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Optimization of Solubilization and Purification Procedures for the Hydroxylase Component of Membrane-Bound Methane Monooxygenase from *Methylococcus capsulatus* strain M

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Abstract—The hydroxylase component of membrane-bound (particulate) methane monooxygenase (pMMO) from *Methylococcus capsulatus* strain M was isolated and purified to homogeneity. The pMMO molecule comprises three subunits of molecular masses 47, 26, and 23 kD and contains three copper atoms and one iron atom. In solution the protein exists as a stable oligomer of 660 kD with possible subunit composition $(\alpha\beta\gamma)_6$. Mass spectroscopy shows high homology of the purified protein with methane monooxygenase from *Methylococcus capsulatus* strain Bath. Pilot screening of crystallization conditions has been carried out.

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Key words: particulate methane monooxygenase, isolation, purification, activity assay

The importance of methane oxidation is directly linked to the possibility of using methane as an energy source because the C–H bond in the methane molecule is one of the most highly energetic (104 kcal/mol); the importance of methane oxidation is also linked to the putative role of atmospheric methane in processes of global warming of the Earth [1, 2].

Methylotrophs are the group of bacteria that can utilize methane as the major source of carbon and energy for growth process [3]. In addition to methane methylotrophs can oxidize (sometimes stereospecifically) alkenes and alkanes, including chloro-substituted derivatives such as di- and trichloroethylene, which are soil contaminants. This explains interest in methylotrophs for soil bioremediation and fine organic synthesis.

Methane monooxygenase (MMO) is an enzyme catalyzing the key reaction of methane oxidation to methanol by oxygen in air:

$$CH_4 + NADH + H^+ + O_2 \rightarrow CH_3OH + NAD^+ + H_2O.$$

Besides MMO, there is only one enzyme (ammonium monooxygenase) that can activate the very inert C–H bond in methane.

Methylotrophs synthesize two types of MMOs—soluble MMO (sMMO) and particulate (membrane-bound) MMO (pMMO). The latter is synthesized by all methylotrophs, whereas the soluble cytosolic form is produced by several strains only under conditions of low Cu^{2+} content in the growth medium. Synthesis of both forms has been found in the following strains: *Methylococcus capsulatus*, *Methylosinus trichosporium*, *Methylosinus sporium*, *Methylocystis* sp., *Methylomonas methanica* [1, 2, 4, 5].

Soluble MMO from *M. capsulatus* strain Bath is one of the most studied MMOs [6-8]. The structure of this enzyme and the putative mechanism of its action have been elucidated. According to spectroscopic and X-ray data, its active site contains a non-heme binuclear 2Fe-cluster, which has also been found in the other sMMO from *M. trichosporium* OB3b. Binuclear 2Fe-centers were also found in other alkane and alkene monooxygenases, including membrane-bound forms of these enzymes. It is

Abbreviations: BSA) bovine serum albumin; DM) dodecyl-β-D-maltoside; pMMO) particulate methane monooxygenase; sMMO) soluble methane monooxygenase.

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possible that these active site structures are typical for reactions of C–H bond oxidation in methane and higher alkanes. The study of pMMO attracts much interest because the reaction catalyzed by this enzyme might follow a different mechanism.

In contrast to sMMO, the particulate enzyme is characterized by limited substrate specificity; besides methane, pMMO can catalyze hydroxylation of short-chain alkanes (up to C_5) and epoxidation of corresponding alkenes. However, pMMO exhibits higher activity and stereoselectivity in hydroxylation reactions [2].

