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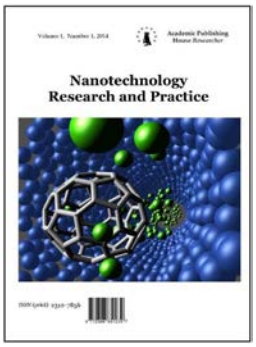

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Introduction of Deuterated Aromatic Amino Acids – [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan into a Molecule of Photochrome Trans-membrane Protein Bacteriorhodopsin

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Abstract

It was carried out the introduction of functionally important deuterated aromatic amino acids – [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan into a molecule of photochrome trans-membrane protein bacteriorhodopsin, synthesized by a photo-organotrophic halobacterium *Halobacterium halobium ET 1001*. The deuterated protein (output 8–10 mg) was isolated from purple membranes by cellular autolysis by distilled water, processing of bacterial biomass by ultrasound at 22 KHz, alcohol extraction of low and high-weight molecular impurities, cellular RNA, carotenoids and lipids, with the subsequent solubilization of final product with 0,5 % (w/v) SDS-Na and fractionation by methanol, gel filtration chromatography on Sephadex G-200, reverse-phase HPLC and EI impact mass-spectrometry of methyl esters of N-Dns-[^{2}H]derivatives of amino acids. Deuterium was detected in all residues of aromatic amino acids. However, the presence in the EI mass spectrum of the BR hydrolysate the peaks $[M]^+$ of semi-deuterated analogues of aromatic amino acids – phenylalanine with $[M]^+$ at m/z = 413–418, tyrosine – with $[M]^+$ at m/z = 428–430 and tryptophan – with $[M]^+$ at m/z = 453–457 with different levels of contributions to the deuterium enrichment of molecules testifies about the conservation of the minor pathways of biosynthesis of aromatic amino acids de novo.

Keywords: *Halobacterium halobium ET 1001*, bacteriorhodopsin, [2,3,4,5,6-$^2H_5$]Phe, [3,5-$^2H_2$]Tyr, [2,4,5,6,7-$^2H_5$]Trp, biosynthesis; EI mass-spectrometry, RP HPLC.

Introduction

Bacteriorhodopsin (BR), named by analogy to the visual apparatus of mammalian protein rhodopsin, was isolated from the cell membrane of extreme photo-organotrophic halobacteria *Halobacterium halobium* in 1971 by W. Stohenius and D. Osterhelt [1]. This photo-transforming

integral trans-membrane protein with the molecular weight ~26.5 kDa represents a chromoprotein determining the purple-red culour of halophilic bacteria, which contains as chromophore group an equimolar mixture of 13-*cis*- and 13-*trans*-retinol C20 carotenoid, bound by the Schiff base (as in the visual animal pigments) with Lys-216 residue of the protein [2]. In halobacteria the BR functions as a light-driven transmembrane proton pump pumping a proton across the membrane. Along with the BR the cell membrane of halobacteria contains a small amount of other related carotenoid pigments, the main of which bakterioruberin determining the stability of halobacteria to solar radiation.

On its structure and location in the cell membrane BR refers to integral trans-membrane proteins, penetrating the cell membrane, which is divided into three separate fractions: yellow, red and purple. Purple fraction comprising 75 % (w/w) of cell membrane consists from carotenoids, phospholipids (mostly phosphoglycerol diesters of phosphatidyl glycerol phosphate (PGP) with a small amount of non-polar lipids and isoprenoids) forms natural two-dimensional crystals which can be investigated using the electron microscopy, X-ray diffraction methods as X-ray crystallography. These methods have established the existence in the BR molecule of seven α-helical protein segments which span the lipid matrix, while in the middle part is symmetrically located a retinal residue being covalently linked to Lys-216 residue in the G helix so that that each protein molecule has one retinal moiety (vitamin A) (Fig. 1). In the native membrane the polypeptide chain is oriented with the amino-end N-terminal in the extracellular medium and the carboxyl C-terminal – inside the cytoplasmic side. The carboxyl C-terminal is non-helical consists of 24 amino acid residues, four of them are negatively charged and two – are positively charged. Other chains connecting the helixes on the cytoplasmic side contain a total of five additional negatively charged residues, but no more positively charged residues. The amino-end N-terminal of the protein is also nonhelical and consists of six residues.

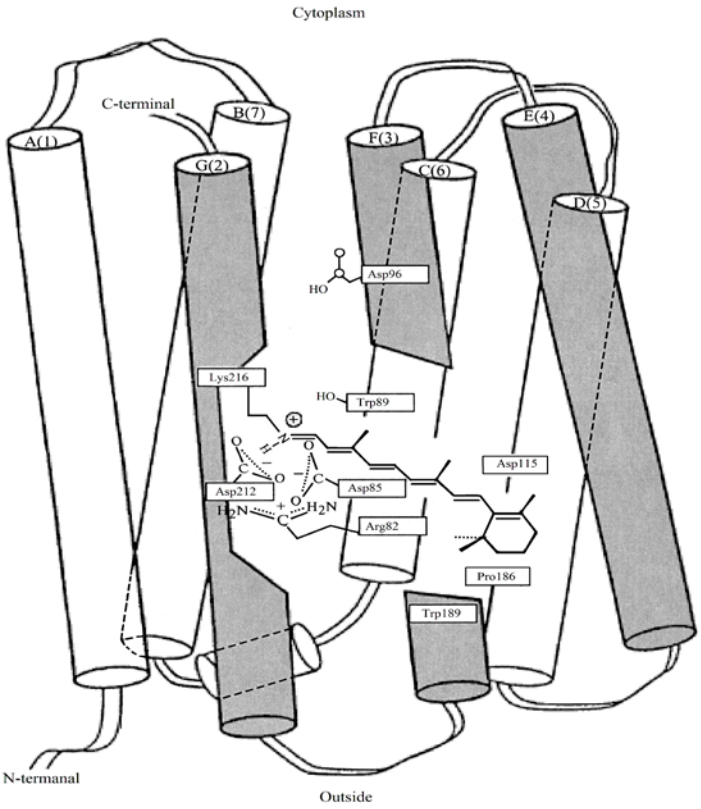


Figure 1: The location of protein and the retinal residue in the BR molecule of halobacterium *H. halobium* according to computer modeling: Latin numerals indicate protein fragments of the BR molecule as 7 α-helical segments (*A*, *B*, *C*, *D*, *E*, *G*, *F*) with exposed amino acid residues; gray color represents the segments responsible for binding of retinal residue to the G-helical segment of BR molecule.

The polypeptide chain of the BR consists of 248 amino acid residues, 67 % of which are hydrophobic, formed with the aromatic amino acids, and 33 % – hydrophilic residues of aspartic and glutamic acids, arginine and lysine; the protein does not contain histidine or cyctein [21]. These residues play the important structural and functional role in the spatial orientation of the α-helical segments of the BR molecule, arranged in PM in an orderly manner forming 7 trimmers with an average diameter ~0,5 μm and a thickness 5–6 nm; each trimmer is surrounded by six others so that to form a regular hexagonal lattice [22]. The BR molecule consists of seven α-helix segments, arranged in a direction perpendicular to the plane of the membrane (Fig. 2). Hydrophobic domains representing trans-membrane segments and hydrophilic domains protruding from the membrane connecting the individual α-helical intra-membranous protein segments of the BR molecules. Along with BR the PM contain lipids, carotenoids and water in their composition.

