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SPECTROSCOPIC ISOLATION OF ES COMPLEXES OF MYOSIN SUBFRAGMENT-1 ATPase BY FLUORESCENCE QUENCHING

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SUMMARY. It was found that the fluorescence of $1,N^6$-ethenoadenosine triphosphate (ε-ATP) bound to myosin subfragment-1 (S-1) is resistant to quenching by acrylamide, while free ε-ATP is effectively quenched. Thus in the presence of acrylamide the bound ε-ATP is still highly fluorescent, while free ε-ATP is much less fluorescent. The Stern-Volmer constants of bound and free ε-ATP are 6.83 and 57.86 M^{-1}, respectively. Therefore it is easy to distinguish spectroscopically the nucleotide-ligated S-1 from nucleotide-free S-1. Moreover acrylamide does not alter the S-1-Mg^{2+}-ε-ATPase behavior.

INTRODUCTION. While in some ATPases a perturbable tryptophanyl residue is conveniently situated near the nucleotide binding site and reports environmental changes resulting from nucleotide binding (1,2), such an intrinsic probe is unlikely to exist in all cases. Moreover, it would be better if the nucleotide itself reported on the environment. Potentially, fluorescent $1,N^6$-ethenoadenosine triphosphate (ε-ATP) is a candidate for such a use because in many reactions it substitutes satisfactorily for ATP (3,4). But usually the difference in fluorescence intensity between ε-ATP in bulk solution and enzyme-bound ε-ATP is too small. It occurred to us that ε-ATP binding could be made to give a very large signal (and thus be an excellent probe for the analysis of ATPases) by the simple expedient of quenching the fluorescence of the bulk ε-ATP with a quencher that minimally affected its enzymology. Acrylamide proved to be a such a quencher.

MATERIALS AND METHODS. Myosin was prepared from rabbit skeletal muscle (5). Myosin subfragment-1 (S-1) containing only light chains 1 or 3, i.e., S-1(LC_1;LC_3) was obtained by digesting myosin with α-chymotrypsin (Sigma

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Chemical) (6). S-1(LC_3) was a gift from Dr. R. Takashi.

S-1-Mg^{2+}-ε-ATPase activities were measured by measuring the absorption change at 340 nm of NADH (7) (initial concentration, 0.2 mM) when the ε-ATPase was coupled to 2 mM phosphoenolpyruvate (PEP), 6 μg/ml pyruvate kinase (PK), and 5 μg/ml lactate dehydrogenase (LDH). ε-ATP was from Molecular Probes. NADH, PEP, and crystaline PK in 2.2 M $(NH_4)_2SO_4$, and LDH in 2.1 M $(NH_4)_2SO_4$ were from Sigma Chemical. Crystalline acrylamide was from Bio-Rad Laboratories.

To estimate the accessibility of bound ε-ATP to a quencher in solution the fluorescence intensity of ε-ATP at 410 nm, when excited at 330 nm, was measured in the presence of acrylamide, with and without S-1. The ε-ATP concentration was varied from 0 to 77 μM by successively adding 5 μl of concentrated ε-ATP to 1.5 ml of a solution containing 20 μg/ml acetate kinase (AK), 5-10 mM acetyl-phosphate (AP), 0-100 mM acrylamide, 0.5 mM DTT, 0 or 20 μM S-1 and solvent. The solvent was 0.15 M KCl, 2 mM $MgSO_4$ and 20 mM TES (pH 7.5). AK-AP (instead of PK-PEP) was used as an ε-ATP-regeneration system, because pyruvate is a fluorescence quencher (8). Since the internal filtering effect became significant above 20 μM ε-ATP, the observed fluorescence intensity with S-1 was corrected by assuming that the filtering effect is the same for ε-ATP molecules bound and not bound to S-1. The fluorescence measurements were made on a Hitachi-Perkin-Elmer Fluorometer MPF-4.

