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2'(3')-O-[N- [2- [3- [5-fluoresceinyl] thioureido] ethyl] carbamoyl] adenosine 5'-triphosphate and its $Cr(H_2O)_4$ and $Co(NH_3)_4$ Complex Derivatives are New Fluorescent Tools for Labelling ATP Binding Sites of Na^+/K^+-ATPase

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Summary

2'(3')-O-[N- [2- [3- [5-fluoresceinyl] thioureido] ethyl] carbamoyl] adenosine 5'-triphosphate (FEDA-ATP), a spectroscopic tool used for studying skeletal muscle myosin ATPase subfragment 1, was applied to Na^+/K^+-ATPase (EC 3.6.1.37). In contrast to the myosin subfragment, we found that FEDA-ATP is not a substrate for Na^+/K^+-ATPase. On the other hand, FEDA-ATP showed an affinity for both the low (E_2, $K_d = 200\,\mu M$) and the high (E_1, $K_d = 22\,\mu M$) affinity ATP-binding sites. When the microscopic affinities of FEDA-ATP were used for calculating the macroscopic affinity in the overall reaction according to $K_i = (K_{dE1} * K_{dE2})^{1/2}$, the experimentally measured inhibition constant of $66\,\mu M$ was obtained. To evoke irreversible binding inhibitors, FEDA-ATP was transferred in its chromium(III) and cobalt(III) complex analogs, which are suitable tools for labelling the ATP binding sites of Na^+/K^+-ATPase in a specific way.

Key words

Na^+/K^+-ATPase – Complex derivatives of ATP – Low-affinity ATP-binding site – High-affinity ATP binding site

Abbreviations

$Cr(H_2O)_4$ATP, β, γ bidentate complex of chromium (III)-tetraaqua-adenosine-5'-triphosphate
$Cr(H_2O)_4$FEDA-ATP, β, γ bidentate complex of chromium (III)-tetraaqua- 2'(3')-O-[N- [2- [3- [5- fluoresceinyl] thioureido] ethyl] carbamoyl] adenosine-5'-triphosphate
$Co(NH_3)_4$ATP, β, γ bidentate complex of cobalt (III)-tetramino-adenosine-5'-triphosphate
$Co(NH_3)_4$FEDA-ATP, β, γ bidentate complex of cobalt (III)-tetramino- 2'(3')-O-[N- [2- [3- [5- fluoresceinyl] thioureido] ethyl] carbamoyl]- adenosine-5'-triphosphate
$Co(NH_3)_4PO_4$, tetramine cobalt(III)phosphate
FITC, fluorescein 5'-isothiocyanate
FEDA-ATP, 2'(3')-O-[N- [2- [3- [5- fluoresceinyl] thioureido] ethyl] carbamoyl] adenosine 5'-triphosphate
EDA-ATP, 2'(3')-O-[amino ethyl carbamoyl] adenosine 5'-triphosphate
E_1ATP site, nucleotide-binding site of Na^+/K^+-ATPase with high affinity for ATP
E_2ATP site, nucleotide-binding site of Na^+/K^+-ATPase with low affinity for ATP

Introduction

Na^+/K^+-ATPase (EC 3.6.1.37) is the biochemical equivalent of the sodium pump of mammalian plasma membranes (Skou and Esmann 1992). The enzyme probably works as a functional $(\alpha\beta)_2$-diprotomer (Schoner *et al.* 1994). Kinetic studies on ATP hydrolysis revealed that phosphorylation of the catalytic α-subunit at Asp-369 occurs in the presence of Na^+ from a high affinity ATP site ($K_d = 1\,\mu M$, E_1ATP site). This process is followed by K^+-activated hydrolysis of the phospho-intermediate. Na^+-dependent phosphorylation of the α-subunit leads to the occlusion of three Na^+ ions. These ions are released into the external medium during the reaction cycle. Occlusion of two K^+ ions, however, takes place in the nonphosphorylated α subunit. To release K^+ at the inner membrane face, ATP has to bind to a low-affinity ATP site (E_2ATP site), which hydrolyzes p-nitrophenylphosphate and supports the binding of ouabain in the presence of P_i (Linnertz *et al.* 1994). Studies with chromium(III) and cobalt(III) complex analogs of ATP revealed that the E_1ATP and E_2ATP sites coexist and that these MgATP complex analogs can be used to differentiate between them. $Cr(H_2O)_4$ATP is an almost irreversible specific inhibitor of the E_1ATP and $Co(NH_3)_4$ATP of the E_2ATP-binding site (Schoner *et al.* 1994, Linnertz *et al.* 1995, Linnertz and Schoner 1996).