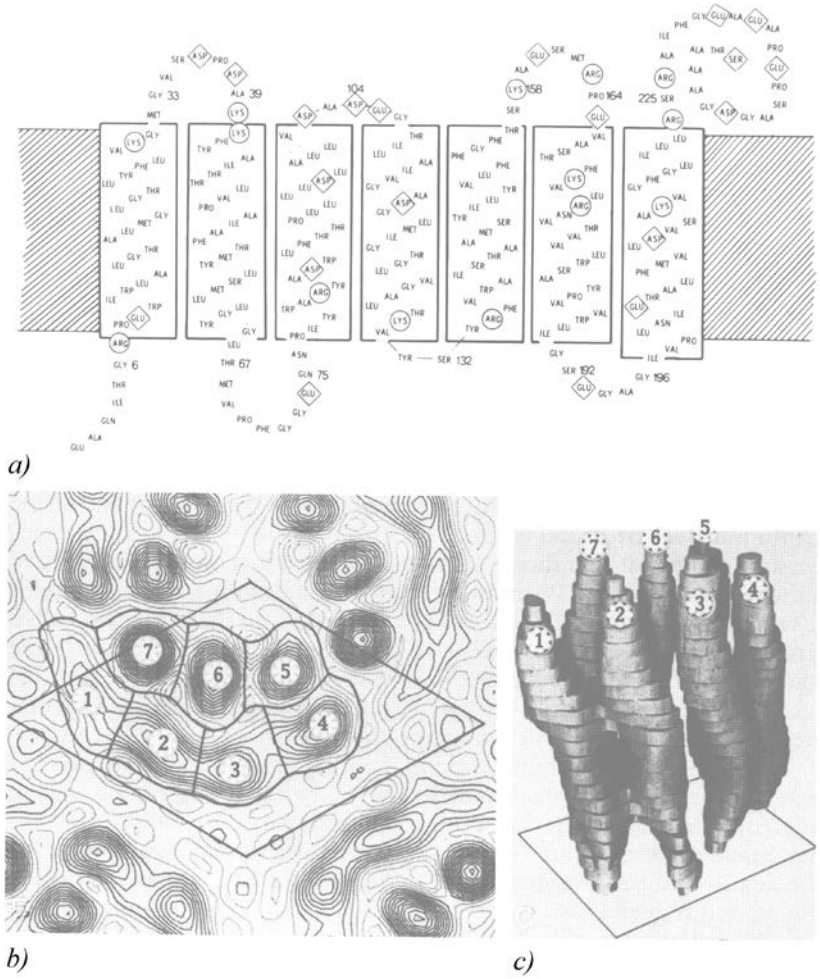


Figure 2: The structure of the BR molecule according to X-ray diffraction analysis:
a) – the primary structure of the BR molecule: amino acids indicated in Latin characters, circles and rhombs show the functionally important amino acids responsible for spatial orientation of α-helical segments of the protein moiety of the BR molecule and the formation of channels for the transfer of protons H^+ across the cell membrane;
b) – the electron density map of PM (a single molecule of the protein is encircled in the center). Numbers 1–7 are designated α-helical segments of the BR molecule: – 1– A-segment; 2 –B-segment; 3 – C-segment; 4 – D-segment; 5 – E-segment; 6 – F-segment; 7 – G-segment;
c) – the spatial structure of the BR molecule: 1 – A-segment; 2 – B-segment; 3 – C-segment; 4 – D-segment; 5 – E-segment; 6 – F-segment; 7 – G-segment.

Owing to the unique structure, the BR molecule acts as a light-dependent proton pump, pumping protons across the cell membrane and generates an electrochemical gradient of H^+ on the surface of the cell membrane, which energy is used by the cell for the synthesis of ATP in the anaerobic photosynthetic phosphorylation. The mechanism of ATP synthesis is denoted as "non-chlorophyll photosynthesis", in contrast to the plant photosynthesis implemented with the participation of chlorophyll. In this mechanism, at absorption of a light photon BR molecule became decolorized by entering into the cycle of photochemical reactions, resulting in the release of a proton to the outside of the membrane, and the absorption of a proton from intracellular space. By the absorption of a light photon is occurred a reversible isomerization of all 13-*tras*-BR (λ_{max} = 548 nm) (the quantum yield 0.03 at t = +20 ^{0}C) into 13-*cis*-BR [(λ_{max} = 568 nm) [3], initiating a cascade of photochemical reactions lasting from 3 ms to 1 ps with the formation of transitional intermediates J, K, L, M, N, and O, followed by separation of H^+ from the retinal residue of BR and its connection from the side of cytoplasm, and finally returns to its 13-*trans*-conformation while remaining bonded to the protein throughout the photo-cycle (Fig. 3). In this process a proton originating at the Schiff base of the retinal residue is passed across by being transferred to the hydrophilic Asp-85 residue lying in sterically favorable positions, to the other side of the cellular membrane; right after that the vacancy is filled up with a proton transferred from Asp-96 residue [24]. As a result, between the internal and external surface of the membrane forms a concentration gradient of H^+, resulting that illuminated by light the halobacteria cells begin to synthesize ATP, i.e. convert light energy into the energy of chemical bonds. This process is reversible and in the dark flows back in the opposite direction, allowing to halobacteria developing in the dark by means of switching of photosynthetic metabolism to the heterotrophic metabolism. Thus, the BR molecule behaves as a photochromic carrier with a short relaxation time – the transition from the excited state to the ground state. The optical characteristics of the BR vary depending on the method of preparation of the PM and the properties of embedded polymer matrix.

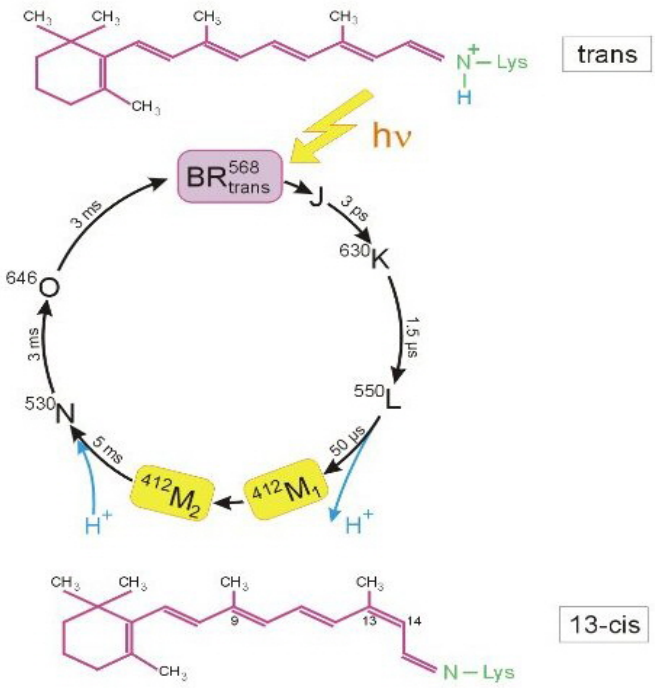


Figure 3: Photocycle scheme of BR (aqueous solution, pH = 7,2, t = +22 ^{0}C). Latin numbers J, K, L, M, N, O denote the spectral intermediants of BR. M_1 and M_2 represent spectral intermediants of *meta*- bacteriorhodopsin with the protonated and deprotonated aldimine bond. The superscripts correspond to the position of the absorption maximum intermediates in photocycle (nm)

The main task for the manufacture of BR-containing nanofilms is the orientation of PM between the hydrophobic and hydrophilic media. Typically, to improve the characteristics of the BR-containing films use multiple layers of the PM that are applied to the surface of the polymeric carrier and dried up, preserving their natural structure. The best results are achieved in the manufacture of nanofilms based on gelatin matrix (Fig. 4). This allows achieve high concentration of BR (up to 50 %) in nanofilms and avoid aggregation of membrane fragments and destruction of BR in the manufacturing process. Being embedded in a gelatin matrix PM fragments are durable (~10^4 h) and resistant to solar light, the effects of oxygen, temperatures greater than +80 ºC (in water) and up to +140 ºC (in air), pH = 1–12, and action of most proteases. The dried PM is stacked on top of each other, focusing in the plane of the matrix, so that a layer with 1 μm thickness contains about 200 monolayers. When illuminated such nanofilms exert the electric potential 100–200 mV, which coincides with the membrane potential of living cells. These factors are of great practical importance for integration of PM into polymeric nanomatrix with keeping photochemical properties.

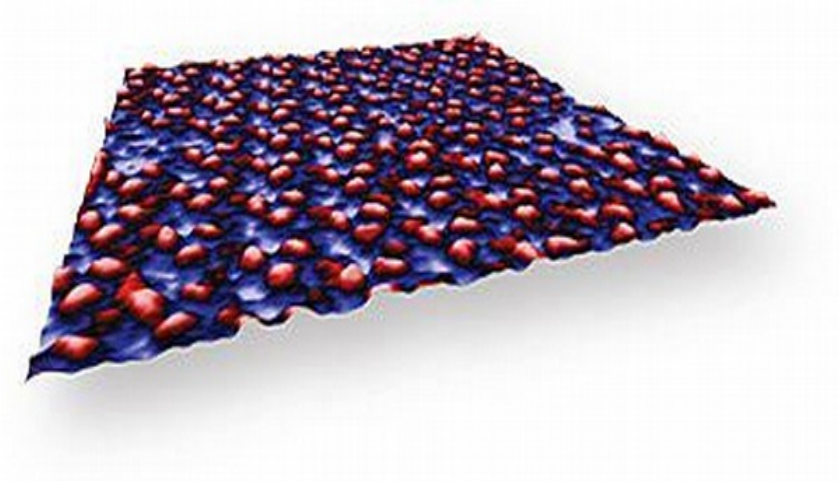


Figure 4: Artificial membrane from the BR-containing PM obtained by the SEM method: scanning area – 100×100 mm, resolution – 50 nm, magnification – 100000 times. PM is shown in purple, BR – in red color

BR is the focus of bio-and nanotechnology mainly because of its high sensitivity and resolution, and is used in molecular bioelectronics as natural photochromic material for light-controlled electrical regulated computer modules and optical systems [4–6]. Additionally, the BR is very attractive as a model for studies of functional activity and structural properties of photo-transforming membrane proteins in the native and photo-converting membranes. For these studies it is useful to enter into the protein molecule a deuterium label (^{2}H), which allows apply for evaluating of the structure by the NMR-method [7]. In this aspect the big scientific and practical interest has the BR labeled with deuterium on the residues of functionally important aromatic amino acids – phenylalanine, tyrosine and tryptophan involved in the hydrophobic interaction of the polypeptide chain of the protein with the lipid bilayer of the cell membrane. ^{2}H-labeled aromatic amino acids can be synthesized in gram scale quntities by isotopic exchange (H-^{2}H) in molecules of the protonated amino acids; for analyzing deuterium enrichment levels the EI mass-spectromerty of N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl) amino acid derivatives may be used [8–10].

