The isolated H_4-H_5 cytoplasmic loop of Na,K-ATPase overexpressed in *Escherichia coli* retains its ability to bind ATP

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Abstract The H_4-H_5 loop of the α-subunit of mouse brain Na,K-ATPase was expressed and isolated from *Escherichia coli* cells. Using fluorescence analogues of ATP, this loop was shown to retain its capability to bind ATP. Isolation of a soluble H_4-H_5 loop with the native ATP binding site is a crucial step for detailed studies of the molecular mechanism of ATP binding and utilisation.

Key words: Sodium,potassium adenosine triphosphatase; Adenosine triphosphate binding site; Protein expression; Etheno-adenosine triphosphate; Time-resolved fluorescence

1. 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 112 000 and responsible for all currently known transport and catalytic functions, and the associated glycoprotein β-subunit (molecular mass about 35 000 excluding oligosaccharides) [1]. There is now common agreement that an $\alpha_2\beta_2$ heterodimer forms in the plasma membrane to create the native structure of the enzyme [2,3]. The α-subunit contains 10 (or eight) transmembrane segments with a large cytoplasmic loop, located between helices H_4 and H_5, where the high-affinity ATP binding site and phosphorylation site are localised [4]. The H_4-H_5 loop of the α-subunit has been recently shown to preserve a rigid and self-supporting structure [5]. Fluorescent derivatives of isothiocyanate have been shown to bind to the ATP binding site at residues Lys^{480} and/or Lys^{501} with highly anisotropic fluorescence of FITC-labelled Na,K-ATPase as well as of the PITC-labelled enzyme. The long rotational relaxation time of PITC indicated not only a rigid ATP binding site on the H_4-H_5 cytoplasmic loop, but a well-defined structure of the whole large H_4-H_5 cytoplasmic loop [5]. Interestingly, the ATP binding site protrudes rather far from the ion transporting domains [6]. This rigid and self-supporting structure may be related to the recent identification of two disulphide bonds between Cys^{452} and Cys^{456}, and between Cys^{511} and Cys^{549} [7].

In the present work, we describe the expression and isolation of the H_4-H_5 loop of the α-subunit of Na,K-ATPase in *Escherichia coli* and we show its capacity to bind ATP. Isolation of this soluble domain with its binding of ATP intact is a first step in an approach to study the structure of the ATP binding site in detail.

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Abbreviations: DTT, dithiothreitol; GST, glutathione *S*-transferase; H_4-H_5 loop, the cytoplasmic loop between transmembrane segments 4 and 5 of the α-subunit of Na,K-ATPase; Na,K-ATPase, Na^+,K^+-transporting ATPase (EC 3.6.1.37); PITC, pyrene 5′-isothiocyanate; TNP-ATP, 2′,3′-*O*-trinitrophenyl-ATP; c_i, pre-exponential amplitude of the *i*th component; τ_i, lifetime of *i*th component; $<\tau>$, average lifetime; χ^2_R, goodness of fit

2. Materials and methods

2.1. Construction of pGEX-H_4-H_5

The expression vector pGEX-2T (Pharmacia Biotech) was digested with *Bam*HI (Promega), the restriction enzyme was then heat-inactivated. The linearised vector was treated with T4 DNA polymerase (Amersham) to obtain blunt ends. Next, the vector was dephosphorylated with calf intestinal alkaline phosphatase (Amersham). The H_4-H_5 sequence was prepared from the sequence of α-subunit of mouse brain Na^+/K^+-ATPase (a gift from Dr. S. Gloor, ETH Zürich) [8]. The pGEM4-α-subunit plasmid was digested with *Hpa*II (Promega) and a fragment of 1201 bp, with the H_4-H_5 sequence (Met^{386} to Ile^{784}), was isolated and purified. This fragment was then digested with S1 nuclease (Promega) to obtain blunt ends. Ligation was performed at 14°C overnight with T4 DNA ligase (Amersham). The ligated DNA was transformed into competent *E. coli* DH5α cells. The *E. coli* transformants were selected on LB agar containing ampicillin (50 μg/ml). The plasmid DNA of all clones obtained was checked and positive clones (with the construct of 6151 bp) were analysed by restriction endonuclease mapping and finally sequenced.