Nevertheless, the structure and composition of metal-containing centers of pMMO are poorly characterized. The main problems are related to difficulties in solubilization and purification of this enzyme. Isolation and purification of membrane-bound proteins are associated with considerable difficulties, and together with crystallization they represent a critical stage in elucidation of the structure of membrane-bound proteins by X-ray analysis. Removal of membrane proteins from hydrophobic membrane environment is usually accompanied by loss of catalytic activity due either to changes in native protein conformation or disruption of interactions with other membrane proteins required for maintenance of physiological function. Even among membrane proteins, pMMO is characterized by increased instability and the properties of electrophoretically homogeneous pMMO significantly differ [2, 9-13]. pMMO was isolated from two bacteria, *M. capsulatus* and *M. trichosporium* OB3b [14, 15]. pMMO from *M. capsulatus* has been better characterized. The hydroxylase component of pMMO consists of three subunits of 26 kD (pmoA or β), 47 kD (pmoB or α), and 23 kD (pmoC or γ); in solution (in the presence of detergent) they form a trimer $(\alpha\beta\gamma)_3$. There is controversy about the content, properties, and functions of metal ions bound to apoprotein of the enzyme; there is evidence that pMMO contains 2-4 copper atoms (some reports give much higher values of 20-29 [2]) and 0-4 Fe atoms [2, 9, 10, 12]. The importance of Cu atoms for catalytic activity is well documented, whereas the putative role of Fe atoms has been questioned in several studies. A recently obtained structure of pMMO from *M. capsulatus* strain Bath with resolution of 2.8 Å could not solve this problem [16]. This structure confirmed the presence of two types of copper-containing centers per αβγ heterotrimer of 100 kD. The putative catalytic center contains a binuclear copper center (type 1), which binds to a mononuclear copper center (type 2) via an electron transport chain formed by His residues; it is possible that this mononuclear center acts as a primary electron acceptor from a physiological donor. Both Cu-containing centers are located on the part of the pmoB subunits oriented to the periplasm. Besides Cu, pMMO contains Zn ion located in the membrane and bound to pmoC; the latter consists of five transmembrane α-helices. Appearance of Zn ion is possibly associated with use of Zn-containing crystallization solution. Fe ions had not been found in the structure, but the authors reported that both enzyme solution used for crystallization and enzyme crystals were inactive. Thus, crystallization of catalytically active enzyme is still an unsolved problem.

So, the goal of this work was optimization of a previously developed method for solubilization of the hydroxylase component of pMMO for subsequent crystallization and optimization of the method for monitoring of catalytic activity of membrane-bound and purified pMMO.

MATERIALS AND METHODS

Periplasmic membranes isolated from cells of *M. capsulatus* strain M was used as the starting material. Membranes were isolated in the group of Structure and Technology of Enzymes, Institute of Problems of Chemical Physics, Russian Academy of Sciences. Membranes were washed in 25 mM Pipes-NaOH buffer, pH 7.25, sedimented by centrifugation for 1 h at 150,000*g*, and were kept until use in liquid nitrogen or at –73°C.

The following chemicals were used in this study: Pipes and dodecyl-β-D-maltoside (DM) from AppliChem (Germany), benzamidine and reagents for protein isolation, purification, and crystallization, and some other reagents from Sigma (USA) and ICN (USA).

Isolation and purification of pMMO. *Solubilization of pMMO.* The frozen membranes were thawed in 25 mM Pipes-NaOH buffer, pH 7.25, and 10% DM solution (w/v) prepared in the same buffer was added dropwise at 4°C with constant stirring up to final DM concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75 mg per mg of protein. The mixture was incubated for 15-60 min and centrifuged at 150,000*g* for 1 h at 4°C to remove insoluble membrane material. Before centrifugation, detergent concentration was decreased to 0.03% by diluting the incubated mixture with 25 mM Pipes-NaOH buffer, pH 7.25, containing 1 mM benzamidine to prevent proteolysis. Solubilization degree was evaluated by assay of protein concentration and pMMO activity in the supernatant. The latter was then aliquoted and kept at –20°C until subsequent pMMO purification.

Ion-exchange chromatography was carried out using an AKTA FPLC chromatograph (Amersham Biosciences, USA) and MonoQ HR5/5 column equilibrated with 25 mM Pipes-NaOH buffer, pH 7.25, containing 0.03% DM and 1 mM benzamidine. Protein was eluted by a linear gradient of NaCl (0-1 M) in the same buffer at a flow rate 0.5 ml/min. The collected fractions were analyzed for protein content, enzyme activity, and by SDS-PAGE.

Gel filtration was carried out using the AKTA FPLC chromatograph and a Superdex^{TM} 200 10/300 GL column with separation range of 10-600 kD. The column was equilibrated with 25 mM Pipes-NaOH buffer,

pH 7.25, containing 0.03% DM, 0.2 M NaCl, and 1 mM benzamidine. Before gel filtration, the protein sample obtained after ion-exchange chromatography was concentrated to final volume of 200 μl. The collected fractions were also concentrated and frozen. For evaluation of molecular mass of the proteins, the column was calibrated with molecular mass markers (Amersham Biosciences).

