Volume 227, number 2, 235-239 FEB 05515 January 1988

Differentiated analysis of the secondary structure of hydrophilic and hydrophobic regions in α- and β-subunits of Na^+,K^+-ATPase by Raman spectroscopy

Yu.A. Ovchinnikov, E.A. Arystarkhova, N.M. Arzamazova, K.N. Dzhandzhugazyan, R.G. Efremov, I.R. Nabiev and N.N. Modyanov

Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, 117871 Moscow, USSR

Received 20 November 1987

Raman spectra of active Na^+,K^+-ATPase from pig kidney and membrane-bound products of its two-stage trypsinolysis, including α-subunit hydrophobic regions as well as the intact β-subunit and hydrophobic regions of α- and β-subunits, were measured to calculate the secondary structure of hydrophilic and hydrophobic regions of the enzyme. Consequent comparison demonstrated unambiguously that (i) membrane-bound hydrophobic parts of polypeptide chains of Na^+,K^+-ATPase subunits are in the α-helical conformation; (ii) essential contents of the α-helix as well as β-sheet are estimated to form the hydrophilic (mainly cytoplasmic) domain of the Na^+,K^+-ATPase α-subunit; (iii) the exoplasmic hydrophilic domain of the β-subunit is shown to include several antiparallel β-pleated sheets and a small amount of the α-helix and unordered conformations. The model of the secondary structure organization of hydrophilic domains as well as 8 hydrophobic transmembrane segments of the enzyme molecule was proposed on the basis of experimental results and predictional calculations.

$(Na^+ + K^+)$-ATPase; Raman spectroscopy; Secondary structure

1. INTRODUCTION

Na^+,K^+-ATPase is an obligate component in plasma membranes of all eukaryotic cells involved in active transport for Na^+ and K^+ across the cell membrane. The enzyme molecule includes equimolar amounts of the catalytic α-subunit (~112 kDa) and glycosylated β-subunit (protein part, ~35 kDa, and the carbohydrate moiety, ~9 kDa) [1]. The molecule as well as each subunit is an integral membrane protein with an asymmetrical structural organization of the subunits: the main part of the α-subunit forms a cytoplasmic domain and the β-subunit forms an exoplasmic hydrophilic region [1–5]. Elucidation of the enzyme amino acid sequence and detailed structural analysis of the exposed domains by two-stage trypsinolysis [12–15] enable one to design a two-dimensional model.

An experimental study on the secondary structure of the enzyme subunit and a comparison of the results with the possible distribution of the regular structures as determined by statistical predictional techniques is an indispensable step in the development of our ideas on the structural organization of Na^+,K^+-ATPase in the membrane. Infrared (IR) and circular dichroism (CD) spectroscopy have revealed [16–18] that Na^+,K^+-ATPase contains a roughly equal mixture of all types of secondary structures unlike some other membrane proteins such as bacteriorhodopsin or porin which exhibit a vast predominance of either α-helix or β-sheet structures [19,20]. This seems reasonable because the molecule is comprised of three distinct domains: water-soluble globular cytoplasmic and exoplasmic regions, and a hydro-

Correspondence address: N.N. Modyanov, Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 GSP Moscow V-437, USSR

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

phobic membrane-spanning region rather than a single predominantly membrane-embedded domain.

Estimates of the protein secondary structure from Raman spectra appeared to be less susceptible to artifacts than those from other optical techniques. It seems possible to proteolytically dissect the molecule in order to determine the nature of different domains by Raman spectroscopy, and this would provide an experimental basis for modelling the protein structure.

In this study Raman spectroscopy was used to characterize the secondary structure of fully active Na^+,K^+-ATPase and its membrane-bound products following a two-stage trypsinolysis [12–15]. Experimental results were used in predictional calculations in mapping the secondary structure distribution of the enzyme domains.

