATP) caused by its binding to HMM was applied to the kinetic measurement of the formation and degradation of the enzyme-substrate complex (2). However, the above two methods are not sufficiently accurate for quantitative measurement of the K_m and V_{max} values.

Recently, Asai and Shimada reported that the absorbance of a fluorescent $1,N^6$-etheno-2-aza-adenosine triphosphate (e-2-aza-ATP) changes greatly when it is bound to actin (3). In this paper, we show that the fluorescence spectrum of e-2-aza-ATP changes greatly when it is bound to HMM. Furthermore, it is shown that various kinetic parameters of e-2-aza-ATP hydrolysis, including the product inhibition constant, can be easily estimated by analysis of the time course of the change in the fluorescence intensity.

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MATERIALS AND METHODS

Materials HMM was prepared from rabbit skeletal muscle myosin by tryptic digestion (4). An ATP regenerating system (pyruvate kinase, PK and phosphoenolpyruvate, PEP) was obtained from Sigma Chemical Co. $MgCl_2$, $CaCl_2$, KCl, and Tris(hydroxymethyl)aminomethane (Tris) were obtained from Wako Junyaku. Ethylenediaminetetraacetic acid (EDTA) was obtained from Dojindo Laboratories. e-2-Aza-ATP was prepared from e-ATP in our laboratory by the method of Tsou et al. (5). The concentration of e-2-aza-ATP was determined using $\varepsilon_{354} = 1530$. The concentrations of the proteins were determined by the method of Lowry et al. (6) The molecular weight of HMM was assumed to be 3.4×10^5 (7).

Methods Fluorescence measurements were done with a Shimadzu double-beam spectrofluorophotometer (model RF-503). For the measurement of the excitation and emission spectra of e-2-aza-ATP in the presence of HMM, an ATP regenerating system was used to regenerate hydrolyzed e-2-aza-ATP. The efficiency of the regeneration of e-2-aza-ATP by the ATP regenerating system was sufficient to maintain an effectively constant concentration of e-2-aza-ATP (see Figure 1B).

The measurement of the change in fluorescence intensity of e-2-aza-ATP in the absence of the ATP regenerating system was done as follows. The reaction was started by adding small aliquots of HMM solution (less than 3% of the total volume) with an adder mixer to the buffer solution containing e-2-aza-ATP. The mixing was completed within 10 seconds. Therefore, even at the lowest concentration (10 μM) of e-2-aza-ATP used, the change in the fluorescence intensity corresponding to the steady-state concentration of HMM-e-2-aza-ATP complex could be measured. The above measurement of fluorescence intensity in the absence of the e-2-aza-ATP regenerating system and the measurement of the difference spectrum (shown in Figure 1B) were done using a reference cell in the double-beam spectrofluorophotometer. The reference cell contained e-2-aza-ATP and buffer solution.

The determination of the amount of P_i was done by the method of Ohnishi et al. (8).

RESULTS

Emission and excitation spectra of e-2-aza-ATP Figure 1A shows emission and excitation spectra of e-2-aza-ATP in the absence and presence of HMM. These spectra were taken under the following conditions: 10 μM e-2-aza-ATP, 5 mM $MgCl_2$, 0.5 M KCl, 10 mM Tris-HCl pH 7.8 (25°C). The concentrations of HMM, PK, and PEP were 10 μM, 1.8 μM, and 1 mM, respectively. In the absence of HMM, the excitation spectrum measured at 480 nm has two maxima at 290 nm and 350 nm. The emission spectra excited at 290 nm and 350 nm have the common maximum at 480 nm. In the presence of HMM and the e-2-aza-ATP regenerating system, the emission spectrum excited at 350 nm has the maximum at 450 nm and the fluorescence intensity at the maximum was 2.8 times greater than that in the absence of HMM. The fluorescence intensity decreased to the same level as that of e-2-aza-ATP in the absence of HMM when all the PEP was consumed (see Figure 1B). This means that the formation of HMM-e-2-aza-ADP complex does not result in any change in the fluorescence intensity as compared with that of e-2-aza-ATP alone.

Figure 1C shows the difference emission spectrum of e-2-aza-ATP between in the presence and in the absence of HMM. The wavelength giving the maximum value of the difference spectrum was 440 nm. We therefore carried out all of the difference fluorescence measurements at 440 nm.

When 5 mM $CaCl_2$ or 5 mM EDTA was used instead of 5 mM $MgCl_2$, no change in the fluorescence intensity was observed, whereas HMM e-2-aza-ATPase activity was observed in the presence of $CaCl_2$ and EDTA.