2'(3')-O-[N- [2- [3- [5-fluoresceinyl] thioureido] ethyl] carbamoyl] adenosine 5'-triphosphate (FEDA-ATP) known from studies of skeletal muscle myosin subfragment 1 (S1) (Conibar *et al.* 1996) makes it possible to determine the activity of single myosin filaments. FEDA-ATP as substrate is hydrolyzed to P_i and FEDA-ADP, which is released with the same rate constant as ADP. As a result of fluorescence changes, it was possible to follow turnover of this ATP analog by fluorescence microscopy (Conibar *et al.* 1996).

The aim of the present work was to ascertain whether FEDA-ATP is a substrate of Na^+/K^+-ATPase and whether it and its MgATP complex analogs provide information about the E_1ATP and/or E_2ATP sites. We therefore studied the interaction of FEDA-ATP with the high-affinity E_1ATP site, which is inactivated by $Cr(H_2O)_4$ATP, and with the low-affinity E_2ATP site, which is inactivated by $Co(NH_3)_4$ATP. Furthermore, FEDA-ATP was transferred in its chromium(III) and cobalt (III) complex analogs, which are suitable tools for labelling the ATP binding sites of Na^+/K^+-ATPase in a specific way.

Materials and Methods

All chemicals were of highest available purity and were obtained from Bio-Rad (Munich, Germany), Boehringer-Mannheim (Mannheim, Germany), E. Merck (Darmstadt, Germany) and Molecular Probes (Eugene, USA). The Lab-Trol protein standard was a product of Merz & Dade (Munich, Germany). [γ^{32}P]ATP was from Amersham Buchler (Braunschweig, Germany).

Synthesis of FEDA-ATP, MgATP- and MgFEDA-ATP-complex analogs

Ethylenediamine was first coupled to ATP according to Cremo *et al.* (1990) to yield 2'(3')-O-[amino ethyl carbamoyl] adenosine 5'-triphosphate (EDA-ATP). This intermediate product was then made to react with fluorescein 5'-isothiocyanate (isomer 1) according to Sowerby *et al.* (1993) to yield 2'(3')-O-[N- [2- [3- [5- fluoresceinyl] thioureido] ethyl] carbamoyl] adenosine 5'-triphosphate (FEDA-ATP). The concentration of FEDA-ATP was determined assuming an absorbance coefficient at pH 9.0, A_{495} of 75 000 M^{-1} cm^{-1}. $Cr(H_2O)_4$ATP, $Co(NH_3)_4$ATP, $Co(NH_3)_4$FEDA-ATP and $Cr(H_2O)_4$FEDA-ATP complex analogs were prepared by the aniline procedure of Cleland and coworkers (De Pamphilis and Cleland 1973, Cornelius *et al.* 1977).

Isolation of Na^+/K^+-ATPase

Na^+/K^+-ATPase was isolated from pig kidney according to the method of Jørgensen (1974) and assayed as described earlier (Schoner *et al.* 1994, Linnertz *et al.* 1995).

Inhibition of ATP hydrolysis by FEDA-ATP

Various concentrations of FEDA-ATP (0–100 μM) were used in 1 ml of the optical assay mixture (Schoner *et al.* 1967) and ATP concentration range from 25 to 500 μM.

Protective action of FEDA-ATP against the inactivation by MgATP analogs

Affinities of FEDA-ATP for the E_1ATP and E_2ATP binding sites were determined from the protective effect of FEDA-ATP as a function of its concentration (0–500 μM). Na^+/K^+-ATPase (0.5 units, 20 units/mg protein) was incubated in a total volume of 250 μl at 37 °C, 15 mM Tris/HCl buffer (pH 7.5) and various concentrations of $Cr(H_2O)_4$ATP (0–100 μM) or $Co(NH_3)_4$ATP (0–500 μM). Residual Na^+/K^+-ATPase activity was measured at the time indicated by transferring aliquots of 30 μl to the optical assay (Schoner *et al.* 1967).