2.2. Purification of GST-H_4-H_5

An overnight culture (3 ml, *E. coli* DH5α cells transformed with pGEX-H_4-H_5) was diluted into 1 l of fresh LB medium containing ampicillin (50 μg/ml) and incubated to an A_{600} of 0.8 (5 h) at 37°C. After addition of IPTG (0.1 mM) the culture was incubated overnight at 30°C. 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 $20\,000\times g$ 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 [9,10]. 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.

2.3. SDS-PAGE

SDS-PAGE was performed using 10% (w/v) acrylamide gels [11] and stained with Coomassie blue.

2.4. Preparation of fluorescent ATP analogues

Synthesis of etheno-ATP was performed according to [12]. TNP-ATP was purchased from Molecular Probes (Eugene, OR,USA).

2.5. Steady-state fluorescence measurements

All steady-state fluorescence measurements were performed using an SLM-8000 spectrofluorimeter. Excitation was provided using a xenon-mercury arc lamp and a monochromator. For TNP-ATP fluorescence measurements, 17 μg of GST-H_4-H_5 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

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of the free label was subtracted as background [13]. For etheno-ATP measurements fluorescently labelled GST-H_4-H_5 loop fusion protein (17 μg) 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 the free label (etheno-ATP) was subtracted as background.

2.6. Dynamic fluorescence measurements

Time-resolved fluorescence measurements were performed by the time-correlated single photon counting method, using synchrotron radiation as a source of the excitation light. The instrument used on the SA1 beam line of Super-ACO has been described previously [14]. The full width at half maximum of the excitation pulse was routinely 600 ps. The total fluorescence decays were collected with the excitation polariser set to the vertical position and the emission polariser set at 54.7° (magic angle). Excitation and emission bandwidths were 9 nm. A total of 2–3 million counts were collected in each decay. Time sampling was 49 ps/channel and 2048 channels were used. All details of data analysis by the maximum entropy method (FAME, MEDC Ltd, UK) are given elsewhere [15]. The final lifetime distribution $a(\tau)$ is split into as many species as there are peaks separated by two well defined minima, over which the lifetime τ_i and relative integrated pre-exponential amplitude c_i of each species i are computed. The first order average fluorescence lifetime $<\tau>$ is then calculated as $\Sigma c_i\tau_i$. Errors on individual lifetimes τ_i are based on the average width of the corresponding peak in the distribution. Errors on average lifetimes $<\tau>$ are based on estimates of the repeatability of the measurements.

3. Results

3.1. Construction of the E. coli expression vector

The plasmid pGEX-H_4-H_5 was constructed as shown in Fig. 1 by inserting a fragment containing the sequence of the H_4-H_5 loop (Met^{386} to Ile^{784}) in pGEX-2T a cDNA just downstream of the sequence of the thrombin cleavage site. The H_4-H_5 loop sequence was obtained from the sequence of the α-subunit of Na^+/K^+-ATPase using the restriction endonuclease *Hpa*II as described in Section 2.

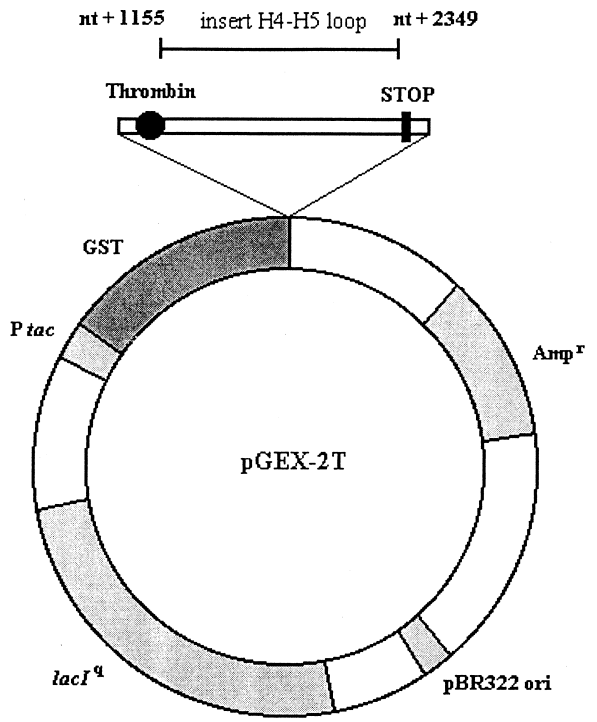

Fig. 1. Plasmid pGEX-H_4-H_5 construction for expression of the GST-H_4-H_5 loop fusion protein. Plasmid pGEX-H_4-H_5 was constructed by inserting a cDNA fragment for the H_4-H_5 loop (from Met^{386} to Ile^{784}) obtained from cDNA of the α-subunit of Na,K-ATPase into the plasmid pGEX-2T (Pharmacia Biotech), using restriction endonuclease *Hpa*II. P*tac*, *tac* promotor; GST, sequence coding for a glutathione *S*-transferase; nt+t, nucleotide number (n) beginning from A of ATG of the first Met in the sequence of the α-subunit of Na,K-ATPase.