2. MATERIALS AND METHODS

2.1. *Preparation of Na^+,K^+-ATPase, membrane-bound products following a two-stage trypsinolysis and lipid*

Purified Na^+,K^+-ATPase was prepared according to Jørgensen et al. [3] from pig kidney (outer medulla). The first (digestion of the α-subunit) and the second (digestion of the β-subunit) stages of hydrolysis were performed as described earlier [12–15]. Then all membrane-bound samples were suspended and homogenized at a protein concentration of 10 mg/ml in aqueous medium (0.3 M sucrose, 30 mM histidine, pH 7.5).

Lipids were extracted from membrane preparations according to [21], then dried, solubilized with 1% Na-cholate in 0.3 M sucrose, 30 mM histidine, pH 7.5, and dialyzed for 4 days at 4°C against the same buffer solution.

2.2. *Raman spectroscopy*

The Raman instrument was described in [22]. Samples were held in quartz cells in 0.3 M sucrose, 30 mM histidine, pH 7.5, at 22°C. The samples retained ATPase activities comparable to those of the original samples prior to spectroscopy.

Spectra of lipids were subtracted from spectra of lipid-protein complexes as described in [23,24].

2.3. *Computational analysis of Raman spectra*

Normalized laser Raman amide I spectra between 1630 and 1700 cm^{-1} were used to compute the contents of regular structures [25]. The secondary structure was calculated with the least-squares fitting (singular value decomposition) and linear programming (simplex method) [25] procedures as well as by an algorithm similar to those recently developed for CD-spectra analysis [26].

2.4. *Recognition of secondary structure and hydrophobic segments with predictional techniques*

The strategy of our secondary structure prediction from the amino acid sequence was as follows. Starting with the protein sequence, the most probable α- and β-segments were predicted by the modified Garnier et al. [27] approach including information on the protein structural class. Such information was obtained by Raman spectra analysis. Next we used Taylor's algorithm to refine the original secondary structure prediction based on the automatic positioning of an ideal $\beta\alpha\beta$ template sequence as well as on incorporation of hydrophobic information [28]. Finally, to outline the boundaries of structural segments more precisely we utilized the modified Chou and Fasman technique [29]. Hydrophobic transmembrane segments of the amino acid sequence were predicted by the Eisenberg et al. [30] and Kyte and Doolittle procedures as described in [12].

3. RESULTS AND DISCUSSION

3.1. *Secondary structure composition of the Na^+,K^+-ATPase*

By applying Provencher's simplex and least-square methods for analyzing the amide I vibrational region (1630–1700 cm^{-1}, fig.1), we determined the percentage of the four classes of secondary structure for Na^+,K^+-ATPase. The differences between the results of these three algorithms were less than 3%. Table 1 lists the estimates obtained using the method of Provencher and Glokner [26], which allows us to maintain the error to be less than 4% for the same protein. It should be noted that the calculations for fragments of the helix structure from the CD and IR spectra reveal the assignment of a distorted α-helix to 'undefined conformation'. This yields an underestimation of the helical content in proteins. We succeeded in correlating the values of the α-helical content in Na^+,K^+-ATPase based on IR and CD spectra analysis and the ordered (bi) α-helical content as probed by Raman technique. The examples of similar underestimation of helical content in Ca^{2+}-ATPase as probed by CD spectroscopy were presented by Williams et al. [24].

3.2. *Secondary structure distribution in hydrophilic and membrane-bound hydrophobic regions of Na^+,K^+-ATPase*

To procure information on the distribution of regular secondary structure regions in hydrophilic and membrane-bound hydrophobic domains of α- and β-subunits, consequent comparison of the protein secondary structure in the native enzyme as well as in membrane-bound products from the two-stage proteolysis [12–15] was made.