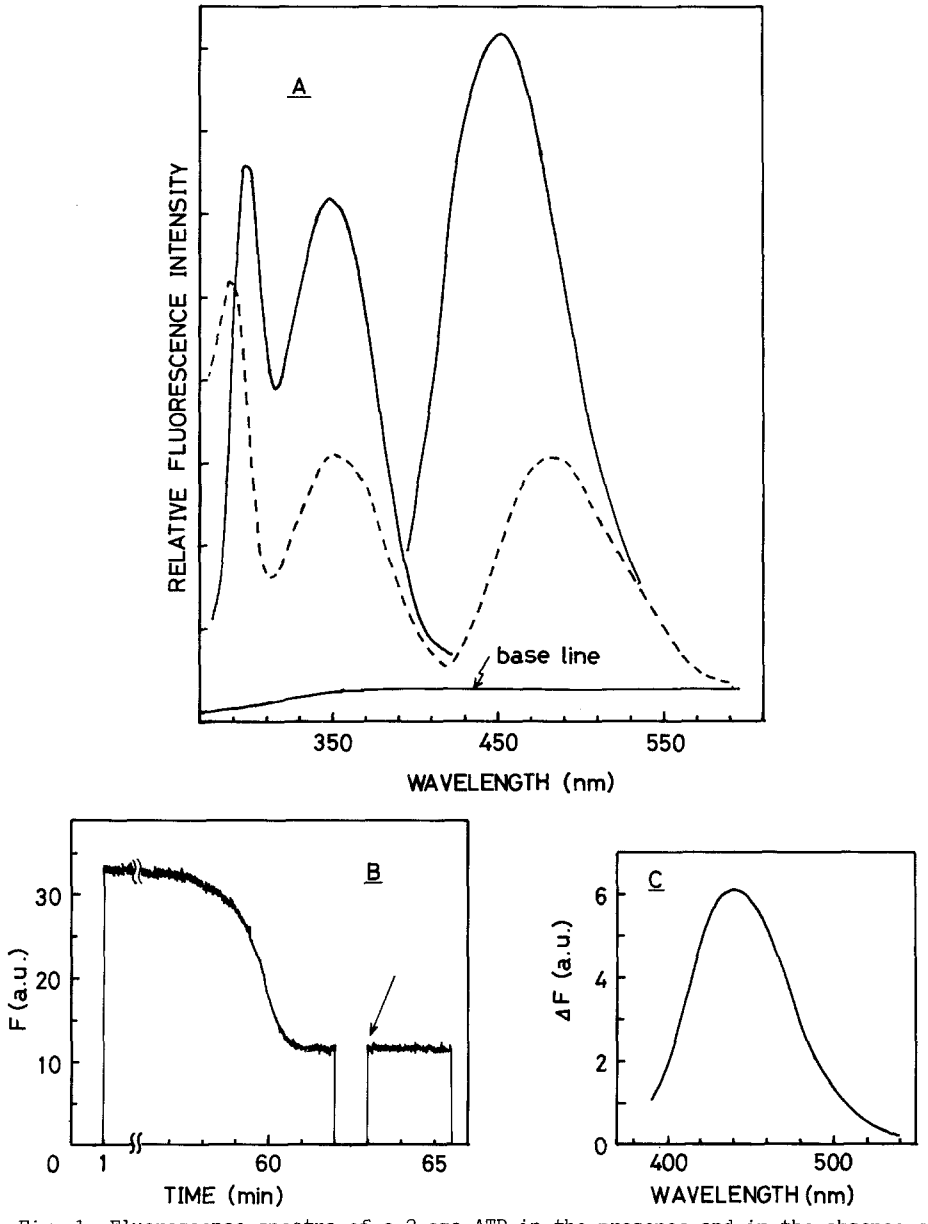

Fig. 1 Fluorescence spectra of e-2-aza-ATP in the presence and in the absence of HMM. Buffer conditions are described in the text. A: Excitation and emission spectra of e-2-aza-ATP in the presence (solid line) and in the absence (dashed line) of HMM. The excitation spectra were taken at 480 nm and the emission spectra were taken with excitaion at 350 nm. Both in the presence and in the absence of HMM, the concentration of e-2-aza-ATP was 10 μM. In the presnce of HMM, 1.8 μM PK and 1 mM PEP were used as an e-2-aza-ATP regenerating system and the concentration of HMM was 10 μM. B: Fluorescence intensity of e-2-aza-ATP in the presence of HMM returning to the same level as that in the absence of HMM (indicated by an arrow) after consumption of all the PEP. C: The difference emission spectrum of e-2-aza-ATP between in the presence and in the absence of HMM. The excitation wavelength was 350 nm.