It was necessary to check the activity of AK in phosphorylating ε-ADP to ε-ATP. Conventional assays were inapplicable because of either low sensitivity or interference, so we used an assay previously devised by one of us (9), based on the differential quenching of ε-ADP and ε-ATP by Tl^+. The reaction was started by adding 5 μl of 10 μg/ml AK (final concentration, 50 ng/ml) to a solution containing 100 μM ε-ADP, 2 mM AP, 0.15 M KCl, 2 mM $MgSO_4$ and 20 mM TES (pH 7.5) at 23°C. An aliquot (0.1 ml) was transfered to 3 ml of solution containing 3 mM $TlCOOCH_3$, 1 mM EDTA and 2 mM Tris (pH 9.0) at 0°C, and then the fluorescence of the ε-ATP/ε-ADP solutions at 410 nm, when excited at 310 nm, was measured. The difference of fluorescence intensity between ε-ATP and ε-ADP was about 18 % under this condition. The AK activity was about 200 μmoles/min·mg AK. ε-ADP was from Molecular Probes. AK and AP were purchased from Sigma Chemical. $TlCOOCH_3$ was from Eastman Kodak.

ANALYSIS AND RESULTS. To test the toxicity of acrylamide for S-1, the effect of acrylamide on the kinetic parameters (V_m and K_m) of the S-1(LC_1;LC_3)-Mg^{2+}-ε-ATPase reaction was measured, while varying the acrylamide concentration from 0 to 0.1 M. In these measurements DTT was required, because acrylamide can slowly react with protein thiols (10). Under our conditions neither V_m nor K_m are affected by acrylamide concentrations less than 0.1 M (Fig.1). Above 0.2 M acrylamide K_m is gradually increased with increasing acrylamide concentration (data not shown). This effect was not changed upon purification of acrylamide by recrystallization and G-10 column chromatography. Therefore the increase of K_m seems to be due to acrylamide itself.

S-1(LC_3)-Mg^{2+}-ε-ATPase yielded K_m and V_m values indistinguishable from those of S-1(LC_1;LC_3). Thereafter S-1(LC_1;LC_3) was not further resolved.

If the kinetics of S-1 ε-ATPase are Michaelian then in a regenerator-maintained steady state, with both total enzyme and total substrate concent-

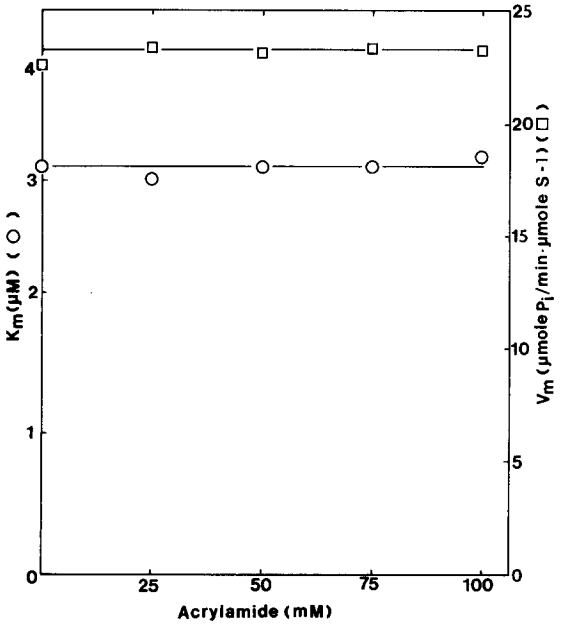
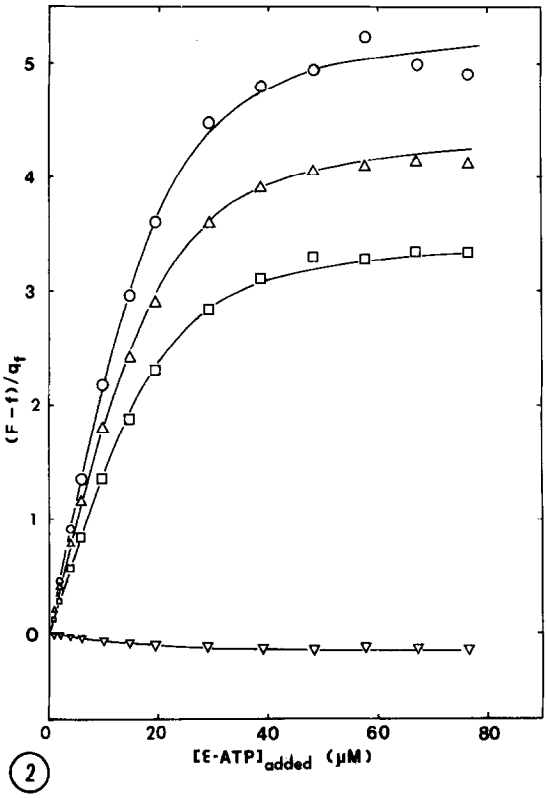


