

# Study of ATP binding in the active site of Na<sup>+</sup>,K<sup>+</sup>-ATPase as probed by ultraviolet resonance Raman spectroscopy

R.G. Efremov, A.V. Feofanov, K.N. Dzhandzhugazyan, N.N. Modyanov and I.R. Nabiev

*Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Ul. Miklukho-Maklaya, 16/10, 117871, Moscow V-437, USSR*

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The ultraviolet resonance Raman (UV RR) spectra of functional ATP/membrane-bound Na<sup>+</sup>,K<sup>+</sup>-ATPase complexes have been obtained. The substrate binding in the enzyme active site has been shown to be accompanied with significant changes in the electronic vibrational structure of the adenine ring. From the spectral analysis of ATP, 8-Br-ATP and 6-NHMe-adenine at various pH values the conclusion was made that N1 and the NH<sub>2</sub> group and, probably, N7 of the substrate adenine part, interact with the protein surroundings via hydrogen bonds.

Na<sup>+</sup>,K<sup>+</sup>-ATPase; ATP; Raman spectroscopy; Ultraviolet resonance Raman spectroscopy

## 1. INTRODUCTION

Na<sup>+</sup>,K<sup>+</sup>-ATPase, an integral membrane protein, is an universal system of the ATP-dependent active transport of Na<sup>+</sup> and K<sup>+</sup> ions across the membrane ensuring the nonequilibrium constancy of the ion distribution between the cell and the medium [1]. The study of nucleotide-protein interaction in the enzyme active site plays an important role in understanding the molecular mechanisms of ion transport.

The enzyme investigation by using ATP analogs [1] allowed identification of residues (Asp<sup>369</sup>, Lys<sup>501</sup>, Asp<sup>710</sup>, Asp<sup>714</sup>, Lys<sup>719</sup>) involved in the ATP-binding site formation. Stewart et al. [2] succeeded in determining the spatial conformation of analogs of ATP bound to the protein in the presence of mono- and bivalent cations. There are also some data concerning the Tyr or Cys residues of Na<sup>+</sup>,K<sup>+</sup>-ATPase located in the vicinity of the ATP adenine ring [3].

On the basis of Raman data we have recently calculated the secondary structure of three distinct domains: water-soluble globular cytoplasmic and exoplasmic regions, and a hydrophobic membrane-spanning region of Na<sup>+</sup>,K<sup>+</sup>-ATPase [4] as well as secondary structure changes induced by binding of monovalent cations [5]. This paper deals with UV resonance Raman (UV RR) spectroscopy application to the investigation of ATP binding in the Na<sup>+</sup>,K<sup>+</sup>-ATPase active site.

## 2. EXPERIMENTAL

Isolation of Na<sup>+</sup>,K<sup>+</sup>-ATPase from pig kidney outer medulla,

*Correspondence address:* R.G. Efremov, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Ul. Miklukho-Maklaya, 16/10, 117871, Moscow

measurements of ATPase activity and concentrations were performed as described in [6]. ATPase activity of the enzyme exceeded 36 units. To record UV RR spectra the samples (1.2 ml) were prepared in buffer (25 mM Tris·HCl, 5 mM EDTA, pH 7.5) at an enzyme concentration of 2–3 mg/ml. ATP/Na<sup>+</sup>,K<sup>+</sup>-ATPase complexes were obtained when mixing the equimolar (20 μM) solutions of the components. ATP from Reanal was used without additional purification. 8-Br-ATP was synthesized by S. Lutsenko. 6-NHMe-adenine and 3-methyladenine was obtained from Prof. N. Zheltovsky.

Experimental set-up, details of spectra recording and processing were described earlier [7]. Each spectrum was recorded at least in three independent experiments to test the reproducibility of the results. Stability of the enzyme upon the experimental conditions was checked by measurements of ATP-splitting activity after UV RR spectra recording.