The purpose of this research was to study the micro preparative biosynthesis of the BR labeled with deuterium on the residues of functionally important aromatic amino acids – [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan, for the reconstruction of artificial membranes, as well as to study of the levels of deuterium enrichment of the BR molecule by EI mass spectrometry in combination with RP HPLC.

Material and methods

Bacterial strain

As a BR producer was used a carotenoid strain of extreme photo-organotrophic halobacterium *Halobacterium halobium ET 1001*, obtained from Moscow State University (Russia) [11]. The strain was modified by selection to individual colonies on solid 2% (w/v) agarose media with peptone and 4,3 M NaCl.

Chemicals

For preparation of growth media were used *D,L*-amino acids ("Reanal", Hungary), AMP, UMP, biotin, folic acid and vitamin B_{12} – $2 \cdot 10^{-5}$ purchased from "Sigma Corp." (Sigma Aldrich, USA). Organic salts were obtained from Reanal ("Reanal", Hungary). The buffer components were from Reachim-Pharm Ltd. (Russia). All solvents were of HPLC grade. Other chemical reagents were of analytical reagent grade. Filtrated water was provided by the Milli-Q-Plus water filtration system (Millipore, Bedford, MA, USA).

Synthesis of L-[2,3,4,5,6-$^2H_5$] phenylalanine

40 g of phenylalanine was dissolved in 300 ml of 85% (v/v) $^2H_2SO_4$ (in ^2H_2O) and heated with water reflux condenser at 50–60 ^{0}C under stirring for 3 days. Upon completion the reaction mixture was cooled, neutralized with 30% (v/v) NH_4ON to pH = 5.5. The product was extracted with ethanol. Yield, 24 g (58,7%), T_m = 271–273; $[\alpha]_d^{25}$ = 4,47 (5 M HCl); pK_a = 2,20 (COOH), 9,31 (NH_2); R_f = 0.6 (A). UV-spectrum (0,1 M HCl): λ_{max} nm [(ε M^{-1} $см^{-1}$)]: 257,5 (ε 195).^{1}H-NMR spectrum ($^2H_2SO_4$ in ^2H_2O) (δ, ppm): δ 3,25 (2H, Hβ), δ 4,4 (1H, Hα), δ 7,2–7,4 (0,07H), 95 atom.% ^{2}H. EI mass spectrum $[M]^+$ *m/z* (*I*, %): 165 (34), methyl ester of N-Dns-[2,3,4,5,6-$^2H_5$]Phe: 417 (14), 418 (6).

Synthesis of L-[3,5-$^2H_2$]tyrosine

100 g of tyrosine was dissolved in 150 ml of 3 M $^2H_2SO_4$. The reaction mixture was heated for 2 days at 40–50 ^{0}C with water reflux condenser under a slow stream of dry nitrogen. The solution was neutralized with 30% (v/v) NH_4OH to pH = 4,5 and cooled 1 day at +4 ^{0}C. The crystalline product was filtered, washed with ^2H_2O and dried at 10 mm Hg. Yield, 90 g (86,5 %); T_m = 316–317; $[\alpha]_d^{25}$ = 10,03 (5 M HCl); pK_a = 2,20 (COOH), 9,21 (NH_2); R_f = 0,45 (A). UV-spectrum (0,1 M HCl) λ_{max} nm [(ε M^{-1} $см^{-1}$)]: 223 (ε 8200), 274,5 (ε 1340). ^{1}H-NMR spectrum (1M ^{2}HCl) (δ, ppm): δ 3,32 (2H), δ 4,35 (1H), δ 6,9 (1H), δ 7,2 (2H), 96 atom.% ^{2}H. EI mass spectrum $[M]^+$ *m/z* (*I*, %): 181 (21), methyl ester of N-Dns-[3,5-$^2H_2$]Tyr: 429 (15), 430 (5).

Synthesis of L-[2,4,5,6,7-$^2H_5$] tryptophan

100 ml of 40% (v/v) ^2H_2O was plases in a round bottom flask and cooled in an ice bath. 80 ml of trifluoroacetic anhydride (0,5 mol) was added dropwise with stirring. The reaction mixture was kept for 2 h at +4 ^{0}C, then 25 g of tryptophan was added added portionwise. The reaction mixture was kept for 3 days in the dark at 22 ^{0}C, the solvent was removed at 10 mm Hg., neutralized with 30% (v/v) NH_4OH to pH = 5,9, and cooled for 1 day at 4 ^{0}C. The crystalline product was filtered, washed with ^2H_2O and dried at 10 mm Hg. Yield, 19 g (60,3%); T_m = 283–285; $[\alpha]_d^{25}$ = 2,8 (1 M HCl); pK_a = 2,46 (COOH), 9,41 (NH_2); R_f = 0,5 (A). UV-spectrum (0,1 M HCl) λ_{max} nm [(ε M^{-1} $см^{-1}$)]: 218 (ε 3350), 278 (ε 5550), 287.5 (ε 4550). ^{1}H-NMR spectrum (CF_3COOH in ^2H_2O) (δ, ppm): δ 3,4 (2H, Hβ), δ 4,4 (1H, Hα), δ 7,3 (1H, Hε), δ 7,2–7,4 (0,1H, In-H), 98 atom.% ^{2}H. EI mass spectrum $[M]^+$ *m/z* (*I*, %): 204(28), methyl easter of N-Dns-[2,4,5,6,7-$^2H_5$]Trp: 455(9), 456(11).

Synthesis of N-Dns-[^{2}H]amino asids

To 4 mg of the dry hydrolyzate BR 1 ml of 2 M $NaHCO_3$ (pH = 9–10) was added portionwise with stirring 25,6 mg of Dns-chloride in 2 ml of acetone. The reaction mixture was kept for 1 h under stirring at +40 ^{0}C, acidified with 2 N HCl to pH = 3,0 and extracted with ethyl acetate (3 times × 5 ml). The combined extract was washed with distilled ^2H_2O to pH = 7,0 (2 times × 2 ml), dried over anhydrous Na_2SO_4. The solvent was removed at 10 mm Hg. Yield, 15,3 mg (78%).

Synthesis of methyl esters of N-Dns-[²H]amino acids

3 g of wet N-nitroso-N-methylurea was added to 20 ml of 40% (v/v) KOH in 40 ml of diethyl ether and then stirred on a water bath with ice for 15–20 min for obtaining diazomethane. After the completion of gas release, the ether layer was separated, washed with distilled water to pH = 7,0, dried with anhydrous Na_2SO_4, and used for the treatment of N-Dns-amino acids. Yield, 17,4 mg (69%).

Biosynthesis of BR

The bacterial growth was carried out on a synthetic medium (SM) containing (g/l): *D,L*-alanine – 0,43; *L*-arginine – 0,4; *D,L*-aspartic acid – 0,45; *L*-cysteine – 0,05; *L*-glutamic acid – 1,3; *L*-lycine – 0,06; *D,L*-histidine – 0,3; *D,L*-isoleucine – 0,44; *L*-leucine – 0,8; *L*-lysine – 0,85; *D,L*-methionine – 0,37; *D,L*-phenylalanine – 0,26; *L*-proline – 0,05; *D,L*-serine – 0,61; *D,L*-threonine – 0,5; *L*-tyrosine – 0,2; *D,L*-tryptophan – 0,5; *D,L*-valine – 1,0; AMP – 0,1; UMP – 0,1; NaCl – 250; $MgSO_4 \cdot 7H_2O$ – 20; KCl – 2; NH_4Cl – 0,5; KNO_3 – 0,1; KH_2PO_4 – 0,05; K_2HPO_4 – 0,05; Na^+-citrate – 0,5; $MnSO_4 \cdot 2H_2O$ – $3 \cdot 10^{-4}$; $CaCl_2 \cdot 6H_2O$ – 0.065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $FeSO_4 \cdot 7H_2O$ – $5 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$; Na-citrate – 0,5; glycerol – 1,0; biotin – $1 \cdot 10^{-4}$; folic acid – $1,5 \cdot 10^{-4}$; vitamin B_{12} – $2 \cdot 10^{-5}$. The growth medium was autoclaved for 30 min at 0,5 atm, the pH value was adjusted to 6,5–6,7 with 0,5 M KOH. The bacterial growth was performed in 500 ml Erlenmeyer flasks (volume of the reaction mixture 100 ml) for 4–5 days at t = +35 ^{0}C on a shaker ("Birad Labs", Hungary) under intense aeration and monochromatic illumination by light fluorescent lamps LDS-40-2 (40 W) ("Alfa-Electro", Russia) (3 lamps × 1,5 lx) [12]. Bacterial growth was studied by optical density of the bacterial suspension measured at a wavelength λ = 620 nm on a spectrophotometer Beckman DU-6 ("Beckman Coulter", USA). All further manipulations with BR were carried out with the use of a photomask lamp equipped with an orange light filter PCM -1X (75×50 cm) ("Marbel", Germany).