During the first stage, only extramembrane do-

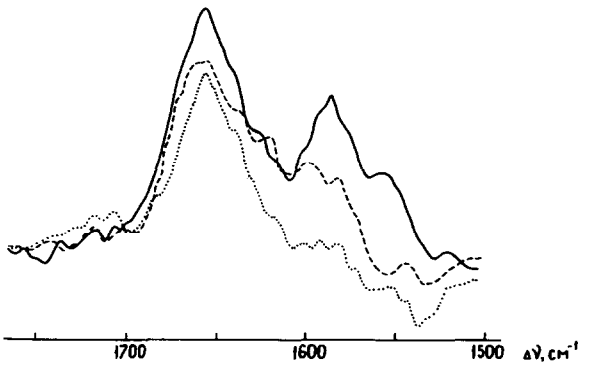

Fig.1. Raman spectra of Na^+,K^+-ATPase (——) and the membrane-bound products of its first (– – –) and second (···) stages of proteolysis.

mains of α-subunit were digested (fig.2b). During the second, the membrane-bound part of the hydrolysate was treated in turn with β-mercaptoethanol and trypsin (fig.2c). Comparison of secondary structures of the native enzyme and its first membrane-bound product (fig.2b) calculated from Raman spectra demonstrated that the hydrophilic fragments of the α-subunit are characterized by the high content of ordered α-helix and β-structure (up to 280 and 200 amino acid residues, respectively, table 2). A small decrease in the ordered α-helical content of such a product seems to be induced by helix-coil transition of some polypeptide chain regions upon hydrolysis. From the results in table 2 it follows that the membrane-bound preparation after the first stage of hydrolysis has a large portion of amino acids forming the β-structure (up to 180 residues). Proteolysis of the β-subunit hydrophilic domain resulted in the disappearance of the β-structure in the membrane-bound parts of α- and β-subunits of Na^+,K^+-ATPase. So, the intramembrane parts of the polypeptide chains of Na^+,K^+-ATPase subunits are of an α-helical conformation and the exoplasmic hydrophilic domain of the β-subunit consists mainly of β-pleated sheets (table 2). Table 2 shows that nearly 230 amino acid residues form transmembrane α-helical rods in the Na^+,K^+-ATPase molecule (ordered and disordered α-helix).

Table 1

Composition of the secondary structure of Na^+,K^+-ATPase

Method	Secondary structure				
	α-Helix (%)		β-Structure %	β-Turn (%)	Random (%)
	Bi-	Mono-			
Raman spectroscopy	28 ± 4	14 ± 4	30 ± 4		28 ± 4
CD spectroscopy	29 ± 7		30 ± 7	10 ± 7	31 ± 7
IR spectroscopy [16]	20 ± 8		25 ± 8		55 ± 8
IR spectroscopy [17]	22 ± 8		22 ± 8		56 ± 8
CD spectroscopy [18]	28		32	9	31

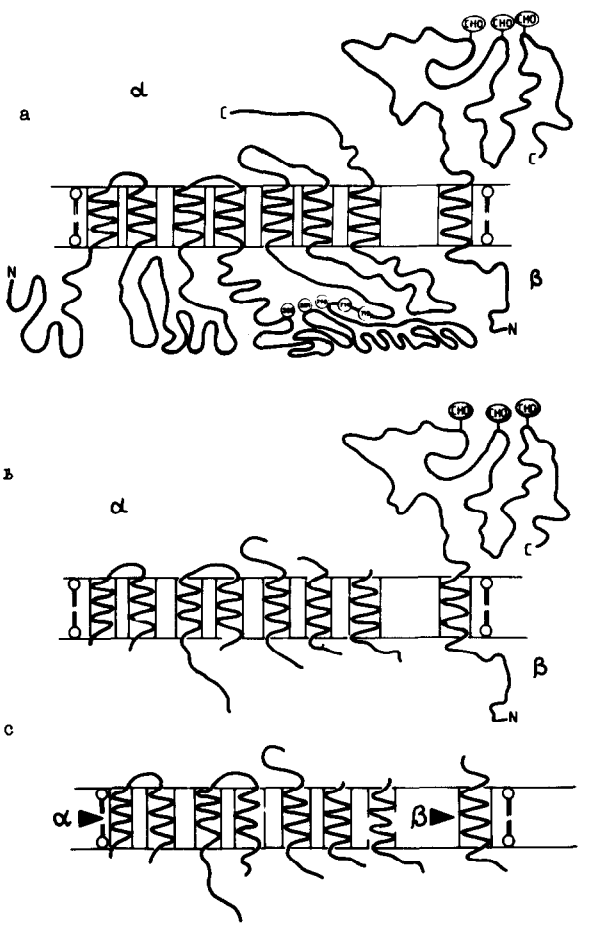

Fig.2. The model for the spatial organization of the enzyme molecule (a) and the membrane-bound products of its first (b) and second (c) stages of proteolysis.