Inactivation with $Cr(H_2O)_4$FEDA-ATP and $Co(NH_3)_4$FEDA-ATP

One unit of Na^+/K^+-ATPase was incubated in a total volume of 1 ml at 37 °C with 20 mM Tris/HCl buffer at pH 7.25, 15 mM NaCl and different concentrations of the inhibitor. Residual activity was measured by transferring 50 μl aliquots to the optical assay mixture (Schoner *et al.* 1967).

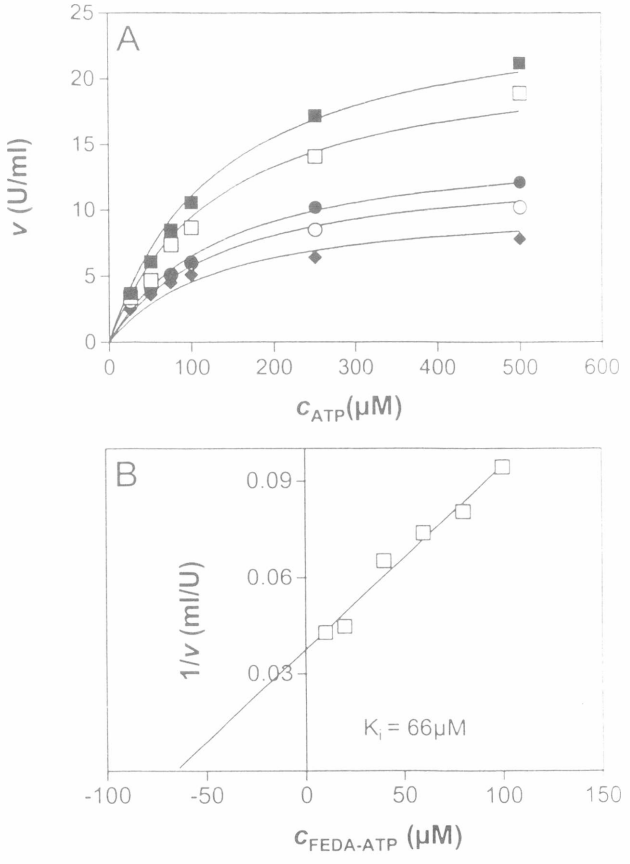

Fig. 1. *Inhibition of ATP hydrolysis by FEDA-ATP. (A) ATP hydrolysis was measured in 1 ml of the optical assay mixture (Schoner et al. 1967) containing 25–500 μM ATP and various concentrations of FEDA-ATP (full square 0 μM, open square 20 μM, full circle 40 μM, open circle 60 μM and full rhombus 100 μM). We detected a reduction of the maximum velocity of ATP-hydrolysis. (B) The inhibition of ATP-hydrolysis by FEDA-ATP results in an inhibition constant of 66 μM and a maximum velocity of 26.7 U/ml.*

Results

Pig kidney Na^+/K^+-ATPase (1 U/ml) was incubated with 0, 20, 40, 60 and 100 μM FEDA-ATP and rates of ATP hydrolysis were measured by the optical assay. Hydrolysis of ATP was inhibited by FEDA-ATP (Fig. 1A), at 100 μM FEDA-ATP, being about 25 % of the control. The inhibition constant K_i was found to be 66 μM (Fig. 1B). However, this macroscopic affinity of FEDA-ATP of 66 μM in the overall reaction is the result of the microscopic affinities of the substance for the E_1ATP and E_2ATP binding sites (Thoenges *et al.* 1997). To establish whether FEDA-ATP binds preferentially to a specific ATP site, its protective effects against inactivation with the specific inhibitor of the E_1-site, namely $Cr(H_2O)_4ATP$, was studied. Na^+/K^+-ATPase (1 U/ml) was incubated in a mixture of 20 mM Tris/HCl (pH 7.25) and 15 mM NaCl at 37 °C with 0 μM, 50 mM and 100 mM $Cr(H_2O)_4ATP$ and the enzyme inhibition curves were measured (Fig. 2).