Isolation of purple membranes (PM)

The row biomass (1 g) was washed with distilled water and pelleted by centrifugation on a T-24 centrifuge ("Carl Zeiss", Germany) (1500 g, 20 min). The precipitate was suspended in 100 ml of dist. H_2O and kept for ~3 h at +4 ^{0}C. The reaction mixture was centrifuged (1500 g, 15 min), the pellet was re-suspended in 20 ml dist. H_2O and disintegrated by infrasound sonication (22 kHz, 1 min) in an ice bath (0 ^{0}C). The cell homogenate after washing with dist. H_2O was re-suspended in 10 ml of a buffer containing 125 mM NaCl, 20 mM $MgCl_2$, and 4 mM Tris-HCl (pH = 8,0), then 5 mg of RNA-ase (2–3 units of activity) was added. The mixture was incubated in the dark for ~2 h at t = +37 ^{0}C. Then 10 ml of the same buffer was added and kept for 10–12 h at +4 ^{0}C. The aqueous fraction was separated by centrifugation on T-24 centrifuge ("Carl Zeiss", Germany) (1500 g, 20 min), the PM precipitate was treated with 50% (v/v) EtOH (5 times × 5 ml) at +4 ^{0}C followed by separation of the solvent. This procedure was repeated 5 times to give colorless washings. The protein content in the samples was determined spectrophotometrically on a Beckman DU-6 VIS/UV-spectrophotometer ("Beckman Coulter", USA) by the ratio D_{280}/D_{568} ($\varepsilon_{280} = 1,1 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$; $\varepsilon_{568} = 6,3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [13]. Regeneration of PM was performed as described in [14]. The output of PM fraction makes up 120 mg (80–85%).

Isolation of BR

Fraction of the PM (in H_2O) (1 mg/ml) was dissolved in 10 ml of 0,5% (w/v) SDS-Na, and incubated for ~5–7 h at t = +37 ^{0}C followed by centrifugation (1200 g, 15 min). The precipitate was separated, after that MeOH was added to the supernatant in divided portions (3 times × 2 ml) at 0 ^{0}C. The reaction mixture was kept for ~14–15 h in ice bath at t = +4 ^{0}C and then centrifuged (1200 g, 15 min). The fractionation procedure was performed three times, reducing the concentration of 0,5% SDS-Na to 0,2 and 0,1% (w/v). The crystal protein (output, 8–10 mg) was washed with cold distilled ^2H_2O (2 times × 1 ml) and centrifuged (1200 g, 15 min).

Purification of BR

A protein sample (5 mg) was dissolved in 10 ml of buffer solution and placed on a calibrated chromatography column (150×10 mm) with stationary phase – Sephadex G-200 ("Pharmasia", USA) (specific volume packed beads – 30–40 units per 1 g dry Sephadex), and equilibrated with buffer containing 0,1% (w/v) SDS-Na and 2,5 mM ETDA. The device was e***quiped with Waters*** 2487 dual absorbance (***UV/VIS***) d***etector with the w***avelength range at λ = 190-700 nm. Elution proceeded by 1 mM Tris-HCl buffer, pH = 7,4 at a flow rate 10 ml/cm^{2}·h. The combined protein fraction was subjected to freeze-drying, sealed in glass ampoules (10×50 mm) and stored in frost camera at t = -10 ^{0}C.

Electrophoresis of BR

The procedure was performed in 12,5% (w/v) polyacrylamide gel (PAAG) containing 0,1% (w/v) SDS. The samples were prepared for electrophoresis by standard procedures (LKB protocol, Sweden). The electrophoretic gel stained with Coomassie blue R-250 was scanned on a CDS-200 laser densitometer ("Beckman", USA) for quantitative analysis of the protein level.

Hydrolysis of BR

For hydrolysis of BR 4 g of dry protein was placed into glass ampoules (10×50 mm), and 5 ml of 4 N $Ba(OH)_2$ was added. The mixture was kept at +110 ^{0}C for 24 h. The reaction mixture was suspended with 5 ml of hot distilled water and neutralized with 2 N H_2SO_4 to pH = 7,0. The precipitate of $BaSO_4$ was removed by centrifugation at 200 g for 10 min, and the supernatant was evaporated in a rotor evaporator at +40 °C.

RP HPLC

RP HPLC was carried out on a liquid chromatograph Knauer Smartline ("Knauer", Germany) equipped with a pump Gilson ("Gilson Inc.", USA), UF-2563 detector and integrator CR-3A ("Shimadzy", Japan) using a column (250×10 mm) with a stationary reverse phase Separon C18 ("Kova", Slovakia), eluent: (A) – acetonitrile-trifluoroacetic acid 100:0,1–0,5 % (v/v) and (B) – acetonitrile – 100 % (v/v); elution rate – 1,5 ml/min; gradient: from 0 to 20 % B – 5 min, 20 to 100 % B – 30 min, 100 % B – 5 minutes, 100 % B to 0 – 2 min 0 % B – 10 min.

EI mass spectrometry

EI mass spectra of methyl esters of N-Dns-amino acid derivatives were obtained by electron impact on MB-80A device ("Hitachi", Japan) at energy of ionizing electrons 70 eV, accelerating voltage of 8 kV and the cathode temperature +180–(+200) ^{0}C. Scanning was carried out at a resolution of 7500 relative units using 10 % sharpness.

Proton translocation

The proton translocation was recorded in visicles with a gel-filled pH electrode (Sensorex No. SG900C) combined with PHM85 Radiometer pH meter. The data were collected (0,8 s per time interval) and analyzed using Lotus Measure (Lotus Development). Initial proton pumping rates were determined over 10 s interval. Changes in pH were calibrated using 1μm of 10 mM HCl.

Preparation of apomembranes (AP)

50 mg of PM was suspended in 50 ml of 1 M NH_2OH (pH = 6,0). The reaxion mixure was kept for 10 h with stirring in ice bath (+4 ^{0}C) under illumination with a xenon lamp. The precipitate was separated by centrifugation (1000 g, 10 min), washed twice with distilled water and centrifuged. AP fraction was resuspended in 2 ml of 5 mM 2-(N-morpholino)-ethane sulfonyl amide in 100 mM NaCl.

Regeneration of AP with 13-trans-retinal

To 2 ml suspension of AP ($2 \cdot 10^{-5}$ mol) in a quartz cuvette was added with stirring 0.1 ml of 2 mM solution of 13-*trans*-retinal in methanol and kept for 6–8 h in the dark at t = +40 ^{0}C. The degree of regeneration of PM was determined by spectrophotometry on CDS-200 laser

densitometer ("Beckman", USA) by the ratio: $D_{nat.280} \cdot D_{nat..568} / D_{reg..280} \cdot D_{reg.568}$, where D_{280} and D_{568} – the absorbance of a suspension of native and regenerated PM at λ = 280 and λ = 568 nm.