Fig.1: Effect of acrylamide on the V_m (□) and K_m (○) of the S-1-Mg^{2+}-ε-ATPase reaction. The ε-ATPase was assayed by the absorbance change at 340 nm of NADH (0.2 mM initial) when the ε-ATPase is coupled with 2 mM (initial) PEP, 6 μg/ml PK and 5 μg/ml LDH in 0.3 μM S-1, using various concentrations of ε-ATP, 0.15 M KCl, 2 mM $MgSO_4$, 20 mM TES (pH 7.5) and 0.5 mM DTT at 23°C.

rations (M_o and ε_o, respectively) conserved, the concentration of enzyme-substrate complex (X) is the lesser root of $X^2-(M_o+\varepsilon_o+K_m)X+M_o\varepsilon_o=0$, and that of unbound substrate is $\varepsilon_o - X$. If $\bar{f}$ and q are the molar fluorescence and quenching factor for free (subscript f) and enzyme-bound (subscript b) substrate fluorophore, then the fluorescence intensity from such a system is proportional to $F = q_b\bar{f}_bX + q_f\bar{f}_f(\varepsilon_o - X)$. From a similar system with enzyme omitted it is proportional to $f = q_f\bar{f}_f\varepsilon_o$. Therefore, F-f is proportional to $(q_b\bar{f}_b - q_f\bar{f}_f)X$. Since M_o and ε_o are known, and K_m is independently obtainable from conventional enzyme kinetics measurements, the curve $X(\varepsilon_o)$ can be constructed from the aforementioned quadratic equation. For $\varepsilon_o \longrightarrow \infty$, X approaches M_o asymptotically. X reaches its half-saturation value, $M_o/2$, when $\varepsilon_o = (M_o/2) + K_m$. Therefore, at this special value, $\varepsilon_o = \varepsilon_o^*$, F - f is proportional to $(q_b\bar{f}_b - q_f\bar{f}_f)M_o/2$.

Quenching is conventionally described by the constant K in the Stern-Volmer equation, $(1/q) = 1 + K[Q]$, where Q is the quenching substance.



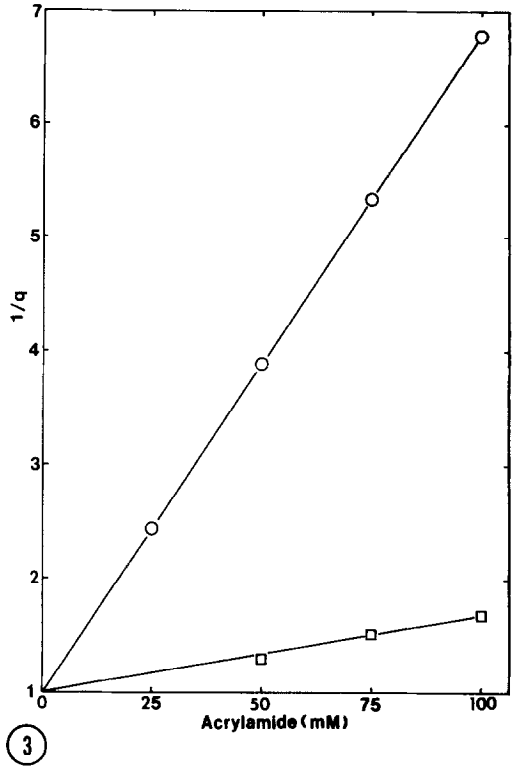


Fig.2: Titration of the nucleotide-binding site of S-1 (20 μM) by ε-ATP in various concentration of acrylamide (▽: 0 mM, □: 50 mM, △: 75 mM, ○: 100 mM), 0.15 M KCl, 2 mM $MgSO_4$, 20 mM TES (pH 7.5) and 0.5 mM DTT. To maintain the ε-ATP concentration, a regenerator consisting of 20 μg/ml AK and 5-10 mM AP was present. The fluorescence was measured at 410 nm when excited at 330 nm. The ordinate scale depends on each acrylamide concentration (see text).

Fig.3: The Stern-Volmer plots for acrylamide quenching of free (○) and bound (□) ε-ATP in 0.15 M KCl, 2 mM $MgSO_4$, 20 mM TES (pH 7.5) at 23°C. The data of bound ε-ATP were derived from Fig.2. 1/q equals to the ratio of the fluorescence intensity in the absence of acrylamide to that in the presence of acrylamide.