3.3. *The model of the enzyme secondary structure organization*

Raman spectroscopy data for native and consequently dissected membrane-bound preparations of Na^+,K^+-ATPase provide an experimental basis for reasonable modelling of the enzyme's secondary structure in different protein domains. Fur-

Table 2

Calculated secondary structures in Na^+,K^+-ATPase and the membrane-bound products following two-stage trypsinolysis (fig.2)

Preparation	Secondary structure						
	Raman data				Predictional techniques		
	α-Helix (%)[a]		β-Structure[a] (%)	Random[a] (%)	α-Helix (%)	β-Structure (%)	Random (%)
	Bi-	Mono-					
Na^+,K^+-ATPase (fig.2a)	42 ± 4 (554 ± 52)	28 ± 4 (369 ± 52) / 14 ± 4 (185 ± 52)	30 ± 4 (395 ± 52)	28 ± 4 (369 ± 52)	39	22	39
Membrane-bound product of the first stage of trypsinolysis (fig.2b)	48 ± 4 (282 ± 24)	15 ± 4 (88 ± 24) / 33 ± 4 (193 ± 24)	32 ± 45 (188 ± 24)	20 ± 4 (117 ± 24)	42	22	36
Membrane-bound product of the second stage of trypsinolysis (fig.2c)	70 ± 4 (230 ± 12)	24 ± 4 (79 ± 12) / 46 ± 4 (151 ± 12)	0	30 ± 4 (99 ± 12)	64	4	28

[a] The number of amino acid residues forming the corresponding secondary structure is given in parentheses (details in text)

thermore, we applied several techniques recently developed for water-soluble parts of α/β-proteins [27,28]. It is noteworthy that utilization of these techniques for membrane-embedded protein domains seems to be questionable [31]. Raman data collected in this work demonstrate unambiguously that the membrane-bound hydrophobic parts of polypeptide chains of Na^+,K^+-ATPase subunits are of an α-helical conformation (fig.2c; table 2). Moreover, we have succeeded in estimating the α-helix, β-sheet and β-turn contents in hydrophilic domains by means of Raman spectroscopy. The information obtained was used to refine the predictional results.

Within the frame of the model for Na^+,K^+-ATPase's transmembrane organization including 8 α-helical transmembrane segments [14], Raman data indicate a reasonable value of 28 amino acid residues per segment. It should be stressed that the high probability of the long α-helical segments was calculated by statistical analysis of Ca^{2+}- [32] and Na^+,K^+-ATPase amino acid sequences.