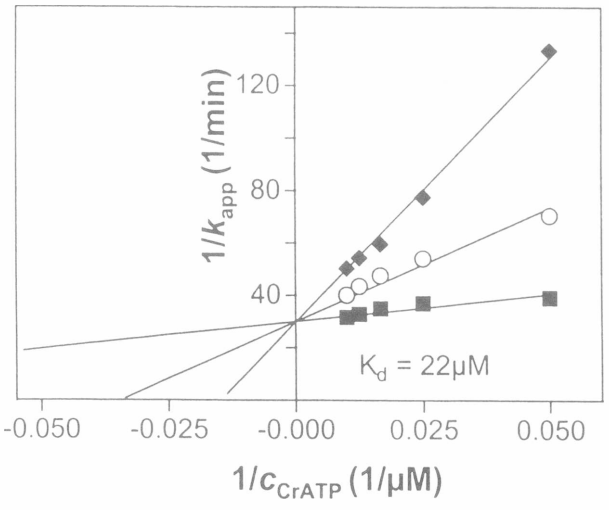

Fig. 2. *Protective effect of different FEDA-ATP concentrations against the inactivation by $Cr(H_2O)_4ATP$. One unit Na^+/K^+-ATPase was incubated in a total volume of 250 μl with different concentrations of $Cr(H_2O)_4ATP$ (0, 20, 40, 60, 80 and 100 μM) and FEDA-ATP (full square 0 μM, open circle 50 μM and full rhombus 100 μM). k_{app} are the apparent velocity constants.*

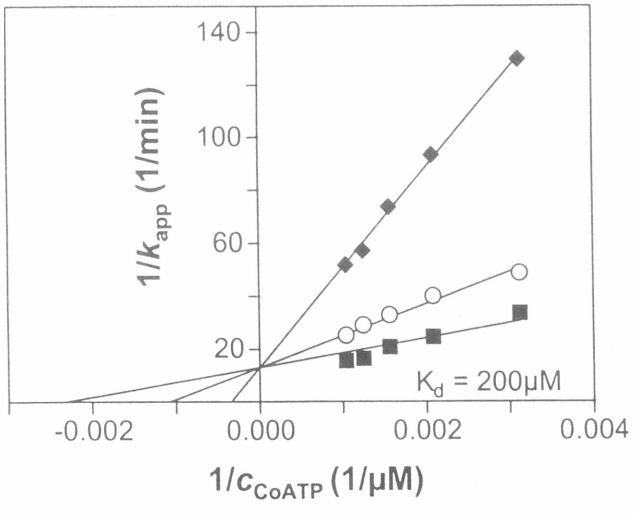

Fig. 3. *Protective effect of different FEDA-ATP concentrations against the inactivation by $Co(NH_3)_4ATP$. One unit Na^+/K^+-ATPase was incubated in a total volume of 250 μl with different concentrations of $Co(NH_3)_4ATP$ (0, 320, 480, 640, 800 and 960 μM) and FEDA-ATP (full square 0 μM, open circle 250 μM and full rhombus 500 μM). k_{app} are the apparent velocity constants.*

Apparently, FEDA-ATP exhibited a competitive and protective effect against the enzyme inactivation by $Cr(H_2O)_4ATP$. The dissociation constant K_d for the high-affinity ATP-binding site (E_1-site) was calculated as 22 μM.

A similar experiment was performed for the low-affinity ATP-binding site (E_2-site). In this case, the Na^+/K^+-ATPase inhibition curves were determined with increasing concentrations of $Co(NH_3)_4ATP$ in the absence and in the presence of 250 μM and 500 μM FEDA-ATP. Similarly to $Cr(H_2O)_4ATP$, a competitive and protective effect of FEDA-ATP against $Co(NH_3)_4ATP$ was observed (Fig. 3). The calculated dissociation constant in this case (low affinity site), however, was by about one order of magnitude higher (K_d = 0.2 mM).

The extinction coefficient of FEDA-ATP in 20 mM Tris/HCl buffer (pH 9) was A_{490} = 78,000 M^{-1} cm^{-1}. The fluorescence measurements in 20 mM Tris/HCl buffer (pH 7.25) result in an λ_{ex} = 495 nm and λ_{em} = 520 nm. We titrated a solution of 1 μM FEDA-ATP with increasing concentrations of Na^+/K^+-ATPase but were not able to detect any significant change of the steady-state fluorescence intensity even with a 10-fold excess of the enzyme (data not shown).

