The Isolated H4-H5 Cytoplasmic Loop of Na,K-ATPase Overexpressed in it Escherichia coli Retains Its Ability to Bind ATP

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Key words: ATPase, fluorescence, ATP analogues

Abstract. The H4-H5 loop of the alpha-subunit of mouse brain Na,K-ATPase was expressed and isolated from E coli cells. Using fluorescence analogues of ATP this loop was shown to retain its capability to bind ATP. Isolation of a soluble H4-H5 loop with the native ATP binding site is a critical step for detailed studies of the molecular mechanism of ATP binding and utilisation.

Introduction

Na,K-ATPase (EC 3.6.1.3) is a plasma membrane protein consisting of at least two major subunits: the catalytic α-subunit (with molecular mass about 112000 and all presently known transport and catalytic functions) and the associated glycoprotein β-subunit (molecular mass about 35000 excluding oligosaccharides) (Jørgensen 1986). There is now a common agreement that an α₂β₂ heterodimer is associated in the plasma membrane to create the enzyme native structure. The α-subunit has ten (or eight) transmembrane segments with a huge H4-H5 cytoplasmic loop where the high affinity ATP-binding site and phosphorylation site are localised. The H4-H5 loop of the α-subunit has been recently shown to preserve a rigid and self-supporting structure. Fluorescent derivatives of isothiocyanate have been shown to bind to the ATP-binding site at Lys480 and/or Lys501 and the fluorescence of FITC-labelled Na,K-ATPase as well as PITC-labelled enzyme was highly anisotropic. The long lifetime of the excited state of PITC indicated not only a kind of immobilised ATP-binding site on the H4-H5 cytoplasmic loop, but a firm structure of the whole large H4-H5 cytoplasmic loop. Interestingly, the ATP-binding site is rather far protruding from the ion transporting domains and the rigid and self-supporting structure may be connected with the recent identification of two disulfide bonds between Cys452 and Cys456, and between Cys511 and Cys549.

In the present work, we expressed and isolated the H4-H5 loop of the α-subunit of Na,K-ATPase in Escherichia coli and proved its capability to bind ATP. Isolation of such a soluble domain with its retaining capacity to bind ATP was a first step in an original approach to study the structure of the ATP binding site in detail.
Materials and Methods

Construction of pGEX-H₄-H₅

The expression vector pGEX-2T (Pharmacia Biotech) was digested with BamH I (Promega), the restriction enzyme was then heat-inactivated. The linearized vector was treated with T4 DNA polymerase (Amersham) to obtain the blunt ends. Then the vector was dephosphorylated with calf intestinal alkaline phosphatase (Amersham). The H₄-H₅ sequence was prepared from the sequence of α-subunit of mouse brain Na⁺/K⁺-ATPase (a gift from Dr. S. Gloor, ETH Zurich). The pGEM4-α-subunit plasmid was digested with Hpa II (Promega) and the fragment of 1201 bps, with H₄-H₅ sequence (Met⁸⁶ to Ile⁷⁸⁴) was isolated and purified. Then this fragment was digested with S1 nuclease (Promega) to obtain the blunt ends. Ligation was performed at 14 °C overnight with T4 DNA ligase. The ligated DNA was transformed into competent *Escherichia coli* DH5α cells. The *E. coli* transformants were selected on LB agar containing ampicillin (50 μg/ml). The plasmid DNA of clones obtained was checked and positive clones (with construct of 6151 bps) were analysed by restriction endonuclease mapping and finally sequenced.

Purification of GST-H₄-H₅

An overnight culture (3 ml, *E. coli* DH5α cells transformed with pGEX-H₄-H₅) was diluted into 1 litre of fresh LB medium containing ampicillin (50 μg/ml) and incubated to an A₀₀ of 0.8 (5 hours) at 37 °C. Then the IPTG was added (0.1 mM) and culture was incubated overnight at 30 °C. The cells were collected by centrifugation and resuspended in TENG buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 1% NP-40, 1 mM DTT). The suspension was sonicated for 5 min with on/off periods of 20 s each and then the suspension was centrifuged at 20000 g for 45 min. The supernatant was loaded on a glutathione-Sepharose column equilibrated with TENG buffer and rinsed with this buffer. The fusion protein was eluted by switching the buffer to 50 mM Tris-HCl, pH 7.5, containing 10 mM glutathione. HPLC gel filtration of the fusion protein was performed on a Shodex SB-804 HQ column equilibrated in 50 mM Tris-HCl, pH 7.5.

SDS/PAGE

SDS/PAGE was performed with 10% (w/v) gels (Laemmli 1970). The gels were stained with coomassie blue.