Results and discussion

The strategy for biosynthesis of ^2H-labeled BR using a strain of extreme photo-organotrophic halobacterium *Halobacterium halobium ET 1001* was determined by the study of the principal possibility for obtaining ^2H-labeled photochrome transmembrane proteins in semi-micropreparative quantities for reconstruction of artificial membranes in ^2H_2O and BR-containing nanofilms. [2,3,4,5,6-$^2H_5$]phenyIalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan play important role in hydrophobic interacting of BR molecule with the lipid bilayer of the cell membrane. They are resistant to the isotopic exchange (H-^2H) reactions in aqueous solutions under growth conditions and may be easily detected by EI spectrometry after the chemical derivatization to the methyl esters of N-Dns-amino acids. ^2H-labeled BR obtained by the growth of halobacterium *H. halobium ET 1001* on synthetic medium (4,3 M NaCl) with [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan. Under optimal growing conditions (incubation period 4–5 days, temperature +35 ^{0}C, illumination with monochromatic light at λ = 560 nm) in cells is synthesized the purple carotenoid pigment, the spectral ratio of protein and chromophore molecule fragments D_{280}/D_{568} = 1,5 : 1,0 in which is identical to the natural BR. The growth of the halobacterium on a synthetic medium (Fig. 5*b*) was practically as the same as in the control (Fig. 5*a*) on the protonated growth medium, that significantly simplifies the optimization of conditions for the biosynthesis of ^2H-labeled BR, which consists in the equivalent replacing of protonated aromatic amino by their deuterated analogues – [2,3,4,5,6-$^2H_5$]phenylalanine (0,26 g/l), [3,5-$^2H_5$]tyrosine (0,2 g/l) and [2,4,5,6,7-$^2H_5$]tryptophan (0,5 g/l).

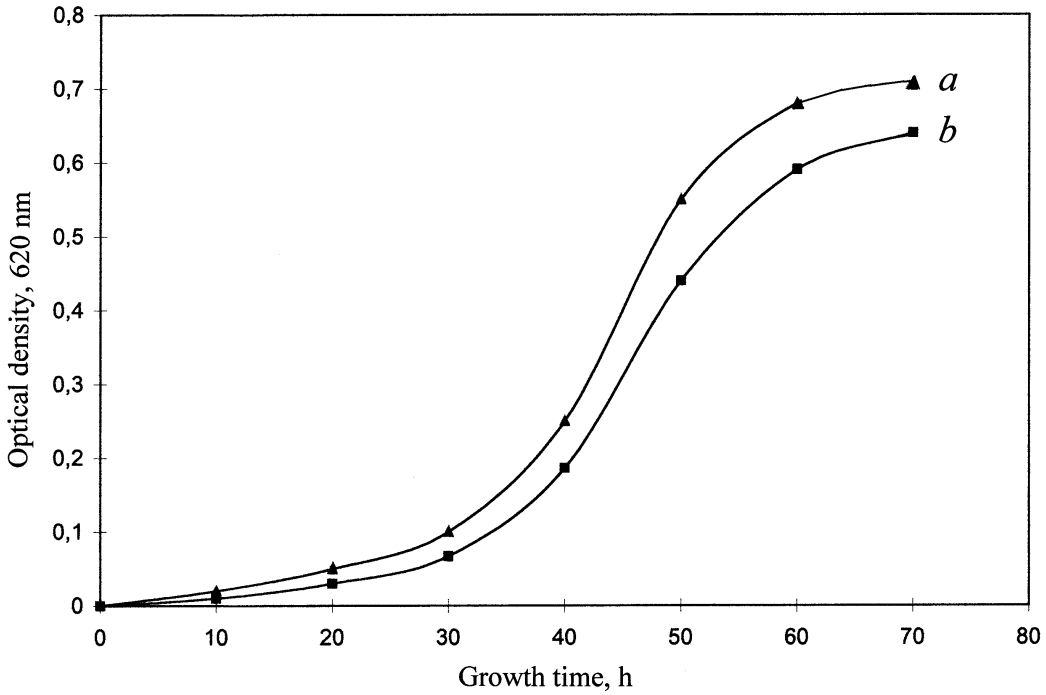


Figure 5: Growth dynamics of *H. halobium ET 1001* under various experimental conditions: (*a*) – protonated synthetic medium; (*b*) – synthetic medium with [2,3,4,5,6-$^2H_5$]Phe (0,26 g/l), [3,5-$^2H_2$]Tyr (0,2 g/l) and [2,4,5,6,7-$^2H_5$]Trp (0,5 g/l). The incubation period: 4–5 days, temperature: t = +35 ^{0}C, illumination under monochrome light at λ = 560 nm.

The main stages of the experiment were: growing of a strain-producer *H. halobium ET 1001* on synthetic medium with [2,3,4,5,6-$^2H_5$] phenylalanine (0,26 g/l), [3,5-$^2H_2$] tyrosine (0,2 g/l) and [2,4,5,6,7-$^2H_5$] tryptophan (0,5 g/l), the separation of cell content, isolation of purple membrane fraction (PM), the separation of low-and high-molecular impurities, cellular RNA, pigments (preferably carotenoids) and lipids, fractionation of solubilized in 0,5 % (w/v) SDS-Na protein by

methanol, purification on Sephadex G-200, electrophoresis 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na. Because protein is localized in the PM, the release of low molecular weight impurities and intracellular contents was reached by osmotic shock of cells with distilled water in the cold after the removal of 4,3 M NaCl and the subsequent destruction of the cell membrane by ultrasound at 22 kHz. For the destruction of cellular RNA the cellular homogenate was treated with Rnase I. The fraction of PM along with the desired protein in a complex with lipids and polysaccharides also contained impurity of related carotenoids and proteins. Therefore, it was necessary to use special methods of fractionation of the protein without damaging its native structure and dissociation. That required applying the special methods of purification of carotenoids and lipids, and the subsequent gel permeation column chromatography on Sephadex G-200. The removing of carotenoids, consisting in repeated treatment of the PM with 50 % (v/v) EtOH at +4 ^{0}C, was a routine but necessary step, in spite of the significant loss of chromoprotein. It was used five treatments by 50 % (v/v) EtOH ethanol to obtain the absorption VIS/UV-spectrum of purified from carotenoids PM suspension (4) and (5) (the degree of chromatographic purity, 80–85 %), as shown in Figure 6 at various processing stages (*b*) and (*c*) relative to the native BR (*a*). The formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption VIS/UV-spectrum of the PM (Fig. 6*c*) – the main bandwith (1) with the absorption maximum at λ = 568 nm caused by the light isomerization of the chromophore by the C13=C14 bond, is determined by the presence of 13-*trans*-retinal residue in BR_{568}; the additional low-intensity bandwith (2) at λ = 412 nm characterizes a minor impurity of a spectral form of *meta*-bacteriorhodopsin M_{412} (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwith (3) with λ = 280 nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for native BR, D_{280}/D_{568} = 1,5 : 1,0).

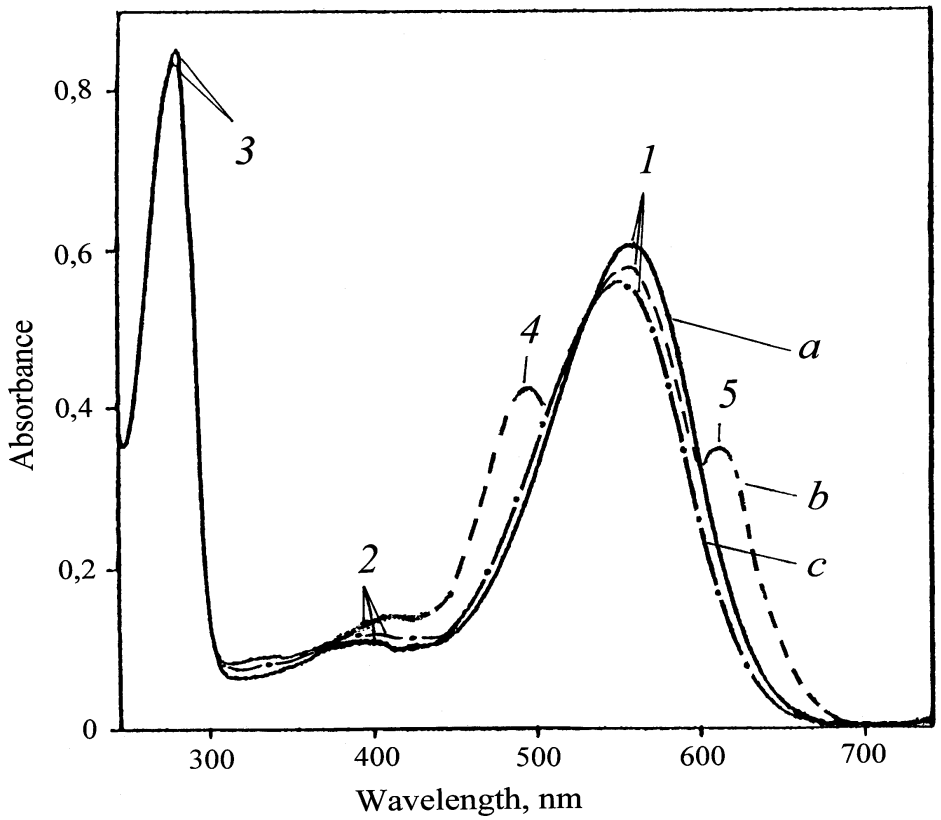


Figure 6: The VIS/UV-absorption spectra of the PM (50 % (v/v) EtOH) at various stages of processing: (*a*) – natural BR; (*b*) – PM after intermediate treatment; (*c*) – PM purified from carotenoids. The bandwith (1) is the spectral form of BR_{568}, (2) – impurity of spectral form of *meta*-bacteriorhodopsin M_{412}, (3) – the total absorption bandwith of aromatic amino acids, (4) and (5) – extraneous carotenoids. As a control was used the native BR

The fractionation and chromatographic purification of the protein was a further necessary next step of the BR purification. For obtaining protein from a biological material in purified, homogeneous state are being used various detergents, assisted to the cleavage of protein-lipid complexes and the rupture of protein-protein bonds. In particular, for the better release of proteins (enzymes) which are firmly connected with biomembranes or other subcellular structures used Triton X-100, sodium dodecyl sulfate (SDS) and sodium deoxycholate as detergents.