Enzyme activity assay. Activity of pMMO was evaluated by measuring rate of propylene oxidation to propylene oxide as described in [2, 13] with minor modification. Dried and purified from ethanol traces NADH and duroquinol were used as reductants. Standard reaction mixture contained 25 mM Pipes-NaOH buffer, pH 7.25, 15 mM NADH or 30 mM duroquinol, 50 μM $CuSO_4$, and pMMO (periplasmic membranes or enzyme samples obtained during purification). Protein concentration in pMMO samples was 40 mg/ml, and final volume of the reaction medium was 300 μl. The mixture was placed into a septum-sealed vial (7 ml) for gas chromatography. The reaction was initiated by substitution (through the septum) of 3 ml air for 3.5 ml propylene using a syringe. The mixture was incubated in thermostat at 45°C under constant stirring. Samples of 0.5 ml were taken from the gaseous phase after 1, 2, 3, 5, 10, 15, and 20 min with the syringe. The samples were analyzed using a CHROM 5 gas chromatograph (Laboratori Pristroje, Czechia) equipped with a katharometer as the detector and nitrogen as carrier gas. Temperatures of evaporator, thermostat, and detector were 120-150°C. Propylene oxide content in the gas samples was determined from a calibration curve. Specific activity of pMMO was expressed as nanomoles of propylene oxide formed per min per mg of protein (pMMO).

Duroquinol was obtained as described [9]. Commercially available duroquinone (0.2 g) was dissolved in 20 ml of ethanol and then sodium dithionite (0.28 g) was added. After mixing (5-7 min), sodium borohydride (0.07 g) was added. The solution bleached within 1 min and was stirred for 15 min. The resultant cloudy solution was filtered using a paper filter; the sediment was washed with 200-250 ml of distilled water. The collected filtrate was dried using a vacuum centrifuge Centrivap Console (Labconco, USA) and kept at −20°C.

Analytical methods. Purity of pMMO was evaluated by SDS-PAGE using the Laemmli system [17]. Protein solutions mixed with standard sample buffer were incubated for 30 min without boiling [9, 10].

Protein concentration in the presence of detergent was determined by the method of Lowry et al. [18] using Bio-Rad Protein Assay kit (USA) and BSA in the presence or absence of DM for calibration curves.

Metal content in purified protein was determined using inductively coupled plasma mass spectrometry on an ELAN 6100 DRC ISP MS spectrometer (Perkin Elmer, USA). Fungal copper-containing laccase from *Coriolus hirsutus* and bovine heart cytochrome *c* (Sigma) were used as control proteins.

Mass spectra were obtained using an Ultraflex MALDI time-of-flight mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the mode of positive ion masses ranging from 500 to 8000 daltons, which were calibrated using known peaks of trypsin autolysis. Gel treatment, trypsin hydrolysis, and peptide extraction were carried out according to protocols [19] with some modification. A sample (0.5 μl) was mixed on the mass-spectrometric matrix with the same volume of 2,5-dihydrobenzoic acid (Sigma) solution (10 mg/ml) in 20% acetonitrile containing 0.1% trifluoroacetic acid and dried in air. Proteins were identified using the Mascot program (Matrixscience (www.matrixscience.com), USA) and the database of the National Center of Biotechnological Information (USA). Accuracy of mass detection was about 0.01% (assuming possibility of modification of cysteine residues with acrylamide and methionine oxidation).

Crystallization. A solution of pMMO in 25 mM Pipes-NaOH buffer, pH 7.25, containing 0.03% DM, 1 mM benzamidine, and 0.2 M NaCl was concentrated up to 20 mg/ml using an ultrafiltration cell (Millipore, USA) and membranes with cut-off 30 kD. For screening of crystallization conditions, we used a Crystal Screen Index kit (Hampton Research, USA). Crystallization was carried out using all 96 positions of the kit at room temperature. A drop of 2 μl contained 1 μl of the protein solution and 1 μl of a reservoir solution.

RESULTS AND DISCUSSION

Optimization of the method of pMMO activity assay. Activity of pMMO was assayed using the reaction of propylene oxidation to propylene oxide. Good evidence now exists that in the membrane the hydroxylase component of pMMO forms a complex with NADH-dependent reductase; using NADH oxidation, the latter reduces membrane quinones, which transfer electrons to the hydroxylase component of pMMO [2, 11, 20]. Therefore, NADH was used for determination of pMMO activity in the membrane fraction. For optimization of activity assay protocol, we investigated the effects of NADH and Cu^{2+} concentrations on kinetics of propylene oxidation. Variation of NADH concentrations within 5-30 mM had minor influence on the initial rate of this reaction; observed changes did not exceed 10% with maximum at 15 mM NADH, but significantly influenced maximal level of propylene conversion (Fig. 1).