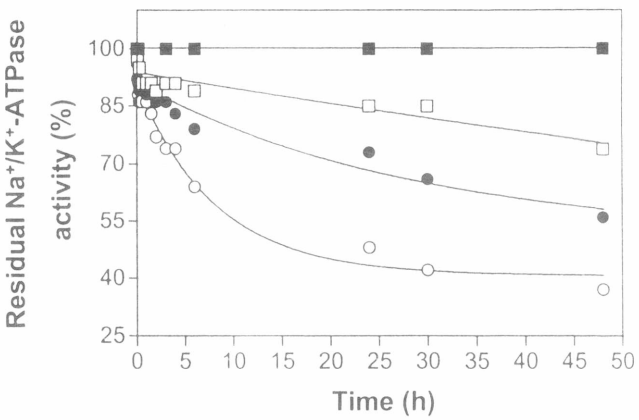

Fig. 4. *Inactivation of Na^+/K^+-ATPase activity by $Cr(H_2O)_4$FEDA-ATP. One unit of Na^+/K^+-ATPase was incubated in a total volume of 1 ml at 37 °C with 20 mM Tris/HCl-buffer pH 7.25, 15 mM NaCl and various concentrations of the inhibitor (full square control, open square 0.3 μM, full circle 1.3 μM and open circle 5.0 μM $Cr(H_2O)_4$FEDA-ATP). Residual activity was measured by transferring aliquots of 50 μl to the optical assay mixture (Schoner et al. 1967).*

To bring about irreversible binding, we prepared analogs of FEDA-ATP with chromium(III) and cobalt (III) and studied their inactivation effect on Na^+/K^+-ATPase. One unit of the enzyme was incubated in 20 mM Tris/HCl (pH 7.25), 15 mM NaCl at 37 °C with increasing concentrations of $Cr(H_2O)_4$FEDA-ATP (Fig. 4) and $Co(NH_3)_4$FEDA-ATP (Fig. 5). Aliquots of 50 μl were taken from the incubation medium for the residual Na^+/K^+-ATPase activity measurement by the optical assay. The Mg^{2+} complex derivatives of FEDA-ATP behaved similarly to those of ATP: 5 μM $Cr(H_2O)_4$FEDA-ATP inactivated by 55 % in 3 h (Fig. 4), while 6 μM $Co(NH_3)_4$FEDA-ATP inactivated by 30 % only (Fig. 5).

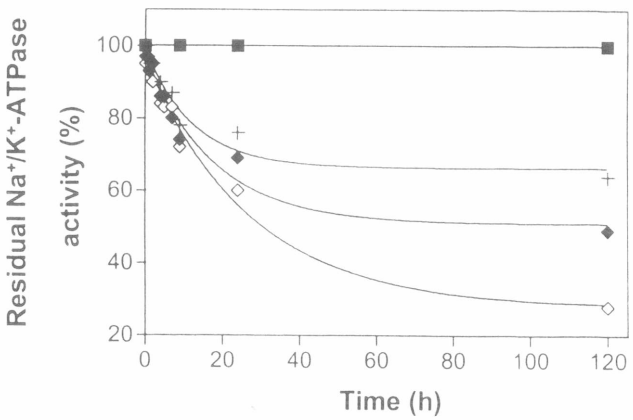

Fig. 5. *Inactivation of Na^+/K^+-ATPase activity by $Co(NH_3)_4$FEDA-ATP. One unit of Na^+/K^+-ATPase was incubated in a total volume of 1 ml at 37 °C with 20 mM Tris/HCl-buffer pH 7.25, 15 mM NaCl and various concentrations of inhibitor (full square control, cross 3.0 μM, full rhombus 6.0 μM and open rhombus 12.5 μM $Co(NH_3)_4$FEDA-ATP). Residual activity was measured by transferring aliquots of 50 μl to the optical assay mixture (Schoner et al. 1967).*

Discussion

FEDA-ATP does not appear to be a substrate of Na^+/K^+-ATPase isolated from the pig kidney but rather a reversible competitive inhibitor of ATP hydrolysis. The presence of FEDA-ATP resulted in enzyme inhibition but the steady-state fluorescence intensity was not changed significantly. This is in contrast with previous reports on myosin fragment S1 (Conibar *et al.* 1996) where FEDA-ATP was reported as a substrate with changed fluorescence properties after binding. This could indicate differences in the microenvironments of the binding sites of the two systems. Nevertheless, additional steady-state and dynamic fluorescence studies will have to be performed.