## 3. RESULTS AND DISCUSSION

The strongest bands in the UV RR spectra of Na<sup>+</sup>,K<sup>+</sup>-ATPase and its complex with ATP (fig. 1) correspond to C=C stretching modes of lipids due to a high lipid/protein ratio (~300:1) as well as a preresonance enhancement of lipid vibrations in the 250–254 nm range. For the ATP/enzyme complex a signal of ATP (Trp/nucleotide ratio is equal to 12) is revealed at 1336 and 1481 cm<sup>-1</sup>. The ATP bands near 1600 cm<sup>-1</sup> overlap with intensive lines of Trp and lipids.

Comparison of spectra of the bound and free substrate leads to the conclusion that the active site of Na<sup>+</sup>,K<sup>+</sup>-ATPase is accompanied with changes of the electronic-vibrational structure of the adenine ring (fig. 2). According to estimations reported in [8] about 70% of ATP are bound to enzyme. So, the spectrum shows features due to the bound and free substrate. Fig. 2 shows that the 1305 cm<sup>-1</sup> band is significantly increased in intensity upon complex formation, whereas in the spectrum of ATP in solution it looks like a small

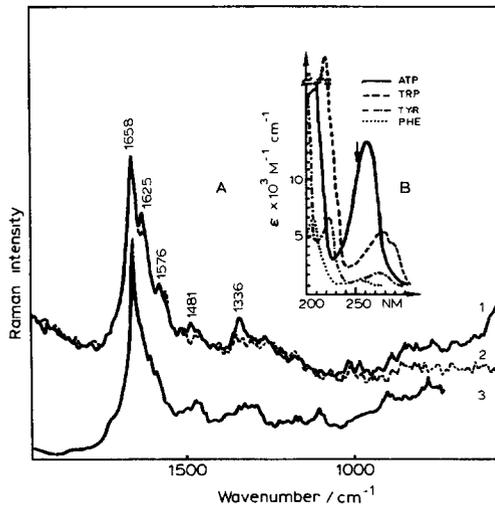


Fig.1. (A) UV RR spectra of ATP bound to  $\text{Na}^+, \text{K}^+$ -ATPase (1), membrane-bound  $\text{Na}^+, \text{K}^+$ -ATPase (2), and lipids from membranes (3) with excitation at 252 nm. (B) Absorption spectra of aromatic amino acids and ATP. Arrow shows UV RR excitation wavelength.

shoulder of the  $1338 \text{ cm}^{-1}$  band. In the ATP/enzyme spectrum the  $1338 \text{ cm}^{-1}$  band undergoes a low-frequency shift; the  $1470 \text{ cm}^{-1}$  band is shifted down from  $1486 \text{ cm}^{-1}$  and its relative intensity is increased. Such features have been reproduced at various excitation wavelengths (fig.2). Relative intensity of the doublet  $1305/1334 \text{ cm}^{-1}$  as well as long-wavelength shift of the  $1486 \text{ cm}^{-1}$  band correlates with the portion of bound nucleotide. When the enzyme is fully inactivated by long-term UV irradiation, the difference spectrum of the complex becomes quite similar to the ATP spectrum in aqueous solution (fig.2). The band located in the  $1380\text{--}1400 \text{ cm}^{-1}$  region, perhaps, appears due to an enhancement and downshift of the  $1365 \text{ cm}^{-1}$  adenine mode, whereas in the spectrum of free ATP it strongly overlaps with the  $1338 \text{ cm}^{-1}$  band.

The observed spectral features can occur due to the following reasons (i) influence of the hydrophobic environment in the active site; (ii) equilibrium shift between *anti*- and *syn*-ATP conformations upon binding to  $\text{Na}^+, \text{K}^+$ -ATPase; (iii) hydrogen bonding between the adenine moiety and the enzyme or partial protonation of the adenine ring.

From the analysis of ATP in water, propanol and water/propanol solutions, we concluded that the spectral changes differ from those in enzyme/substrate complexes. So, the change from hydrophilic to hydrophobic surroundings is not responsible for the UV RR features observed upon ATP binding to  $\text{Na}^+, \text{K}^+$ -ATPase. *Anti*→*syn* conformational transition also shows no influence on the electron density distribution in the adenine ring and cannot alter the UV RR spectrum of ATP [9].