Maximal yield of propylene oxide was detected at 15 mM NADH. Increase or decrease in NADH concentration resulted in lowering propylene conversion level. Putative reasons for this phenomenon can be attributed to a deficit of reducing equivalents at low NADH concentrations and to inhibition of this reaction by excess of NADH

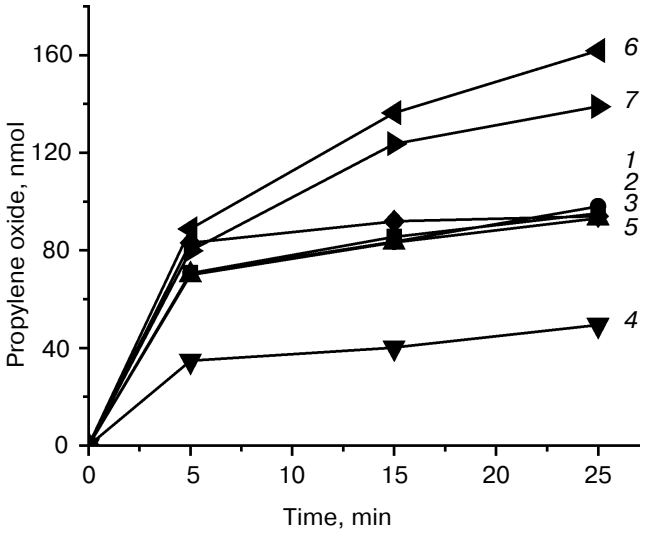

Fig. 1. Dependence of the rate of propylene oxidation catalyzed by pMMO on duroquinol and NADH concentrations in the reaction medium. Duroquinol concentrations (mM): *1*) 12; *2*) 30; *3*) 60; *4*) 70. NADH concentrations (mM): *5*) 5; *6*) 15; *7*) 30.

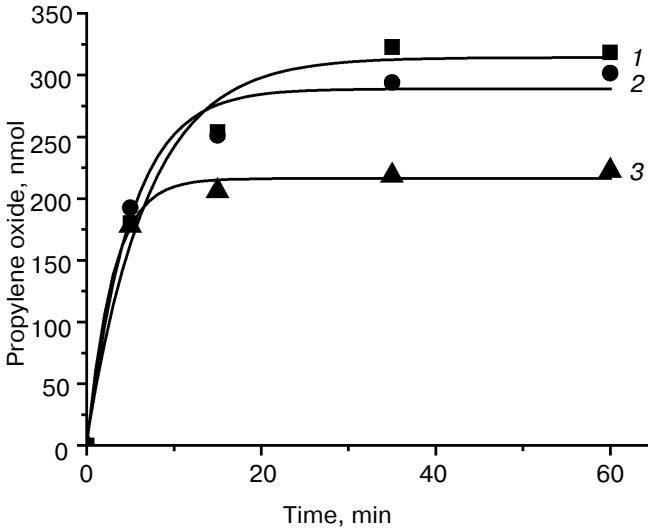

Fig. 2. Dependence of reaction rate of propylene oxidation catalyzed by pMMO on propylene concentration in the reaction medium. *1*) Air (3 ml) was substituted for 3.5 ml propylene; *2*) air (1.5 ml) was substituted for 2 ml propylene; *3*) air (4 ml) was substituted for 4.5 ml propylene.

at high NADH concentrations. Enzyme inactivation by propylene oxide, a toxic reaction product, is also possible.

Changes in copper ion concentrations from 0.05-0.2 mM did not influence the reaction rate, but in the absence of this ion this reaction is negligible.

The ratio of propylene and oxygen concentrations influenced the reaction rate. Maximal enzyme activity was observed with 3.5 ml propylene injected into a working volume of 7 ml (Fig. 2).

The temperature optimum of the reaction catalyzed by pMMO was 40-45°C. In some studies, pMMO activity was assayed in buffer containing 0.15-0.20 M NaCl. We investigated the effect of NaCl on pMMO activity. Figure 3 shows that increasing the NaCl concentration is accompanied by a decrease in pMMO activity; this may be attributed the decrease in propylene solubility with increase in ionic strength of the reaction medium.