Clearly, in the system without enzyme or quencher, when ε_o assumes its half-saturation value the observed fluorescence is proportional to $\bar{f}_f\varepsilon_o^*$, or equal to it if the (otherwise arbitrary) units of fluorescence are so chosen. For any added quencher concentration, [Q], f will be $q_f([Q])\bar{f}_f\varepsilon_o^*$; if the q_f s so obtained are inverted and plotted against [Q], the slope of the resulting line will be K_f. For $\varepsilon_o = \varepsilon_o^*$, $F - f = q_f\bar{f}_f\{a([Q]) - 1\}M_o/2$, where $a([Q]) = q_b\bar{f}_b/q_f\bar{f}_f$. An experimental value of F - f in the absence of quencher thus permits the evaluation of $a(0) = \bar{f}_b/\bar{f}_f$; since $\bar{f}_f$ is known, $\bar{f}_b$ can be found. Finally, $a(0)/a([Q]) = q_f/q_b$. Since the function, $1/q_f = 1 + K_f[Q]$, has been

established, multiplying it by $a(0)/a([Q])$ gives the remaining unknown, $1/q_b = 1 + K_b[Q]$, and therefore K_b.

Fig.2. shows F - f measurements plotted against ε_o for various [Q]s; the lines are calculated by the foregoing analysis, making use of a K_m value inferred from independent ε-ATPase measurements. Fig.3 shows Stern-Volmer plots, $1/q([Q])$ vs. [Q], for free and enzyme-bound fluorophore, calculated according to the same analysis. K_f was 57.9 M^{-1}, and K_b was 6.8 M^{-1}.

DISCUSSION. It was found here that ε-ATP bound to S-1 is protected from collisions with acrylamide. In the presence of 0.1 M acrylamide the increase in the fluorescence intensity of ε-ATP on binding to S-1 is 3.72-fold, i.e., it is increased by nearly 400 %. Thus when the transition from free to bound is used as a signal this signal change is enormous compared to the corresponding changes in absorption or fluorescence of perturbable tryptophan residues of enzymes, or changes in the fluorescence of ATP analogs as conventionally used; until now these are 30 %, at most. The large signal change obtained with the present system makes it easier and more accurate to measure reaction rate constants. Importantly, acrylamide (up to 0.2 M) is not toxic to the ATPase. The large signal change also allows detection of concentration fluctuations in the steady state. From fluctuation measurements we can estimate reaction rate constants without perturbing the reaction system (11, 12, 13). For the study of ATPases kinetics in vivo wherein large perturbations are hard to apply, fluctuation analysis of the chemical signal is a powerful alternative. The chemical signal provided by the present fluorescence quenching method may make such fluctuation analysis feasible.

In closing we would like to emphasize that in any ATPase the susceptibility to quenching of ε-ATP bound to enzyme is probably less than that of free ε-ATP. If so, the quenching method proposed here would be applicable to ATPases other than the contractile ATPase.

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REFFERENCES

1. Morita, F. and Yagi, K. (1966) Biochem. Biophys. Res. Comn. 22, 297-302.
2. Werber, M.M., Szent-Györgyi, A.G., and Fasman, G.D. (1972) Biochemistry 11, 2872-2883.
3. Secrist III, J.A., Barrio, J.R., and Leonard, N.J. (1972) Science 75, 646-647
4. Onishi, H., Ohtsuka, E., Ikehara, M., and Tonomura, Y. (1973) J. Biochem. 74, 435-450.
5. Tonomura, Y., Appel, P., and Morales, M.F. (1966) Biochemistry 5, 515-521.
6. Weeds, A.G., and Taylor, R.S. (1975) Nature 257, 54-56.
7. Imamura, K., Tada, M., and Tonomura, Y. (1966) J. Biochem. 59, 280-289.
8. Ando, T. (submitted to Anal. Biochem.)
9. Ando, T., and Asai, H. (1980) J. Biochem. 88, 255-264.
10. Cavins, J.F., and Friedman, M. (1968) J. Biol. Chem. 243, 3357-3360.
11. Magde, D. (1976) Quart. Rev. Biophys. 9, 35-47.
12. Magde, D., Elson, E.L., and Webb, W.W. (1974) Biopolymer 13, 29-61.
13. Morales, M.F. (1982) Proc. Nat. Acad. Sci. USA 79, 1126-1128.