To explain the spectra of bound ATP (fig.2) we obtained the spectra of ATP in aqueous solution at

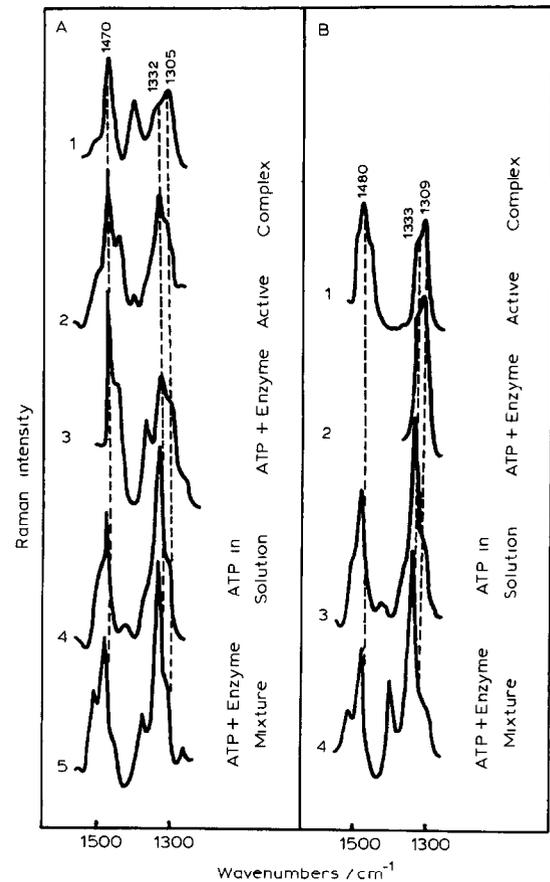


Fig.2. (A) Subtractive UV RR spectra of ATP bound to  $\text{Na}^+, \text{K}^+$ -ATPase (ATP/ $\text{Na}^+, \text{K}^+$ -ATPase minus  $\text{Na}^+, \text{K}^+$ -ATPase) under different ratios of substrate/enzyme: 1/1 (1), 2/1 (2,3). Spectrum of ATP in water (4). Subtractive UV RR spectra of ATP mixed with nonactive  $\text{Na}^+, \text{K}^+$ -ATPase (5). Excitation wavelength is 252 nm. (B) Subtractive UV RR spectra of ATP bound to  $\text{Na}^+, \text{K}^+$ -ATPase (ATP/ $\text{Na}^+, \text{K}^+$ -ATPase minus  $\text{Na}^+, \text{K}^+$ -ATPase) at substrate/enzyme ratio 1/1 (1,2). Spectrum of ATP in water (3). Subtractive UV RR spectra of ATP mixed with inactivated  $\text{Na}^+, \text{K}^+$ -ATPase (4). Excitation wavelength is 254 nm.

various pH values (a model of H-bonding through N1), 3-methyl- and 6-NHMe-adenines (models of interaction through N3 and the  $\text{NH}_2$  group, respectively) as well as the spectra of 8-Br-ATP (a model of interaction through the imidazole ring).

From the analysis of 3-methyladenine spectra at different pH we concluded that neither H-bonding via N3 position nor interaction through two sites N3 and N1 simultaneously can explain the spectral features of bound ATP.

The increase of the  $1309 \text{ cm}^{-1}$  band intensity compared with the  $1333 \text{ cm}^{-1}$  band observed upon binding of ATP (fig.2) is revealed in spectra of 6-NHMe-adenine at acidic pH (fig.3) when N1 is protonated. This was also detected upon 8-Br substitution of the ATP (fig.3) but to a lower extent. Protonation of only N1 (ATP at low pH) or methylation of the  $\text{NH}_2$  group (6-NHMe-adenine at neutral pH) do not induce the