Steady-state fluorescence measurements

All steady-state fluorescence measurements were performed using a SLM-8000 spectrophotometer. Excitation was provided using a xenon-mercury arc lamp and a monochromator. For TNP-ATP fluorescence measurements 17 μg of GST-H₄-H₅ loop fusion protein dissolved in 50 mM Tris-HCl, pH 7.5 was titrated with increasing concentrations of TNP-ATP at 37 °C. Excitation and emission wavelengths were 408 nm and 540 nm, respectively. Fluorescence intensity of free label was subtracted as a background (Moutin et al.) For etheno-ATP measurements fluorescently labelled GST-H₄-H₅ loop fusion protein (17 mg) dissolved in 50 mM Tris-HCl, pH 7.5 was titrated with increasing concentrations of ATP at 37 °C. Excitation and emission wavelength were 290 nm and 415 nm, respectively. Fluorescence intensity of free label (etheno-ATP) was subtracted as a background.
Results

Construction of the E. coli expression vector

The plasmid pGEX-H4-H5 was constructed by inserting into pGEX-2T a cDNA fragment containing the sequence of H4-H5 loop (Met$^{386}$ to Ile$^{784}$) just downstream from the sequence of the thrombin cleavage site. The H4-H5 loop sequence was obtained from the sequence of a-subunit of Na$^+$/K$^+$-ATPase using restriction endonuclease Hpa II as described in Methods.

Production of the GST-H4-H5 fusion protein

The recombinant fusion protein GST-H4-H5 loop was produced. The protein was homogeneous by the criteria of SDS/PAGE electrophoresis and ran with a molecular mass of 70 kDa. The fusion protein was purified in two steps using affinity chromatography on a glutathione-Sepharose column followed by HPLC gel filtration.

Prove for ATP binding to the fusion protein

a. Quantum yield of TNP-ATP increases after binding to the fusion protein. The fusion protein (0.8 μM) was titrated with increasing concentration of TNP-ATP in 50 mM Tris-HCl, pH 7.5 at 37°C and fluorescence intensity TNP-ATP was determined when an equilibrium was reached (about three minutes at each step). Increase of fluorescence intensity of TNP-ATP incubated with the protein was observed comparing to fluorescence intensity of the TNP-ATP in buffer. Two independent experiments with nine determinations at each step were performed and presence of the fusion protein increased the quantum yield of TNP-ATP which indicated binding of the fluorescent ATP analog. In a control experiment where the fusion protein was substituted with GST only, titration with TNP-ATP resulted in the same slope of fluorescence intensity as in the absence of any protein. Thus, the same value of the TNP-ATP quantum yield in buffer as in the presence of GST indicating no binding to the GST protein. Consequently, the observed increase of the quantum yield of TNP-ATP after binding to our fusion protein was due to the binding of the fluorescent ATP analog to the H4-H5 loop of the a-subunit.

b. Binding etheno ATP to the fusion protein. EthenoATP, another fluorescent analog of ATP, was used to prove ATP binding to isolated H4-H5 loop. The fusion protein (0.3 mM) was incubated for 60 min with 0.2 mM ethenoATP in 50 mM Tris-HCl, pH 7.4 at 37°C. After incubation, the steady-state fluorescence intensity of ethenoATP increased compared to its fluorescence intensity in buffer only indicating an increase of the quantum yield of ethenoATP after binding to the fusion protein. Subsequently, the fluorescently labelled fusion protein was titrated with non-fluorescent ATP. Fluorescence intensity of the ethenoATP labelled fusion protein was quenched with increasing concentration of ATP indicating a competitive effect of nonfluorescent ATP. In a control experiment, no decrease of fluorescence intensity of ethenoATP in buffer after titration with nonfluorescent ATP was observed. Binding of ethenoATP to the fusion protein should be reflected also by an increase of the lifetime of the excited state of ethenoATP.
Discussion

The H4-H5 loop of the α-subunit of Na,K-ATPase was expressed and isolated from E. coli. The large cytosolic loop was shown to bind ATP. This followed both from the steady-state experiments with TNP-ATP and ethenoATP as well as from the dynamic experiments. A sharp increase of steady-state fluorescence intensity of TNP-ATP when the fusion protein GST-H4-H5 was added clearly indicated binding of the fluorescent ATP analog. This fluorescence intensity increase was similar like in the native enzyme. Binding of TNP-ATP was obviously to the H4-H5 loop and not to the GST part because no similar intensity increase was observed in the control experiment when only GST protein was titrated with the fluorescent ATP-analog. Alternatively, another fluorescent ATP analog, etheno-ATP, showed a similar pattern. Steady-state fluorescence increase was clearly observed when the fusion protein GST-H4-H5 was added to the fluorescent analog. In addition, steady-state fluorescence intensity decreased (was quenched) when the labelled fusion protein GST-H4-H5 loop was incubated with increasing concentration of ATP and, thus, subsequently replaced by non-fluorescent ATP.

In conclusion, the large cytoplasmic loop of the α-subunit of Na,K-ATPase maintains a firm and self-supporting structure and the H4-H5 loop is possible to express with an intact ATP binding site. Isolation of the soluble segment of the Na,K-ATPase with an intact ATP-binding site stream toward the crystallisation experiments with the aim to visualise the ATP-binding site which is rather conserved in P-type ATPases. The isolated H4-H5 loop of the α-subunit of Na,K-ATPase will serve as an important model for study of the high- and low-affinity ATP-binding sites and can, thus, significantly contribute to understanding of the molecular mechanism of the enzyme function.

Acknowledgements. This work was supported by Copernicus Program, Barrande Exchange Program and by grants No. A7011801 of the Grant Agency of CAS and No. 204/98/0468 of the Grant Agency of the Czech Republic.

References