As the BR, being an integral membrane protein intricately penetrates bilipid layer in form of seven α-helices, the use of ammonium sulfate and other conventional agents to salting out did not give a positive result for isolation of the protein. The resolving was in the translation of the protein to a soluble form by the colloidal dissolution (solubilization) in appropriate ionic detergent. Using as the ionic detergent SDS-Na was dictated by the need of carrying out the more accomplished solubilization of the protein in a native, biologically active form in complex with 13-*trans*-retinal, because BR solubilized in 0,5 % (w/v) SDS-Na retains a native α-helical configuration [15]. However SDS-Na is a strong detergent and its usage for protein fractionation is justified by a limited range of concentration (from 0,1 to 0,5 % (w/v)). In addition SDS-Na seems to be more effective for lipid removal that other conventional detergents. Therefore, there was no need the use organic solvents as acetone, methanol and chloroform for purification of phospholipids, while precipitation and delipidization stages being combined in one single step, which significantly simplifies the further fractionation of the protein and reduces its losses during the precipitation procedure. A significant advantage of this method is that the isolated protein in complex with phospholipids and detergent was distributed in the supernatant, while other high molecular weight impurities – in the precipitate, which can be easily separated by centrifugation. Fractionation of the solubilized in 0,5 % (w/v) SDS-Na protein and its subsequent isolation in crystalline form was performed at 0 ^{0}C in three steps precipitating procedure with MeOH, slowly reducing the concentration of SDS-Na from 0,5; 0,25 and 0,1 % (w/v). The final stage of the BR purification involved the separation of the protein from low-molecular-weight impurities by GPC. For this purpose the fractions containing BR were passed twice through a chromatography column with dextran Sephadex G-200 balanced with buffer (pH = 8,35) containing 0,1 % (w/v) SDS-Na and 2,5 mM EDTA. The data on purification of the BR are shown in Table 1.

Table 1: The data on purification stages of the BR by various methods

Sample	mol PM/mol BR	Phospholipid removal, %	BR yield*, %
PM	15.5	–	–
PM washed with EtOH			
1 wash	4.9	65	93
2 wash	4.1	70	90
3 wash	3.5	76	88
4 wash	3.2	81	84
5 wash	2.8	84	80
BR crystallized from MeOH	1.9	86	75
BR from GPC on Sephadex G-200	1.2	92	86

* Note:
Percentage yield relative to the BR solubilized in SDS-Na before the purification procedure

As was shown in Table 1, 88% of phospholipids was removed by five washes (65 and 76 % was removed by 1st, 2nd and 3nd wash respectively). When the sample was solubilized in 0,1 % (w/v) SDS-Na and applied to the GPC on Sephadex G-200, a chromatogram shown in Figure 6 was obtained. The total endogenous phospolipid removal on the BR peak was 92 % relative to the native PM. The absorbance profile shows the separated protein. For smaller BR loads (0.1 mg), the analytical column (size) worked equally well.

The homogeneity of the isolated BR satisfies to the requirements for reconstruction of native membranes, and was confirmed by electrophoresis in 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na and *in vitro* regeneration of AP with 13-*trans*-retinal. The degree of regeneration of PM was determined by the ratio: $D_{nat.280} \cdot D_{nat.568} / D_{reg.280} \cdot D_{reg.568}$ (D_{280} and D_{568} – the absorbance of a suspension of native and regenerated PM at $\lambda = 280$ and $\lambda = 568$ nm) was 65 mol.%. The output of the crystalline protein makes up approximately 5 mg. The isolated protein was washed with cold dist. ^2H_2O, centrifuged (1200 g, 15 min), subjected to freeze-drying, sealed into glass ampoules and stored in frost camera at t = -10 ^{0}C.

The conditions of further hydrolysis of ^{2}H-labeled protein were determined to prevent the isotopic (H–^{2}H) exchange of hydrogen by deuterium in the molecules of aromatic amino acids, as well as to retain tryptophan in the protein hydrolysate. Therefore, we considered two alternatives variants - acid and alkaline hydrolysis. Acid hydrolysis of the protein in standard conditions (6 N HCl or 8 N H_2SO_4, t = +110 ^{0}C, 24 h) is known to induce complete degradation of tryptophan and partial degradation of serine, threonine, and several other amino acids in the protein [16], which do not play a significant role for this study. Modification of this method consists in adding to the reaction mixture of phenol, thioglycolic acid, β-mercaptoethanol, can save up to 80–85 % tryptophan [17]. Using of p-toluenesulfonic acid with 0,2 % (v/v) 3-(2-aminoethyl) indole or 3 M mercapto sulfoacid is also effective to maintain a tryptophan (up to 93 %) [18]. However, these methods are possess an essential disadvantage, because during acid hydrolysis at high speed occurs isotopic (H–^{2}H) exchange of aromatic protons (deuterons) in molecules of tryptophan, tyrosine and histidine as well as protons at C3 position of aspartic and C4 glutamic acid [19]. Therefore, even carrying out of hydrolysis in deuterated reagents (6N ^{2}HCl, 4N $^2H_2SO_4$ in ^2H_2O) does not derive the real data about the inclusion of the deuterium into the protein.

Under conditions of alkaline hydrolysis (4N $Ba(OH)_2$ or 4N NaOH, t = +110 ^{0}C, 24 h) the reactions of isotopic (H–^{2}H) exchange were almost not occurred (except for a proton (deuteron) at C2 atom of histidine, and tryptophan not destroyed. These factors determined the choice of this method of hydrolysis in our research. Simplification of the procedures for the allocation of amino acid mixture from protein hydrolizate due to the neutralization of H_2SO_4 (in ^2H_2O) was the reason for choosing as hydrolysing agent 4N $Ba(OH)_2$. The possible *D,L*-amino acid racemization by alkaline hydrolysis did not affect the result of the subsequent mass spectrometric study of the level of deuteration of amino acid molecules.

To study the deuterium labelling of BR molecule the EI mass spectrometry was used after modification of amino acid mixture of the BR hydrolyzate into methyl esters of N-Dns-[^{2}H]amino acids. In order to obtain reproducible results on the deuteration of ^{2}H-labeled protein first was recorded a total scan EI mass spectrum of the mixture of methyl esters of N-Dns-[^{2}H]amino acids derived from the hydrolyzate BR. The level of deuteration was calculated from the peak of the molecular ion $[M]^+$ of amino acid derivatives relative to the control, obtained in a protonated medium. Then, the separation of methyl esters of N-Dns-[^{2}H] aromatic amino acids was performed by by RP HPLC to record EI mass spectra for each individual amino acid derivates.

The El mass spectrum of the mixture of methyl esters of N-DN-amino acids as shown in Figure 7 (scanning at m/z = 50–640, the base peak at m/z = 527, 100 %), is characterized by continuity: the peaks in the range at m/z = 50–400 on the scale of the mass numbers are fragments of metastable ions, low molecular weight impurities, as well as products of chemically modified amino acids. The analyzed [^{2}H]-labeled aromatic amino acids occupying scale mass numbers at m/z = 415–456, are represented by mixtures of deuterated molecules containing various numbers of deuterium atoms. Therefore, the molecular ions $[M]^+$ were polymorphously split into individual clusters displaying a statistical set of m/z values depending on a number of hydrogen atoms in the molecule. Taking into account the effect of isotopic polymorphism, the level of deuterium enrichment in [^{2}H]amino acid molecules was determined using the most commonly encountered peak of the molecular ion $[M]^+$ in each cluster recorded by mass-spectrograph with mathematically averaged value of $[M]^+$ (Fig. 7). As is shown in Figure 7 the molecular ion peak of phenylalanine was determined by $[M]^+$ at m/z = 417, 14 % (instead of the $[M]^+$ at m/z = 412, 20 % for non-labeled derivative (unlabeled peaks of amino acid derivatives are not shown)), tyrosine – $[M]^+$ at m/z = 429, 15 % (instead of $[M]^+$ at m/z = 428, 13 %), tryptophan – $[M]^+$ at m/z = 456, 11 % (instead of $[M]^+$ at m/z = 451, 17 %). The level of deuterium enrichment of aromatic amino acid molecules