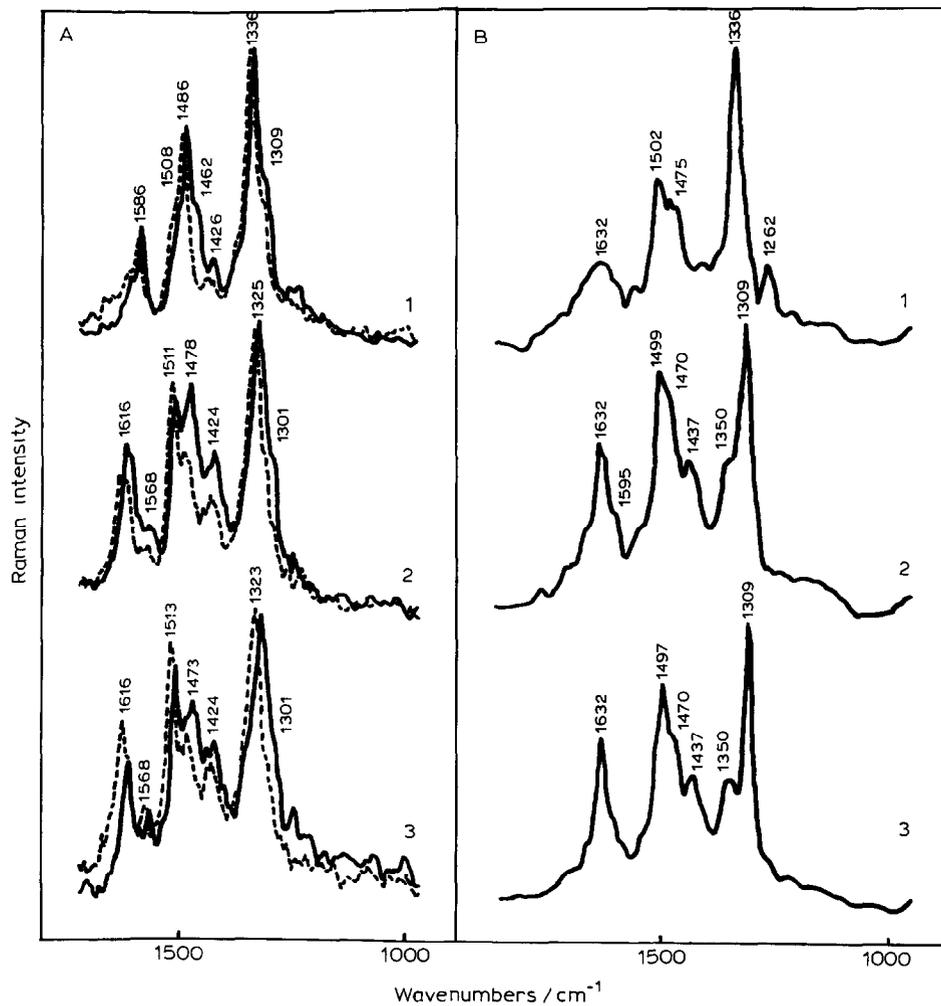


Fig.3. (A) UV RR spectra of ATP in water (broken line) and 8-Br-ATP in water (solid line) at different pH-values: 1, pH = 7.8; 2, pH = 3.0; 3, pH = 1.3. Wavenumbers are shown for 8-Br-ATP vibrational modes. (B) UV RR spectra of 6-NHMe-adenine in water at different pH-values: 1, pH = 8.0; 2, pH = 3.2; 3, pH = 1.7. Excitation wavelength is 252 nm.

spectral feature discussed. The  $I_{1309}/I_{1333}$  ratio characteristic to bound ATP (fig.2) is considerably lower than that of N1 protonated 6-NHMe-adenine (fig.3B), because the spectrum shows features due to the bound (about 70%) and free substrate (about 30%).

Intensity of the  $1470\text{ cm}^{-1}$  mode is increased in spectra of the methylated analog. At the same time the  $1486\text{ cm}^{-1}$  band of 8-Br-ATP is more pH sensitive than ATP, and it shifts down to  $1473\text{ cm}^{-1}$  at N1 protonation. So, binding through N1,  $\text{NH}_2$  as well as via the imidazole ring N7 can explain the appearance of the  $1470\text{ cm}^{-1}$  band in spectra of bound ATP.

It is necessary to note that the protonation of 8-Br-ATP at the N1 and N3 positions is complicated ( $\text{p}K \leq 2$ ) compared with ATP, and according to calculations [10] the order of protonation is  $\text{N1} > \text{N3} > \text{N7}$ . Hence, involvement of the ATP imidazole ring to interaction decreases a probability of the protonation and hydrogen bonding through N1 and N3 positions.