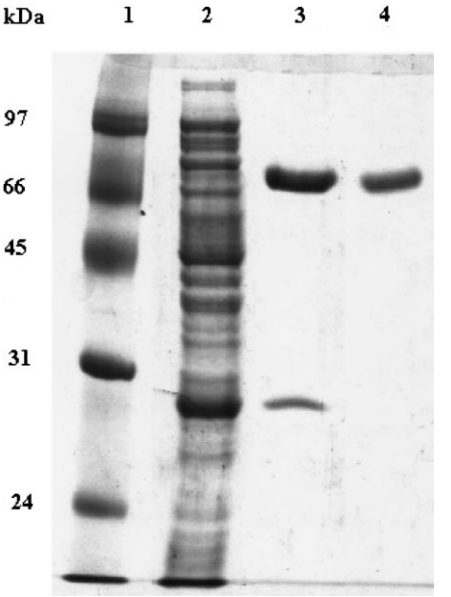

Fig. 2. SDS-PAGE gel electrophoresis of the various steps of GST-H_4-H_5 loop fusion protein production. SDS-PAGE (10% polyacrylamide) with in lane 1: molecular weight standards; in lane 2: bacterial sonicate of the induced culture of *E. coli* DH5α cells transformed with pGEX-H_4-H_5; in lane 3: purified GST-H_4-H_5 loop fusion protein after chromatography on a glutathione-Sepharose column; in lane 4: purified GST-H_4-H_5 loop fusion protein after HPLC gel filtration on a Shodex SB-804 HQ column.

3.2. Production of the GST-H_4-H_5 fusion protein

The recombinant fusion protein GST-H_4-H_5 loop can be produced by the protocol described here. The fusion protein was purified in two steps using affinity chromatography on a glutathione-Sepharose column followed by HPLC gel filtration (see Section 2). The resulting protein is homogeneous by the criteria of SDS-PAGE electrophoresis and it displays a molecular mass of 70 kDa (Fig. 2).

3.3. Proof of ATP binding to the fusion protein

3.3.1. Quantum yield of TNP-ATP increases after binding to the fusion protein. The fusion protein (0.8 μM) was titrated with increasing concentrations of TNP-ATP in 50 mM Tris-HCl, pH 7.5 at 37°C and the fluorescence intensity of TNP-ATP was determined when an equilibrium was reached (about 3 min at each step). We observed an increase of fluorescence intensity of TNP-ATP incubated with the protein, compared to the fluorescence intensity of the TNP-ATP in buffer. Two independent experiments with nine measurements at each step were performed (Fig. 3). The presence of the fusion protein increased the quantum yield of TNP-ATP, which indicates binding of the fluorescent ATP analogue. 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 (data not shown). Thus, the same value of the TNP-ATP quantum yield

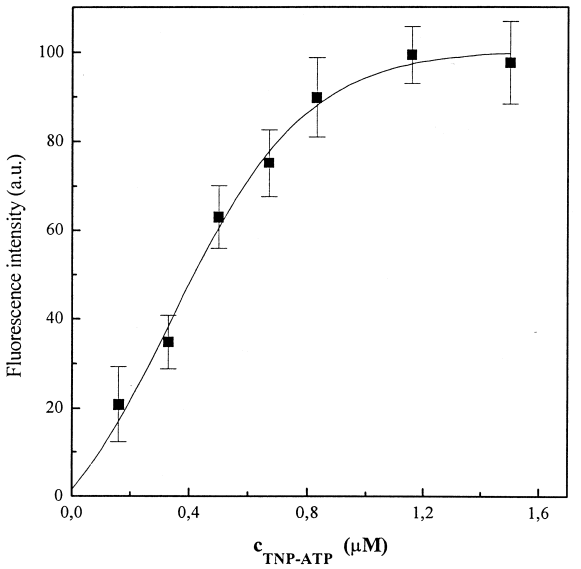

Fig. 3. Titration of GST-H_4-H_5 loop fusion protein with TNP-ATP. 17 μg of GST-H_4-H_5 loop fusion protein dissolved in 50 mM Tris-HCl, pH 7.5 was titrated with increasing concentrations of TNP-ATP at 37°C. Excitation and emission wavelengths were 408 nm and 540 nm, respectively. Fluorescence intensity of free label was subtracted as background. All values are averages of three independent measurements.