The highest specific activity of pMMO detected in membranes and calculated from the initial rate of propylene oxidation in 25 mM Pipes-NaOH buffer in the presence of 15 mM NADH and 0.1 mM copper chloride was 45 ± 5 nmol/min per mg of protein. Specific activity was constant within the range of pMMO concentrations used. Our values of pMMO activity are consistent with those described in the literature: mean 40-70 and range from 10 to 200 nmol/min per mg of protein [2].

Solubilization and purification of pMMO is accompanied by loss of a reductase component and so determination of enzyme activity requires reductants other than NADH, such as reduced quinones. We used duroquinol as the electron donor for assay of solubilized enzyme activity. Duroquinol was obtained by reduction of commercially available duroquinone by dithionite and sodium borohydride. Variation of duroquinol concentrations within 10-60 mM had minor influence on the initial rate of the reaction (Fig. 1). Increasing duroquinol concentration above 70 mM caused almost total inhibition of enzyme activity. It was earlier noted [20] that duroquinol excess also inhibited the NADH-reductase activity assayed by NADH-dependent reduction of duroquinone.

Activity of membranes assayed with 50 mM duroquinone was 40 ± 2 nmol/min per mg of protein; this cor-

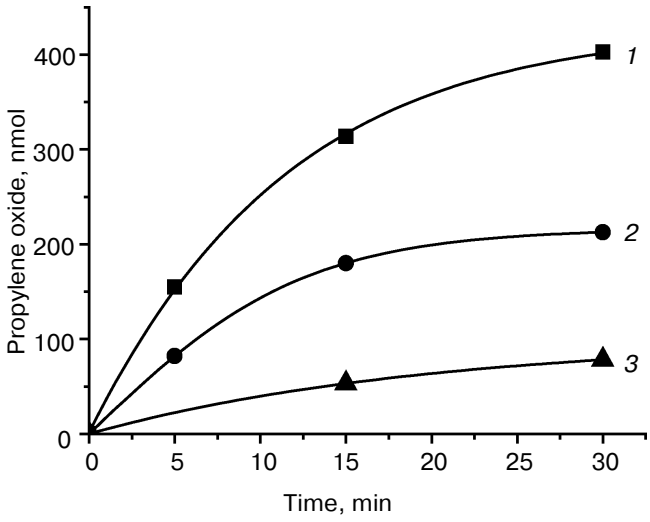

Fig. 3. Dependence of reaction rate of propylene oxidation catalyzed by pMMO on NaCl concentration in the reaction medium: *1*) 0 M NaCl; *2*) 0.2 M NaCl; *3*) 0.5 M NaCl.

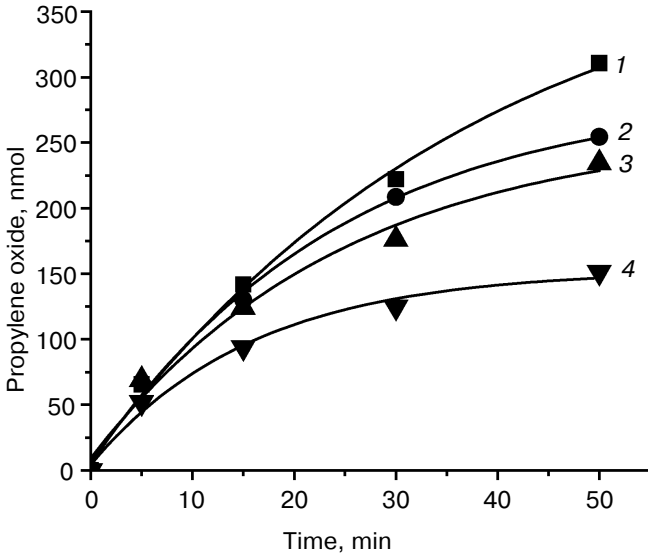

Fig. 4. Dependence of reaction rate of propylene oxidation catalyzed by pMMO on dodecyl-β-D-maltoside (DM) concentration in the reaction medium (%): *1*) 0; *2*) 0.03; *3*) 0.05; *4*) 0.1.

responds to 90% of membranes activity assayed using NADH. Since pMMO solubilization employs DM at concentrations up to 2%, we investigated the effect of DM on pMMO activity. Increasing DM concentration in the reaction medium from 0 to 0.1% decreased the pMMO activity (Fig. 4).