The model of the enzyme's secondary structure

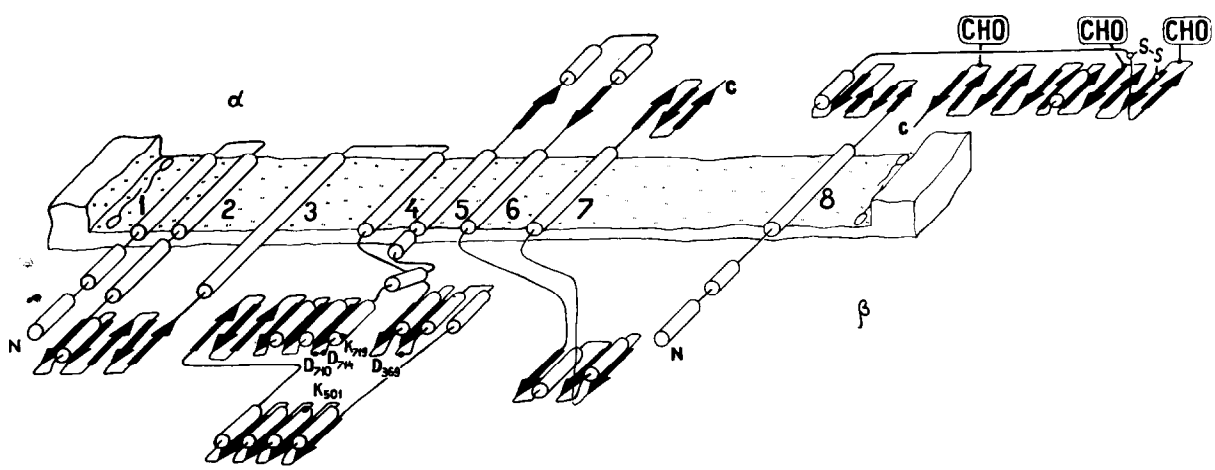

Fig.3. Structural diagram of Na^+,K^+-ATPase in the membrane based on Raman data, trypsin proteolysis and predictional techniques. Cylinders and arrows correspond to α-helical segments and β-pleated sheets, respectively.

organization (including some elements of the super-secondary structure) based on Raman data and predictional calculations is presented in fig.3. Its main features are: (i) compact structural units forming antiparallel β-sheet and $\beta\alpha\beta$-folds in the hydrophilic cytoplasmic domain of the α-subunit; (ii) within the frame of the model it is possible to locate the amino acid residues forming the enzyme ATP-binding center (Asp^{369}, Lys^{501}, Asp^{710}, Asp^{714} and Lys^{719}) in a close spatial vicinity (see [14]); (iii) two α-helical 'stalks' connecting the globular domains to transmembrane hydrophobic segments 2 and 3.

Similar structural elements were shown to be predicted in Ca^{2+}-ATPase [32] which are highly homologous to the α-subunit of Na^+,K^+-ATPase.

At present the only way to determine the spatial folding in Na^+,K^+-ATPase is the combined application of a wide variety of different methodological approaches. We believe that the combination of optical spectroscopy with the consequent proteolysis and predictional techniques will be extremely useful in tuning finely the spatial arrangement of Na^+,K^+-ATPase.

This model may be useful in future analysis of conformational changes in proteins during ATP-dependent ion transport across the membrane.

REFERENCES

[1] Glynn, I.M. (1985) in: The Enzyme of Biological Membranes (Martanosi, N.Y. ed.) pp.35–114, Plenum, New York.

[2] Dzhandzhugazyan, K.N. and Jørgensen, P.L. (1985) Biochim. Biophys. Acta 817, 165–173.

[3] Dzhandzhugazyan, K.N., Modyanov, N.N. and Ovchinnikov, Yu.A. (1981) Bioorg. Khim. 7, 1790–1800.

[4] Ovchinnikov, Yu.A., Demin, V.V., Barnakov, A.N., Kuzin, A.P., Lunev, A.V., Modyanov, N.N. and Dzhandzhugazyan, K.N. (1985) FEBS Lett. 190, 73–76.

[5] Ovchinnikov, Yu.A., Demin, V.V., Barnakov, A.N., Svetlichny, E.V., Modyanov, N.N. and Dzhandzhugazyan, K.N. (1986) Biol. Membrany 3, 519–522.

[6] Ovchinnikov, Yu.A., Arsenyan, S.G., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Aldanova, N.A., Arzamazova, N.M., Arystarkhova, E.A., Melkov, A.M., Smirnov, Yu.V., Guryev, S.O., Monastyrskaya, G.S. and Modyanov, N.N. (1985) Dokl. Akad. Nauk SSSR 285, 1490–1495.

[7] Ovchinnikov, Yu.A., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Kiyatkin, N.I., Arzamazova, N.M., Gevondyan, E.N., Melkov, A.M., Smirnov, Yu.V., Malyshev, I.V., Monastyrskaya, G.S. and Modyanov, N.N. (1986) Dokl. Akad. Nauk SSSR 287, 1491–1496.

