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REVIEW

Quinone-Dependent Alcohol Dehydrogenases and FAD-Dependent Alcohol Oxidases

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Abstract—This review considers quinone-dependent alcohol dehydrogenases and FAD-dependent alcohol oxidases, enzymes that are present in numerous methylotrophic eu- and prokaryotes and significantly differ in their primary and quaternary structure. The cofactors of the enzymes are bound to the protein polypeptide chain through ionic and hydrophobic interactions. Microorganisms containing these enzymes are described. Methods for purification of the enzymes, their physicochemical properties, and spatial structures are considered. The supposed mechanism of action and practical application of these enzymes as well as their producers are discussed.

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Key words: methanol dehydrogenase, ethanol dehydrogenase, quinohemeprotein alcohol dehydrogenase, FAD-methanol oxidase, three-dimensional structure, mechanism of action

There are several types of enzymes that oxidize alcohols. Considering the nature of the cofactor used, these enzymes can be divided into four classes:

1) Enzymes containing pyrroloquinoline quinone (PQQ, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) – PQQ-dependent methanol dehydrogenases, PQQ-dependent ethanol dehydrogenases, PQQ-dependent heme-containing alcohol dehydrogenases, and membrane-bound alcohol dehydrogenases;

2) FAD-containing alcohol oxidases;

3) NAD^+- and $NAD(P)^+$-dependent alcohol dehydrogenases, which are usually subdivided into short-chain, iron-activated, and zinc-containing ones;

4) Enzymes containing heme and alcohol dehydrogenase cofactor F420.

These enzymes differ significantly in the nature of the cofactor, amino acid sequence, spatial structure, and substrate specificity. However, they all catalyze the reaction that can be described as follows: $R\text{-}OH + A_{ox} \rightarrow R{=}O + HA_{red}$, where R is an aliphatic or aromatic group, and A is the electron acceptor in the oxidized (ox) or reduced (red) forms.

This review considers PQQ-dependent alcohol dehydrogenases and FAD-dependent alcohol oxidases, since most of them are well studied, use methanol as the single source of energy, and contain cofactor that is non-covalently bound to the protein. Information on these enzymes is available in the cited scientific and patent literature.

QUINONE-DEPENDENT ALCOHOL DEHYDROGENASES

In the 1980s, the new term quinoprotein appeared in the literature for bacterial enzymes containing the new type of the organic cofactor PQQ [1-9] (Fig. 1a). Biosynthesis of PQQ has been studied in detail [5, 8]. Besides, PQQ serves as the coenzyme in some animal enzymes and may be considered as a vitamin [10, 11].

Quinoproteins also include a number of enzymes containing other quinones, derivatives of tryptophan or tyrosine (tryptophanyl quinone (TTQ), tryptophan tryp-

Abbreviations: FAD, flavin adenine dinucleotide; FMO, FAD-dependent methanol oxidoreductase; PMS, phenazine methosulfate; PQQ, pyrroloquinoline quinone; QEDH, quinoprotein ethanol dehydrogenase; QHADH, quinohemeprotein alcohol dehydrogenase; QMDH, quinoprotein methanol dehydrogenase.