The correspondence of P_i liberation to the time course of the change in the fluorescence intensity Figure 2A shows the time course of the change in the fluorescence intensity of e-2-aza-ATP after the addtion of HMM. Figure 2B shows the time course of P_i liberation measured under the same conditions as used for

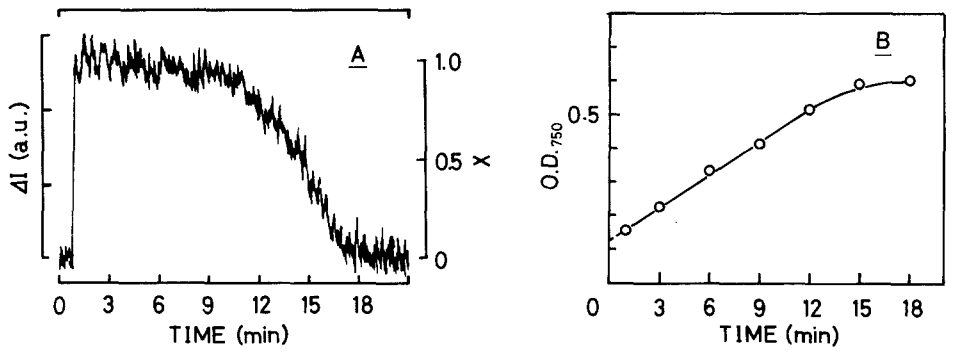

Fig. 2 A: A time course curve of the change in the fluorescence intensity of e-2-aza-ATP in the presence of HMM. Buffer conditions were the same as in Fig. 1. The concentrations of HMM and e-2-aza-ATP were 0.5 μM and 160 μM, respectively. No e-2-aza-ATP regenerating system was used. The right hand scale shows $\Delta I/\Delta I_{max}$, written as "x". B: A time course curve of hydrolysis of e-2-aza-ATP by HMM in terms of the amount of P_i release measured colorimetrically at 750 nm. The conditions were the same as in A.

the measurement in Figure 2A. These two figures clearly indicate that while the HMM e-2-aza-ATPase reaction continued, the fluorescence intensity was higher than the intensity before the addition of HMM, and that when the reaction was completed, the fluorescence intensity returned to the original level. Therefore, the change in the fluorescence intensity ΔI, is proportional to the amount of HMM e-2-aza-ATP complex; $\Delta I/\Delta I_{max} = [ES]/[E_o]$. Here, ΔI_{max} is the maximum change in the fluorescence intensity obtained when an excess amount of e-2-aza-ATP with respect to HMM is added and all of the HMM is bound to e-2-aza-ATP. Using this relation, the amount of ES complex can be directly estimated from the change in the fluorescence intensity.

Analysis of the time course curve As shown in the above section, $\Delta I/\Delta I_{max}$ is a direct indicator of the amount of HMM-e-2-aza-ATP complex. Therefore, the kinetic parameters, V_{max} and K_m, can be estimated from the time course curve shown in Figure 2A. The V_{max} value is estimated from the width of the time course curve, and the K_m value is estimated from the sharpness of the time course curve (decay curve). That is, the smaller the K_m value, the more rapidly the ES complex decays. For details and the principle of the estimation of V_{max} and K_m values, see the **"APPENDIX"**.

Table I shows K_m values and V_{max} values thus obtained at various concentrations of e-2-aza-ATP. The V_{max} obtained from the measurement of P_i liberation is also shown in Table I. The K_m values are dependent on the total concentration of e-2-aza-ATP, suggesting the occurrence of product inhibititon. As shown in the **"APPENDIX"**, the inhibition constant, K_i, and the true K_m value can be obtained from these apparent K_m values. The K_i value and the true K_m value thus obtained are also shown in Table I. The V_{max} values estimated at various total concentra-

TABLE I: K_m and V_{max} values estimated by analysis of the decay curve

$[S_o]$ μM	V_{max}	$\bar{V}_{max}$	V'_{max}	$K_{m\ app}$ μM	true K_m μM	K_i μM
10	20.2		--	0.71		
20	19.8		--	0.94		
30	21.8	21.4	--	1.24	0.57	26
50	22.6		--	1.97		
80	22.6		24.3	2.15		

$[S_o]$ is the total concentration of e-2-aza-ATP. V_{max}, $\bar{V}_{max}$ and V'_{max} are given in the units of [μM P_i/min μM HMM]. $\bar{V}_{max}$ is the mean value of V_{max}. V'_{max} was obtained from measurement of the amount of liberated P_i. $K_{m\ app}$ is an apparent Michaelis constant directly estimated by analysis of the decay curve.

tions of e-2-aza-ATP are almost constant and coincide well with the V_{max} obtained from the measurement of P_i liberation.