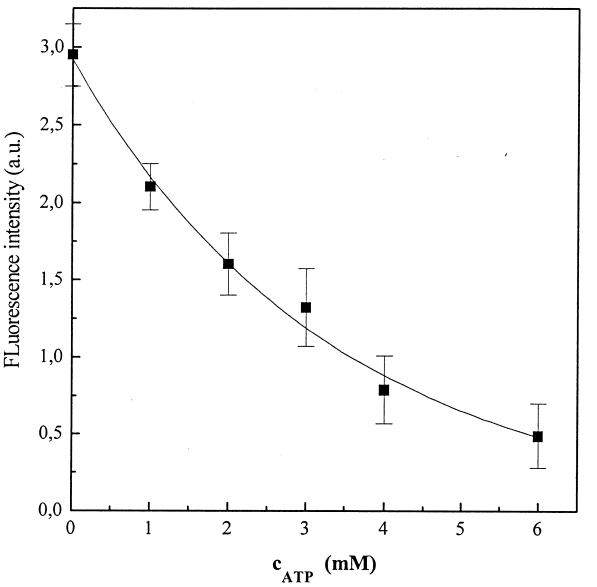

Fig. 4. Titration of fluorescently labelled GST-H_4-H_5 loop fusion protein with ATP. Fluorescently labelled GST-H_4-H_5 loop fusion protein (17 μg) dissolved in 50 mM Tris-HCl, pH 7.5 was titrated with increasing concentrations of ATP at 37°C. Excitation and emission wavelengths were 290 nm and 415 nm, respectively. Fluorescence intensity of free label (etheno-ATP) was subtracted as background. All values are averages of three independent measurements.

in the buffer as in the presence of GST indicates 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 analogue to the H_4-H_5 loop of the α-subunit.

3.3.2. Binding of etheno-ATP to the fusion protein. Etheno-ATP, another fluorescent analogue of ATP, was used to determine ATP binding to the isolated H_4-H_5 loop. The fusion protein (0.3 μM) was incubated for 60 min with 0.2 μM etheno-ATP in 50 mM Tris-HCl, pH 7.4 at 37°C. After incubation, the steady-state fluorescence intensity of etheno-ATP increased compared to its fluorescence intensity in buffer only, indicating an increase of the quantum yield of etheno-ATP after binding to the fusion protein. Subsequently, the fluorescently labelled fusion protein was titrated with non-fluorescent ATP (Fig. 4). Two independent experiments with 30 determinations at each step were performed. Fluorescence intensity of the etheno-ATP-labelled fusion protein was quenched with increasing concentration of ATP indicating a competitive effect of non-fluorescent ATP. In a control experiment, no decrease of fluorescence intensity of etheno-ATP in buffer after titration with non-fluorescent ATP was observed.

Binding of etheno-ATP to the fusion protein should be also reflected by an increase of the lifetime of the excited state of etheno-ATP. Therefore the fluorescence lifetime of the excited state of etheno-ATP was determined by the time-correlated single photon counting method using synchrotron radiation (see Section 2). First, the lifetime of the excited state of etheno-ATP (0.2 μM) was determined in 50 mM Tris-HCl, pH 7.5, 37°C. The excitation wavelength was 290 nm and emission was 415 nm. The fluorescence lifetime distribution includes mostly two major peaks (Table 1), with a dominating 76% amplitude of a 3.7 ns component and 18% of a long lifetime of 23 ns, the average fluorescence lifetime being 7.1 ns. The origin of the lifetime heterogeneity of etheno-ATP in buffer solution is unclear, but could arise either from the conformational freedom of this large molecule, or from small aggregation of the dye. In the next experiment, 0.3 μM of the fusion protein was incubated with 0.2 μM of fluorescent ATP

Table 1
Analysis of the fluorescence intensity decay of the etheno-ATP-labe-led Na^+,K^+-ATPase