On the other hand, FEDA-ATP showed the affinity for both the low (E_2, K_d = 200 μM) and the high (E_1, K_d = 2 μM) affinity ATP-binding sites. Apparently, the FEDA-ATP behaves like ATP in these reactions (Schoner *et al.* 1994). When the microscopic affinities of FEDA-ATP were used to calculate the macroscopic

affinity in the overall reaction according to $K_i = (K_{dE1} * K_{dE2})^{1/2}$ given by Thoenges *et al.* (1997), the experimentally measured inhibition constant of 66 μM was obtained.

In addition, the chromium(III) and cobalt(III) analogs of FEDA-ATP behaved similarly as the analogs of ATP. The chromium analog of FEDA-ATP inhibited the high affinity ATP-binding site while the cobalt analog of FEDA-ATP was found to inhibit the low-affinity ATP-binding site. These observations open up the possibility for labelling specifically the E_1ATP site with $Cr(H_2O)_4$FEDA-ATP and to introduce another label on the E_2ATP site as a $Co(NH_3)_4$ATP derivative. Since 2'(3')-O-[amino ethyl carbamoyl] adenosine 5'-triphosphate (EDA-ATP) can react with any other isothiocyanate but FITC (Cremo *et al.* 1990), other fluorescent ATP analogs overlapping in their fluorescent spectra with fluorescein are conceivable as $Co(NH_3)_4$complex derivatives. Such analogs may help to determine the distance between the E_1ATP and E_2ATP sites by Förster energy transfer.

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References

CONIBAR P.B., JEFFREYS D.S., SEEHRA C.K., EATON R.J., BAGSHAW C.R.: Kinetic and spectroscopic characterization of fluorescent ribose-modified ATP analogs upon interaction with skeletal muscle myosin subfragment 1. *Biochemistry* **35:** 2299–2308, 1996.

CORNELIUS R.D., HART P.A., CLELAND W.W.: Phosphorus-31 NMR studies of complexes of adenosine triphosphate, adenosine diphosphate, tripolyphosphate, and pyrophosphate with Cobalt(III)amines. *Inorg. Chem.* **16:** 2799–2805, 1977.

CREMO C.R., NEURON J.M., YOUNT R.G.: Interaction of myosin subfragment 1 with fluorescent ribose-modified nucleotides: a comparison of vanadate trapping and SH1-SH2 cross-linking *Biochemistry* **29:** 3309–3319, 1990.

DE PAMPHILIS M.L., CLELAND W.W.: Preparation and properties of chromium(III)-nucleotide complexes for use in the study of enzyme mechanisms. *Biochemistry* **12:** 3714–3724, 1973.

JØRGENSEN P.L.: Purification and characterisation of Na^+/K^+-ATPase III. *Biochim. Biophys. Acta* **356:** 36–52, 1974.

LINNERTZ H., SCHONER W.: Interaction of ATP sites in Na^+/K^+-ATPase is changed by phosphorylation of the E_1ATP site. *Biol. Chem.* **377:** S67, 1995.

LINNERTZ H., THÖNGES D., SCHONER W.: Na^+/K^+-ATPase with a blocked E_1ATP site still allows backdoor phosphorylation of the E_2ATP site. *Eur. J. Biochem.* **232:** 420–424, 1995.

SCHONER W., VON ILLBERG C., KRAMER R., SEUBERT W.: On the mechanism of Na^+ and K^+ stimulated hydrolysis of ATP. *Eur. J. Biochem.* **1:** 334–343, 1967.

SCHONER W. THÖNGES D., HAMER E., ANTOLOVIC R., BUXBAUM E., WILLEKE M., SERPERSU E.H., SCHEINER-BOBIS G.: Is the sodium pump a functional dimer? In: *The Sodium Pump.* E. BAMBERG, W. SCHONER (eds), Springer, New York, 1994, pp. 332–341.

SKOU J.C., ESMANN M.: The Na,K-ATPase. *J. Bioenerg. Biomembr.* **24:** 249–261, 1992.

SOWERBY A.J., SEEHRA C.K., LEE M., BAGSHAW C.R.: Turnover of fluorescent nucleoside triphosphates by isolated immobilized myosin filaments. *J. Mol. Biol.* **234:** 114–123, 1993.

THOENGES D., LINNERTZ H., SCHONER W.: A two-site model of interacting ATP sites. *Ann. N.Y. Acad. Sci.* (in press), 1997.

Reprint requests

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