In all cases, duroquinol concentration was calculated from the weight of the solid material used for preparation of the duroquinol solution. However, it should be noted that duroquinol quantities introduced into the reaction cannot be correctly defined as "duroquinol concentrations". This is because duroquinol is a poorly soluble substance and at a given detergent concentration we worked with a saturated duroquinol solution in buffer. It is more convenient for experimental work and detection of precise concentration to use alcohol solution. However, in this case duroquinol undergoes oxidation within a few hours. Introduction of ethanol solution into the reaction mixture is also accompanied by total inhibition by pMMO activity.

pMMO solubilization. According to literature data, treatment of periplasmic membranes with non-ionic detergent dodecyl-β-D-maltoside (DM) is one of the most effective and gentle methods used for pMMO solubilization [21]. Various procedures for pMMO solubilization have been described [2, 9-14, 22]. For optimization of the solubilization procedure we varied the following parameters: protein/detergent ratio, duration of solubilization, temperature.

In accordance with previous report [22], we found that the optimal DM/protein ratio was 1.2 : 1 (w/w). At lower DM effectiveness of protein extraction from membranes decreased. At higher DM/protein ratio, effectiveness of solubilization remained unchanged but the pMMO became inactive. At high DM concentrations in solution, the pMMO trimer loses a subunit of 22-23 kD, and this is accompanied by loss of its catalytic activity [12]. Enzyme solubilization with high DM concentrations was shown to be accompanied by loss of signals of 8 of 10 copper atoms in the EPR spectra; due to their oxidation or removal from the active site of pMMO [12]. Circular dichroism spectra revealed no influence on secondary structure at DM concentrations up to 2%.

Solubilization depends on temperature and duration of treatment with detergent. According to our data, at 4°C solubilization is complete within 1 h. SDS-PAGE revealed that under our experimental conditions pMMO is the main protein component of the solubilized membrane material (Fig. 5, lanes *1* and *2*).

Purification of pMMO. The purification procedures described in the literature [2, 9-16] are based on the combination of various stages of column chromatography; it includes ion-exchange chromatography, gel filtration, and hydrophobic chromatography. In all cases the final purification product, pMMO comprises three polypeptide chains of 45-47, 26, and 23 kD. However, catalytic activity of the purified enzyme and metal ion (Cu^{2+} and Fe^{3+}) content significantly vary in the various purification schemes. Many authors indicate lack of reproducibility of

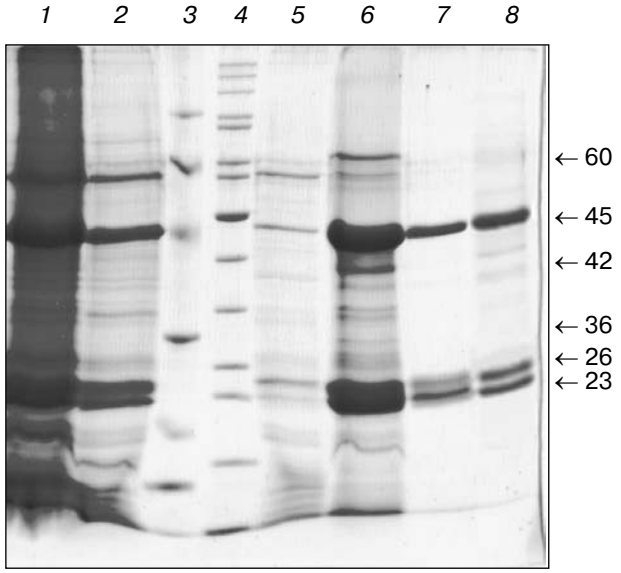

Fig. 5. SDS-PAGE of pMMO purified using a two-stage procedure including anion-exchange chromatography and gel filtration. Lanes: *1*, *2*) two dilutions of solubilized membranes; *3*) protein molecular mass markers (from top to bottom): cellulase (94.6 kD), BSA (66.2 kD), ovalbumin (45 kD), trypsin inhibitor (21.5 kD), lysozyme (14.4 kD); *4*) molecular mass markers of 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, and 10 kD; *5*, *6*, *8*) fractions 2, 3 (after additional concentrating), and 4 obtained after anion-exchange chromatography, respectively; *7*) homogeneous pMMO after gel filtration.

properties of purified pMMO. This may be attributed to various conditions used for cultivation of cells and therefore various combinations of pMMO accompanying proteins. The most successful protocol for pMMO purification was developed in DiSpirito's group [22]. This team obtained a homogeneous pMMO with specific activity of 140 nmol/min per mg of protein, significantly exceeding all previously reported values. The purification procedure including two stages of ion-exchange chromatography did not differ significantly from the protocols described in the literature, but the purification was carried out under anaerobic conditions.