corresponding to the increase of molecular weight was for [^{2}H]tyrosine – 1 (90 atom.% ^{2}H), [^{2}H]phenylalanine – 5 (95 atom.%, ^{2}H) and [^{2}H]tryptophan – 5 (98 atom.% ^{2}H) deuterium atoms. This result coincides with the data on the initial level of deuterium enrichment of aromatic amino acids – [3,5-$^2H_2$]Tyr, [2,3,4,5,6-$^2H_5$]Phe and [2,4,5,6,7-$^2H_5$]Trp, added to the growth medium and indicates a high selectivity of inclusion of aromatic [^{2}H]amino acids into the BR molecule. Deuterium label was detected in all residues of aromatic amino acids (Table 2). However, the presence in the EI mass spectrum peaks $[M]^+$ of semi-deuterated phenylalanine analogues with $[M]^+$ at m/z = 413–418, tyrosine – with $[M]^+$ at m/z = 428–430 and tryptophan – with $[M]^+$ at m/z = 453–457 with different levels of contributions to the deuterium enrichment of molecules testifies about conservation of the minor pathways of biosynthesis of aromatic amino acids de novo, resulting in the dilution of the deuterium label, that evidently is determined by the conditions of biosynthesis of ^{2}H-labeled BR (Table 2).

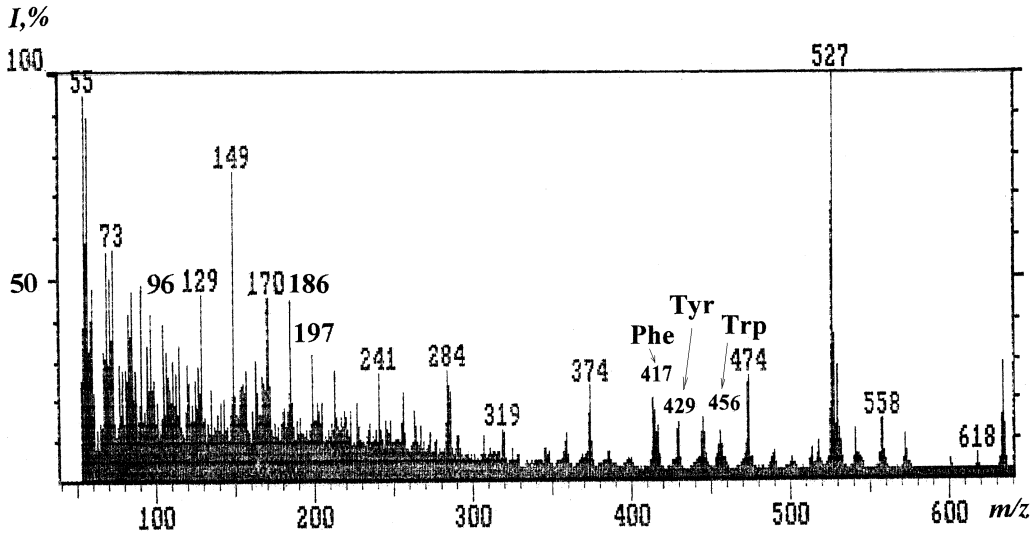


Figure 7: Full scan EI mass spectrum of methyl ester of N-Dns-[^{2}H]derivatives of aromatic amino acids isolated from the BR hydrolyzate, obtained on synthetic medium with [2,3,4,5,6-$^2H_5$]Phe (0,26 g/l), [3,5-$^2H_2$]Tyr (0,2 g/l) and [2,4,5,6,7-$^2H_5$]Trp (0,5 g/l). (energy: 70 eV, accelerating voltage: 8 kV, temperature: 180–200 ^{0}C). Hydrolysis conditions: 4 N $Ba(OH)_2$ (in ^2H_2O), +110 ^{0}C, 24 h. Molecular ion peaks represented by the symbols of amino acids correspond to their derivatives; *I* – relative intensity (%).

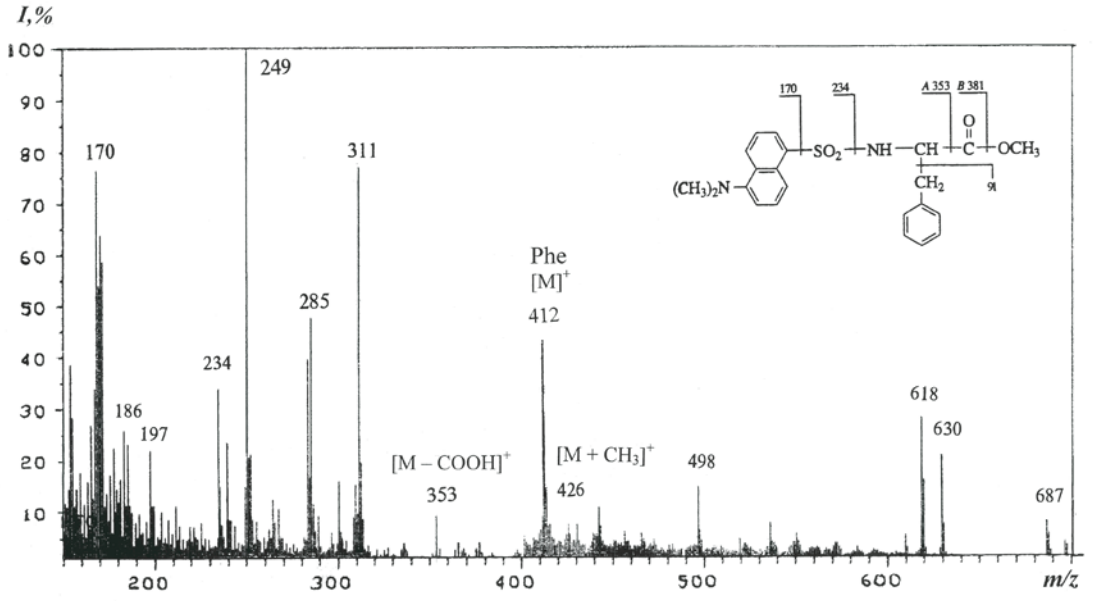
Table 2: The values of the molecular ion peaks $[M]^+$ in the EI mass spectrum of methyl esters of N-Dns-[2,3,4,5,6-$^2H_5$]Phe, N-Dns-[3,5-$^2H_2$]Tyr and N-Dns-[2,4,5,6,7-$^2H_5$]Trp and levels of their deuterium enrichment

Compound	Value of $[M]^+$	Intensity, %	The total number of hydrogen atoms*	Level of deuterium enrichment, % of the total number of hydrogen atoms **
N-Dns-[2,3,4,5,6-$^2H_5$]Phe-OMe	413	7	1	13
	414	18	2	25
	415	15	3	38
	416	11	4	50
	417	14	5	63
	418	6	6	75
N-Dns-[3,5-$^2H_2$]Tyr-OMe	428	12	–	–
	429	15	1	14
	430	5	2	29

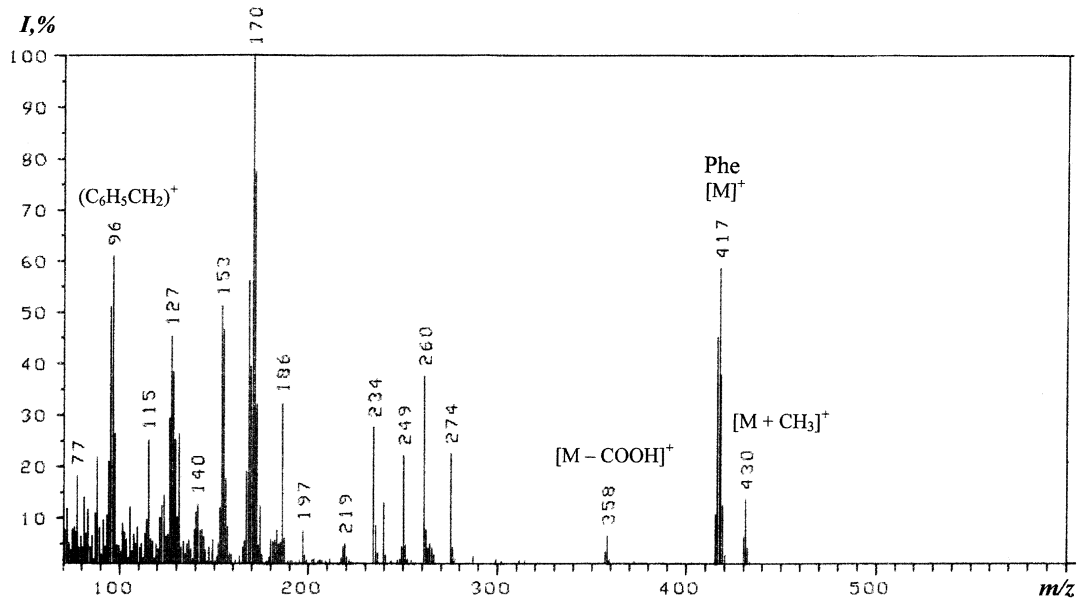
N-Dns-[2,4,5,6,7-$^2H_5$]Trp-OMe	453	5	2	26
	454	6	3	38
	455	9	4	50
	456	11	5	64
	457	5	6	77

Notes:
* A dash means no incorporation of deuterium.
** In calculating the level of deuterium enrichment protons(deuterons) at carboxyl and NH_2-amino groups of amino acids were not considered due to the easily isotopic (H-^{2}H) exchange.