[8] Kawakami, K., Nogushi, S., Noda, M., Takahashi, H., Ohta, T., Kawamura, M., Nojima, H., Nagano, K., Hirose, T., Ynayama, S., Hayasida, H., Miyata, T. and Numa, S. (1985) Nature 316, 733–736.

[9] Nogushi, S., Noda, M., Takahashi, H., Kawakami, K., Ohta, T., Nagano, K., Hirose, T., Ynayama, S., Kawamura, M. and Numa, S. (1986) FEBS Lett. 196, 315–320.

[10] Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.

[11] Shull, G.E., Lane, L.K. and Lingrel, J.B. (1986) Nature 321, 429–431.

[12] Ovchinnikov, Yu.A., Modyanov, N.N., Broude, N.E., Petrukhin, K.E., Grishin, A.V., Arzamazova, N.M., Aldanova, N.A., Monastyrskaya, G.S. and Sverdlov, E.D. (1986) FEBS Lett. 201, 237–245.

[13] Modyanov, N.N., Arzamazova, N.M., Arystarkhova, E.A., Gevondyan, N.M., Gavrilyeva, E.E., Dzhandzhugazyan, K.N., Lutsenko, S.V., Luneva, N.M., Shafieva, G.I. and Chertova, E.N. (1987) in: Receptors and Ion Channels (Ovchinnikov, Yu.A. and Hucho, F. eds) pp.287–1294.

[14] Ovchinnikov, Yu.A., Arzamazova, N.M., Arystarkhova, E.A., Gevondyan, N.M., Aldanova, N.A. and Modyanov, N.N. (1987) FEBS Lett. 217, 269–274.

[15] Arzamazova, N.M., Arystarkhova, E.A., Gevondyan, N.M., Gavrilyeva, E.E., Azizova, G.I., Chertova, E.N., Klimenko, A.S. and Modyanov, N.N. (1987) Biol. Membrany 4, 590–599.

[16] Chetverin, A.B., Brazhnikov, E.V. and Chirgadze, Yu.N. (1979) Biokhimia 44, 949–952.

[17] Chetverin, A.B. and Brazhnikov, E.V. (1985) J. Biol. Chem. 260, 7817–7819.

[18] Gresalfi, T.J. and Wallace, B.A. (1984) J. Biol. Chem. 259, 2622–2628.

[19] Henderson, R., Baldwin, J.M., Downing, K.H., Lepault, J. and Zemlin, F. (1986) Ultramicroscopy 19, 147–178.

[20] Engelman, D.M., Steitz, T.A. and Goldman, A. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 321–353.

[21] Kates, M. (1972) Techniques of Lipidology, Plenum, New York.

[22] Nabiev, I.R., Savchenko, V.A. and Efremov, E.S. (1983) J. Raman Spectr. 14, 375–380.

[23] Yager, P., Chang, E.L., Williams, R.W. and Dalziel, A.W. (1984) Biophys. J. 45, 26–28.

[24] Williams, R.W., McIntyre, J.O., Gaber, B.P. and Fleischer, S. (1986) J. Biol. Chem. 261, 14520–14524.

[25] Williams, R.W. (1986) Methods Enzymol., 311–331.

[26] Provencher, S.W. and Glokner, J. (1981) Biochemistry 20, 33–37.

[27] Busseta, B. and Hospital, M. (1978) Biochim. Biophys. Acta 709, 73–78.

[28] Taylor, W.R. and Thornton, J.M. (1984) J. Mol. Biol. 173, 487–514.

[29] Chou, P.Y. and Fasman, G.D. (1974) Biochemistry 13, 222–230.

[30] Eisenberg, D., Schwarz, E., Komaromy, M. and Wall, R. (1984) J. Mol. Biol. 179, 125–142.

[31] Green, N.M. and Flanagan, M.T. (1976) Biochem. J. 153, 729–732.

[32] MacLennan, D.H., Brandl, C.J., Korczak, B. and Green, N.M. (1985) Nature 316, 696–700.