At the same time in the UV RR spectra of [poly-(dA-dT)]<sub>2</sub> and [poly(rA-rU)]<sub>2</sub> [11], where N1 and  $\text{NH}_2$  of adenine form hydrogen bonds with a thymine or uracil, the spectral changes detected differ from those in our study. This may be explained by stronger interaction between protein groups of  $\text{Na}^+, \text{K}^+$ -ATPase and ATP base.

In short, the interaction of the adenine ring with the protein surroundings in  $\text{Na}^+, \text{K}^+$ -ATPase active site proceeds mainly via N1 and the  $\text{NH}_2$  group. Hydrogen bonding between N1,  $\text{NH}_2$  and the protein hydroxylic groups of Thr, Ser, and Tyr or amide groups of Gln and Asn residues seems to be the most probable explanation. Model experiments which were made cannot clearly answer the question about the involvement of the imidazole ring in the binding process. Studying of other model compounds as well as quantum chemical calculations seem to be necessary steps in solving such a problem. This work is in progress now.

It is interesting to note that similar UV RR spectral changes were obtained upon binding the adenine moiety of NAD<sup>+</sup> and NADH to dehydrogenases [9]. They were interpreted in terms of conformation, hydrogen bonding, and protonation of the adenine ring in the protein surroundings.

Hydrogen bonds provide selectivity of the nucleotide-protein interactions. Such interactions are advantageous if two H-bonds are simultaneously formed [12]. A comparison of the results with the data on the three-dimensional structure of the complexes of adenine mono- and dinucleotides with enzymes reveals the major tendencies in the purine binding to the protein groups. Hydrogen bonding with the hydrophilic side chains of the amino acid residues and peptide groups appears to be the most characteristic [13]. Sometimes a stacking with aromatic residues as well as hydrophobic contacts with the side groups of non-polar amino acids is also possible [12].

To understand the mechanism of the ATP hydrolysis providing the transmembrane ion transport, the spatial location of the substrate and the protein groups forming the nucleotide-binding site should be established. But this problem cannot be solved because no direct experimental results are available. It is noteworthy to mention here the studies performed by Stewart et al. [2]. They calculated the conformation of the substrate bound to the protein and evaluated its location relative to the mono- and bivalent cation binding sites. Unfortunately, the results contain no information about the protein groups/ATP interactions.

Data on the involvement of some amino acid residues in the nucleotide-binding site organization were obtained by biochemical approaches. Thus, two fragments were identified in the polypeptide chain containing structural elements of the active site [1]. One includes Asp<sup>369</sup>, phosphorylated upon ATP hydrolysis. The second containing Lys<sup>501</sup> is localised close to the adenine ring binding site. Asp<sup>710</sup>, Asp<sup>714</sup> and Lys<sup>719</sup> also take part in the active site formation [1]. Asp residues 710 and 714 seem to be localized near the phosphate moiety.

To identify the residues forming hydrogen bonds with the adenine part of a substrate through N1 and the NH<sub>2</sub> group we have compared the amino acid sequences of 15 ATPases from bacteria, animals and plants [1,13]. The residues responsible for specific binding of

the substrates in the enzyme active sites seem to be invariant in all sequences and are located in conservative regions. The residues which satisfy the requirements mentioned above: Ser 215, 732; Thr 240, 258, 371, 373, 375, 610 and Glu 348, 377, 713 (the numbers for Na<sup>+</sup>,K<sup>+</sup>-ATPase from pig kidney [1]).

We understand that the results obtained are only the first step to establish the structural organization of the Na<sup>+</sup>,K<sup>+</sup>ATPase active site. We are planning to perform a detailed quantum-chemical analysis as well as modelling of the changes in UV RR spectra of bound ATP. It would be also interesting to compare primary, secondary and elements of supersecondary structures of ion-pumping ATPase with the nucleotide-binding domains topology in kinases, dehydrogenases and other proteins with the known three-dimensional structure. This will shed light on the mechanism of active ion transport.

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