According to the mass spectrometric analysis the molecular ion peaks $[M]^+$ of methyl esters of N-Dns-[^{2}H] derivatives of aromatic amino acids have a low intensity in EI mass spectra and were polymorphously split, so the areas of of the molecular enrichment were strongly broadened. Moreover, mass spectra of the mixture components are additive, so the mixture can be analyzed only if the spectra of the various components are recorded in the same conditions. These calculations provide for the solution of the system of *n* equations in *n* unknowns for a mixture of *n* components. For components, the concentration of which exceeds 10 mol.%, the accuracy and reproducibility of the analysis makes up ± 0,5 mol.% (at 90% confidence probability). Therefore, to obtain reproducible results it was necessary to chromatographically isolate individual derivatives of [^{2}H] amino acids from the protein hydrolyzate. For this aim was used RP HPLC on octadecylsilane silica gel Silasorb C18, the effectiveness of which was confirmed earlier by separation of a mixture of methyl esters of N-Dns-derivatives [^{2}H]amino acids from other microbial objects as methylotrophic bacteria and microalgae [20].



a)



b)

Figure 8: EI mass spectra of methyl ester N-Dns-[2,3,4,5,6-$^2H_5$]Phe under various experimental conditions: (*a*) – the unlabeled methyl ester of N-Dns-Phe; (*b*) – methyl ester of N-Dns-[2, 3,4,5,6-$^2H_5$]Phe, isolated from the BR hydrolyzate by RP HPLC. Separation conditions: 250×10 mm Column C18 ("Kova", Slovakiya), eluents: (A) – CH_3CN–CF_3COOH (100:0,1–0,5 % (v/v)) and (B) – CH_3CN (100 % (v/v)).

The method was adapted to the conditions of chromatographic separation of a mixture of methyl esters of N-Dns-[^{2}H]amino derivatives of the BR hydrolyzate comprising in optimization of eluent ratios, the gradient type, and the rate of gradient elution from the column. The best separation was achieved by gradient elution with a solvent mixture CH_3CN–CF_3COOH (100:0,1–0,5 % (v/v)). It was possible to isolate the tryptophan and inseparable pair of phenylalanine/tyrosine. The levels of chromatographic purity of methyl esters of N-Dns-[2,3,4,5,6-$^2H_5$]phenylalanine, N-Dns-[3,5-$^2H_2$]tyrosine and N-Dns-[2, 4,5,6,7-$^2H_5$]tryptophan isolated from the protein hydrolysate were 89, 91 and 90 %, respectively with outputs 97–85 %. The result confirmed Figure 8*b* which shows the EI mass spectrum of methyl N-Dns-[2,3,4,5,6-$^2H_5$]phenylalanine isolated from the BR hydrolysate by RP HPLC (scanning at m/z = 70–600, the base peak at m/z = 170, 100 %) (the EI mass spectrum is shown relative to the unlabeled methyl ester of N-Dns-phenylalanine (Fig. 8*a*), scanning at m/z = 150–700, the base peak at m/z = 250, 100 %). The proof for the inclusion of 5 deuterium atoms into the phenylalanine molecule is the presence of "heavy" molecular ion peak of methyl ester of N-Dns-[^{2}H]phenylalanine ($[M]^+$ at m/z = 417, 59 %, instead of $[M]^+$ at m/z = 412, 44 % for non-labeled derivative of phenylalanine) and the deuterium-enriched additional peaks of the benzyl $C_6H_5CH_2$ fragment of [^{2}H]phenylalanine molecule detected at m/z = 96, 61 % (instead of m/z = 91, 55 % in the control (not shown)) (Fig. 8*b*). The peaks of secondary fragments of varying intensity values detected at m/z = 249, 234 and 170 are the secondary decomposition products of the dansyl residue to the 5-(dimetilamino)naphthalene, the low intensity peak $[M - COOCH_3]^+$ detected at m/z = 358, 7 % (instead at m/z = 353, 10 % in the control) is a product of cleavage of a carboxymethyl ($COOCH_3$) group of the methyl ester of N-Dns-[^{2}H]phenylalanine, and a peak $[M + CH_3]^+$ at m/z = 430, 15 % (instead of m/z = 426, 8 % in the control) – the product of further methylation by α-amino group of the [^{2}H]phenylalanine (Fig. 8*b*). According to the EI mass spectrometry data, the difference between the molecular weight of the protonated and deuterated species of peaks $[M]^+$ of methyl ester of N-Dns-phenylalanine makes up 5 units that corresponds with the primary data on the deuterium enrichment level of [2,3,4,5,6-$^2H_5$]phenylalanine added to the growth medium. Mass spectrometric data on deuterium enrichment levels on [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan are also in good correlation and confirmed by ^{1}H NMR.

Conclusions

The experimental data indicate the high efficiency of the incorporation of H-labeled aromatic amino acids into the BR molecule with output of the BR 8–10 mg per 1 g of biomass. The main advantage of this method is that the isolated BR keeps its native configuration in combination with 13-*trans*-retinal, and the ability to photochemical reactions in vitro, as demonstrated by electrophoresis in 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na. The method is also applicable for the preparation of other similar to BR transmembrane proteins of halophilic bacteria – sensory rhodopsin and halorhodopsin. The unique properties of natural bacteriorhodopsins provide a wide range of bio- and nano-technological applications in which may find their application ^{2}H-labeled analogues. In the future we plan obtaining fully deuterated analogs of BR for the reconstruction of functionally active systems of transmembrane proteins in heavy water with purified ^{2}H-labeled fatty acids and other biologically active compounds. These studies will provide an answer to the question of how functions the BR in the native membranes in condition of the complete replacement of protons by deuterium.

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Включение дейтерированных ароматических аминокислот – [2,3,4,5,6-$^2H_5$]фенилаланина, [3,5-$^2H_2$]тирозина и [2,4,5,6,7-$^2H_5$]триптофана в молекулу фотохромного трансмембранного белка бактериородопсина

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Аннотация

Осуществлено включение функционально важных дейтерированных ароматических аминокислот – [2,3,4,5,6-$^2H_5$]фенилаланина, [3,5-$^2H_2$]тирозина и [2,4,5,6,7-$^2H_5$]триптофана в молекулу фотопреобразующего белка бактериородопсина, синтезируемого фотоорганотрофной галобактерией *Halobacterium halobium ET 1001*. Дейтерированный белок (выход 8–10 мг) выделен из пурпурных мембран лизисом клеток дистиллированной водой, обработкой клеточной биомассы ультразвуком при 22 кГц, спиртовой экстракцией низко- и высокомолекулярных примесей, клеточной РНК, каротиноидов и липидов, с последующей солюбилизацией конечного продукта в 0,5 % ДДС-Na и низкотемпературным фракционированием метанолом, гель-проникающей хроматографией на сефадексе G-200, ОФ ВЭЖХ и масс-спектрометрией ЭУ метиловых эфиров N-Dns-[^{2}H]производных аминокислот. Дейтериквая метка детектировалась во всех

аминокислотных остатках белка. Однако, присутствие в масс спектре ЭУ гидролизата БР пиков $[M]^+$ полудейтерированных аналогов ароматических аминокислот – фенилаланина с $[M]^+$ при m/z = 413–418, тирозина – с $[M]^+$ при m/z = 428–430 и триптофана – с $[M]^+$ при m/z = 453–457 с различными вкладами в уровень дейтерированности молекул, свидетельствует о сохранении незначительных путей биосинтеза ароматических аминокислот de novo.

Ключевые слова: *Halobacterium halobium ET 1001*, бактериородопсин, [2,3,4,5,6-$^2H_5$]Phe, [3,5-$^2H_2$]Tyr, [2,4,5,6,7-$^2H_5$]Trp, биосинтез; масс-спектрометрия ЭУ, ОФ ВЭЖХ.