The first stage of our purification procedure of solubilized pMMO included ion-exchange chromatography on Mono Q column equilibrated with 25 mM Pipes-NaOH buffer, pH 7.25, containing 1 mM benzamidine for protease inhibition and 0.03% DM for increasing pMMO solubility and decreasing nonspecific sorption. After enzyme solubilization, DM concentration in the protein solution was decreased by dilution with buffer lacking DM. After protein loading and column washing with the initial buffer (eight column volumes), proteins were eluted by a gradient of NaCl (0-1 M) in the same buffer (Fig. 6). Fractions were collected and analyzed for the presence of pMMO and NADH-reductase activities and also by electrophoresis.

Fraction 1, which did not adsorb on the column, was almost protein free. Fractions 2 and 3 possessed NADH-reductase activity; maximal activity was detected in fraction 3. Besides pMMO, the electrophoregrams of fractions 2 and 3 contained protein bands of molecular masses 70, 60, 42, 36, and 10 kD (Fig. 5). Since there is no generally accepted viewpoint on the nature and molecular mass of NADH-reductase, we analyzed the most probable candidate proteins of 60, 42, and 36 kD using MALDI-TOF mass spectrometry.

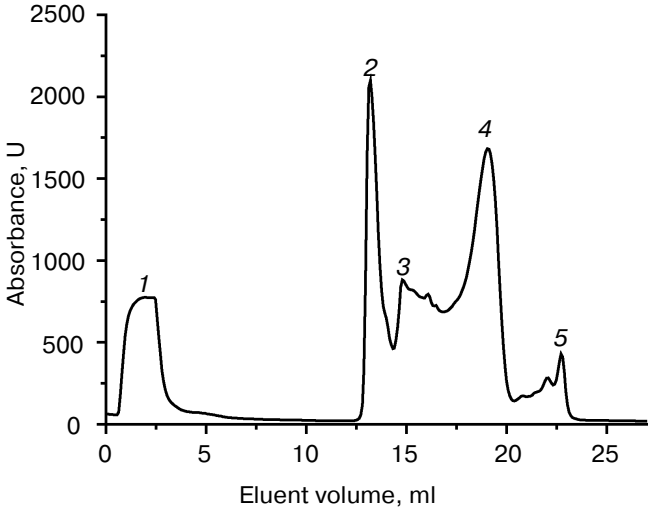

Fig. 6. Anion-exchange chromatography of the solubilized pMMO. Conditions: MonoQ HR5/5 column equilibrated with 25 mM Pipes-NaOH buffer, pH 7.25, containing 0.03% DM and 1 mM benzamidine. Protein was eluted by a gradient of NaCl (0-1 M) in the same buffer.

According to the MALDI-TOF data, the band with molecular mass of 42 kD obviously represents a fragment of the pMMO α-subunit, which is formed due to proteolysis; this fragment is rather tightly bound to pMMO. Neither of the components used for analysis including the protein band of 36 kD shared significant homology with NADH-reductase described in [20]. Some authors believe that in *M. capsulatus* NADH-reductase activity can be attributed not only to the transmembrane protein [20], but also to large subunit of NAD-dependent formaldehyde dehydrogenase [23] or methanol dehydrogenase [11], representing a complex comprising two subunits of 63 and 8 kD. However, in our case the primary structure of 60 kD subunit reveals 80% identity with the soluble regulatory protein, chaperonin, and does not exhibit marked similarity with the methanol dehydrogenase fragment of 63 kD.

According to SDS-PAGE data, fraction 4 (eluted at 0.2 M NaCl) represents almost homogeneous pMMO (Fig. 5, lane *8*), containing the characteristic set of subunits. However, no catalytic activity in the reaction of propylene oxidation was detected.