DISCUSSION

The fluorescence spectrum of e-2-aza-ATP changed greatly upon binding to HMM. This change in the fluorescence spectrum is thought to be caused by burying of the fluorescent group of e-2-aza-ATP in a hydrophobic HMM ATPase site (9,10). This was confirmed by our finding that the fluorescence spectrum of e-2-aza-ATP in 96% ethanol was different from the spectrum in water. That is, the wavelength of the fluorescence maximum excited at 350 nm showed a 12 nm blue shift in ethanol and the maximum value of the fluorescence intensity in ethanol was twice that in water.

By analysis of the time course curve, the V_{max}, true K_m, and K_i values were easily obtained. The V_{max} value agreed well with the value obtained from the measurement of P_i liberation. The true K_m value of 0.57 μM obtained from the apparent K_m values is reasonable, because reported K_m values of ATP to HMM range from 0.4 μM to 0.45 μM (1,11). Moreover, the ratio K_m/K_i, which is equal to 0.57/26, is not greatly different from the ratio conventionally obtained under conditions similar to ours using ATP and ADP as substrate and reaction product, respectively.

The analysis to obtain the K_m value was done by assuming that the two ATPase sites of HMM are identical and that the increase in the fluorescence intensity occurred at either of the two sites. That is, the total concentration of enzyme is equal to twice the total concentration of HMM. This assumption was used to analyze the results of an experiment in which the fluorescence intensity of ES complex was measured at various total concentrations of e-2-aza-ATP in the presence of the e-2-aza-ATP regenerating system. The analysis gave a K_m value which coincides well with the K_m value kinetically obtained. Therefore, the above assumption of two identical sites is a reasonable one.

Elson and Magde (12) have proposed that the kinetic constant of a chemical reaction can be determined by measuring the time correlation function in fluctuations of fluorescence intensity of a fluorophore in solution irradiated by a narrow laser beam, if the chemical reaction of the fluorophore involves a change in fluorescence intensity. This method is known as fluorescence correlation spectroscopy. As described in this paper, the binding of e-2-aza-ATP to HMM causes a significant increase of the fluorescence intensity. Thus, this fluorescent analogue of ATP can be used as a fluorescent probe of fluorescence correlation spectroscopy to determine the kinetic constant of the binding reaction. The ultraviolet light (365 nm) of an Argon laser is suitable for exciation of e-2-aza-ATP.

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APPENDIX

V_{max} for the HMM e-2-aza-ATPase reaction was determined as follows. As described above, the velocity of liberation of product is a function of the concentration of ES complex:

$$d[P]/dt = k_2[ES], \qquad \text{A-1}$$

where k_2 is the rate constant of product formation. This equation can also be represented in the following integrated form:

$$[P] = k_2[E_o]\int_o^t \Delta I/\Delta I_{max}\, dt = V_{max}\int_o^t \Delta I\, dt/\Delta I_{max}. \qquad \text{A-2}$$

Here, we use the relation $[ES]/[E_o] = \Delta I/\Delta I_{max}$ and use V_{max} for $k_2[E_o]$. If the time t is large enough, the final concentration of product becomes equal to the initial concentration of substrate; $[P_{final}] = [S_o]$. Thus, the following expression is obtained:

$$[S_o] = V_{max}\int_o^\infty \Delta I\, dt/\Delta I_{max},$$

where $\int_o^\infty \Delta I\, dt$ is the total area under the time course curve. This relation gives the V_{max} value at any given concentration of substrate.

The procedure for the estimation of K_m and K_i value is as follows. The equation (A-1) can be rewritten as follows:

$$d[P]/dt = V_{max} \cdot x, \qquad \text{A-3}$$

where we put $\Delta I/\Delta I_{max}$ as x. On the other hand, [P] is equal to $[S_o]-[S]-[ES]$, where [S] is the free substrate concentration. According to the conventional Michaelis-Menten kinetics, [S] is equal to $K_m[ES]/([E_o]-[ES])$. Using the last two equations, the equation (A-3) can be rewritten in the form of a differential equation of x against time:

$$-\frac{d}{dt}(K_m x/(1-x)) + [E_o]\frac{dx}{dt} = V_{max} \cdot x.$$

This equation is easily integrated as follows:

$$V_{max}\cdot t = -(1+ K_m/[E_o])\ln x/x_o + K_m/[E_o]\ln(1-x)/(1-x_o) - K_m/[E_o](1/(1-x) - 1/(1-x_o)). \quad \text{A-4}$$

Here, x_o is the value of x at time zero. This equation combines the sharpness of the decay curve at around x = 1/2 with the K_m value.

In the case of the presence of product inhibition, the K_m value in (A-4) is an apparent one and can be represented as follows:

$$K_{m\ app} = K_m(1+[S_o]/K_i).$$

Here, we use $[S_o]$ for $[P]_{x=1/2}$ because at the time when x is equal to 1/2, the concentration of product is almost equal to the initial concentration of substrate.

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