Sample	c_i	τ_i (ns)	$<\tau>$ (ns)	χ^2_R
Etheno-ATP	0.02	0.13 ± 0.05		
	0.04	1.38 ± 0.06		
	0.76	3.7 ± 0.2		
	0.18	23. ± 3.0	7.1 ± 0.1	1.05
Etheno-ATP+fusion protein	0.37	0.14 ± 0.05		
	0.14	1.41 ± 0.05		
	0.10	4.0 ± 0.1		
	0.39	24. ± 1.0	10.1 ± 0.1	1.10

Fluorescence intensity decays were fitted by the maximum entropy method, and pre-exponential amplitudes c_i, individual lifetimes τ_i, and average lifetimes $<\tau>$ were computed from the lifetime distributions as described in Section 2.

analogue for 60 min at 37°C. The fluorescence lifetime distribution is now thoroughly modified, showing four well defined components. The pre-exponential amplitude of the lifetime close to 4 ns, possibly characteristic of the free compound, decreases to 10%, while the amplitude of a 24 ns component increases to 39%. As a result, the average fluorescence lifetime of etheno-ATP in the presence of the fusion protein is increased to 10.1 ns.

4. Discussion

The H_4-H_5 loop of the α-subunit of Na,K-ATPase from mouse brain 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 etheno-ATP, and from dynamic experiments.

A sharp increase of steady-state fluorescence intensity of TNP-ATP when the fusion protein GST-H_4-H_5 was added clearly indicated binding of the fluorescent ATP analogue. This fluorescence intensity increase was similar to that of the native enzyme [6]. Binding of TNP-ATP was obviously to the H_4-H_5 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 analogue. Furthermore, another fluorescent ATP analogue, etheno-ATP, showed a similar pattern. A steady-state fluorescence increase was clearly observed when the fusion protein GST-H_4-H_5 was added to the fluorescent analogue. In addition, steady-state fluorescence intensity decreased (was quenched) when the labelled fusion protein GST-H_4-H_5 loop was incubated with increasing concentrations of ATP and thus subsequently replaced by non-fluorescent ATP.

In fact, steady-state fluorescence intensity changes can reflect several, including trivial, effects. Therefore, dynamic measurements were also performed to assess the binding of the ATP analogue to the H_4-H_5 loop. Indeed, in the presence of the H_4-H_5 loop, the large increase of the long fluorescence lifetime close to 24 ns definitely shows that the micro-environment of the probe has been drastically modified. Considering the excitation and emission wavelengths used, the other shorter components may include or reflect some possible contamination by tryptophan fluorescence from the protein. However, the 24 ns lifetime is well outside the range of possible fluorescence lifetimes of tryptophan, which has a reported radiative lifetime of 21 ns [15], setting a theoretical upper limit, while it never exhibits experimental fluorescence lifetimes significantly in excess of 10 ns. Therefore, this long lifetime is to be specifically ascribed to the fluorescence of the ATP analogue bound to the H_4-H_5 protein.

The preparation and characterisation of the analogous domain from the related sarcoplasmic Ca^{2+}-ATPase has been reported earlier [13]. It was shown capable of binding ATP, though with a somewhat lower affinity than the complete native enzyme. In our present work, the affinity of the ATP binding has not been studied so far. The most important difference with earlier results [13] lies in the fact that our construct gives rise to a soluble protein which does not have to undergo any denaturation-refolding procedures. As far as the Na,K-ATPase is concerned, Kaplan and co-workers [16] have also reported recently the expression of a similar construct.

In conclusion, the large cytoplasmic loop of the α-subunit of Na,K-ATPase maintains a well-defined and self-supporting structure, and the H_4-H_5 loop which we were able to express contains an intact ATP binding site. It is interesting that the H_4-H_5 loop protrudes far from the plasma membrane into the cytosol and the ATP binding site is relatively remote (about 7 nm from the ouabain binding site which is localised extracellularly on the membrane/water interface). Thus, such a rigid structure which can be a consequence of two disulphide bonds between Cys^{452} and Cys^{456} and between Cys^{511} and Cys^{549}, as well as multipole-multipole interactions, could play a key role in the transmission of conformational changes between the ATP binding site and the ion translocation domain.

Furthermore, the isolated H_4-H_5 loop of the α-subunit of Na,K-ATPase will serve as an important model for the study of the high- and low-affinity ATP binding sites and can thus significantly contribute to our understanding of the molecular mechanism of the enzyme function.

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