Gel filtration. Gel filtration was the final stage of pMMO purification. (Fraction 4 obtained after ion-exchange chromatography was used as the initial material for the final purification stage.) The pMMO was eluted as a single symmetrical peak (Fig. 7) of molecular mass of 660 kD (calculated by the calibration curve). Data of SDS-PAGE confirmed the presence of only three bands corresponding to the three subunits of pMMO (Fig. 5, lane *7*). It is possible that the molecular mass value obtained in this study corresponds to $(\alpha\beta\gamma)_6$ subunit composition. Oligomeric composition of pMMO in solution has been discussed in several studies [2, 16, 24]. It was suggested that the most probable state of pMMO is a monomer ($\alpha\beta\gamma$) with molecular mass 220 kD due to bound detergent molecules [12] or dimer $(\alpha\beta\gamma)_2$ with molecular 200 kD [13]. According to X-ray data, the crystal structure of pMMO has $(\alpha\beta\gamma)_3$ subunit composition [16], forming an ordered ring structure. It is suggested [24] that such pMMO trimer is a functional unit of this enzyme in the membrane. In this case it is reasonable to suggest that the molecular mass of 660 kD obtained in our study for pMMO solution in the presence of 0.03% DM is consistent with the oligomeric structure of $[(\alpha\beta\gamma)_3]_2$.

We employed mass spectrometry for additional characterization of the subunits obtained. The protein was tested for the presence of metals, and maintenance of secondary structure was analyzed by CD spectrophotometry.

Characterization of the hydroxylase component of pMMO. According to MALDI-TOF mass spectrometry, the large subunit of pMMO purified in this study repre-

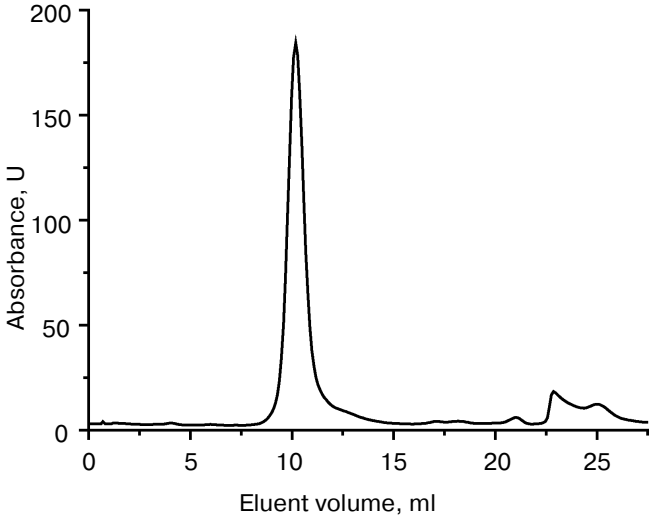

Fig. 7. Gel filtration of fraction 4 obtained during anion-exchange chromatography. Conditions of gel filtration: Superdex^{TM} 200 10/300 GL column equilibrated with 25 mM Pipes-NaOH buffer, pH 7.25, containing 0.03% DM, 0.2 M NaCl, and 1 mM benzamidine.

sents a protein of 47 kD that shares 52% homology with pmoB deduced from the genome of *M. capsulatus* strain Bath. Homology of amino acid sequences for two small subunits pmoA and pmoC, calculated from MALDI data, was 13 and 11%, respectively. These results are consistent with characteristics of corresponding subunits isolated from strain Bath [12, 24].

According to data of metal analysis, homogeneous pMMO contains three copper atoms and one Fe atom per molecular mass of 100 kD. This is consistent with X-ray analysis data [16] demonstrating the presence of three copper atoms and one zinc atom, which probably substitutes for an atom of another metal, most probably an Fe atom.

The CD spectrum demonstrated high content of α-helices in the protein structure (up to 62%); this is consistent with data of X-ray analysis (59% α-helix) [16]. Such coincidence might confirm the preservation of native protein structure during purification and solubilization; however, the protein used in [16] was catalytically inactive, and its structure could significantly differ from the structure of the native protein.

In the process of screening of crystallization conditions using the Crystal Screen Index reagent kit, we obtained a monocrystal of pMMO of 40 × 40 × 100 μm using the following conditions: 0.1 M succinic acid, pH 7.0, and 15% polyethylene glycol 3350 as a precipitant. Now we are examining the quality of this crystal and trying to reproduce this result.

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