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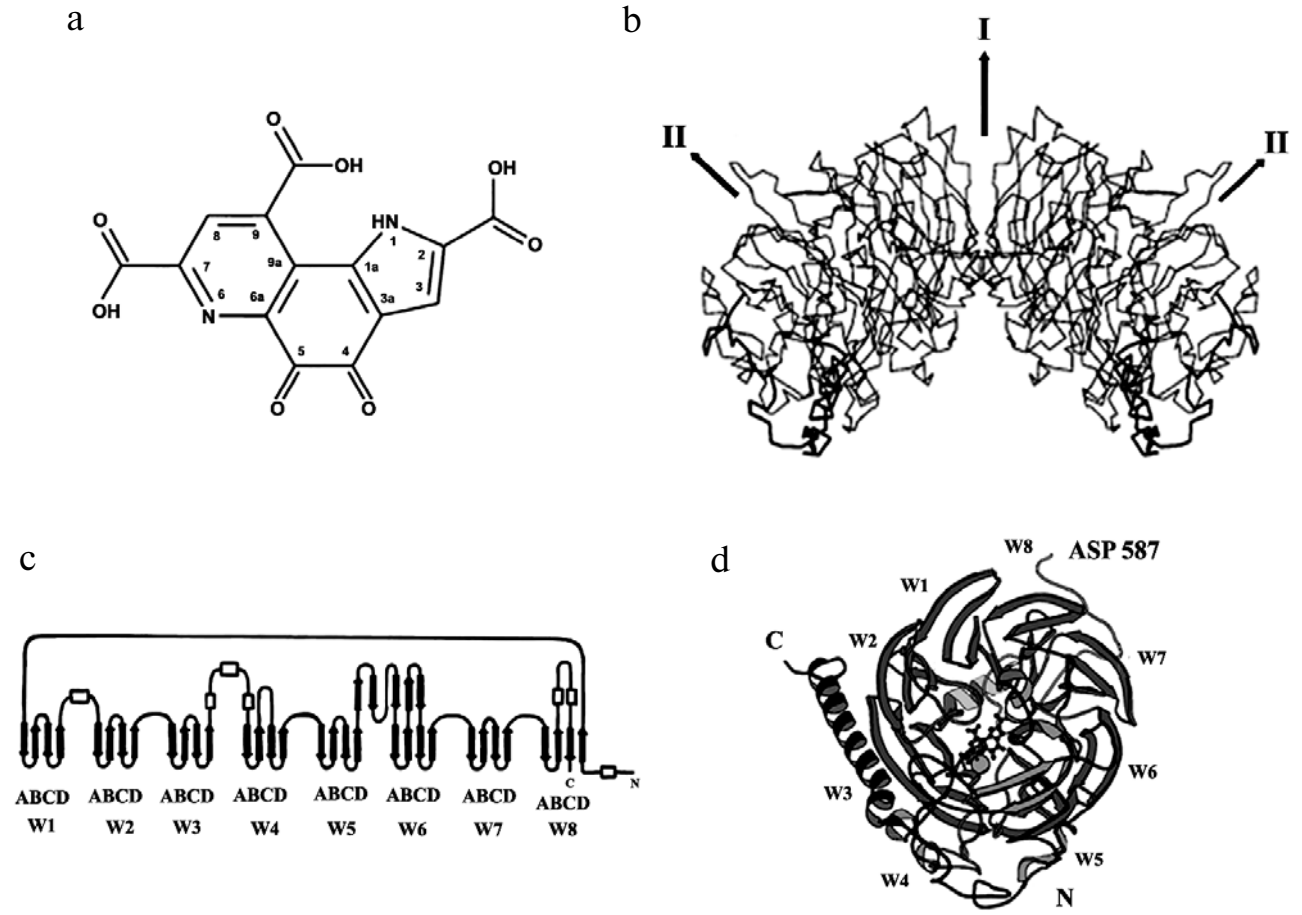

Fig. 1. Structure of PQQ-dependent quinoprotein methanol dehydrogenase (QMDH) from *Methylophilus* W1 3A. a) Chemical structure of pyrroloquinoline quinone [1-9]; b) skeletal model of the tertiary structure of the QMDH tetramer [28]; pseudo axes of two α-subunits and the major axis of the molecule are in the same plane. The angle between their axes is 90°, and small β-subunits are highlighted bold; c) topology of the α-subunit: separate motifs (blades [28]) are designated as ABCD, helical structures are shown by squares; d) ribbon model of the α-subunit of QMDH [19]; C- and N-termini are designated as C and N; the β-subunit is not shown.

tophylquinone, 6-hydroxytriphenylalanine quinone (TPQ), and lysine tyrosyl quinone (LDQ)), which are, in contrast to PQQ, covalently bound to the polypeptide chain of the protein, and they constitute part of the active site [12]. PQQ is contained in quinoprotein methanol dehydrogenases (QMDHs, EC 1.1.99.8), which are found in gram-negative methylotrophic bacteria and in most of methanotrophs. QMDHs are the largest class of PQQ-containing enzymes [13]. They are usually soluble proteins that are located in the cytoplasm of bacteria.

PQQ is also present in some enzymes that oxidize methanol poorly but oxidize ethanol and more complex alcohols with high rates [8, 13-18].

QMDH producers and methods of isolation. The group of bacteria producing QMDH includes *Pseudomonas*, *Diplococcus*, *Paracoccus*, and a large group of methane-oxidizing bacteria [19]. Recently, quinoprotein alcohol dehydrogenases have been isolated from other bacteria [20, 21]. The genes encoding QMDH in a number of methylotrophs have been cloned and sequenced [22-26].

QMDH is usually isolated from cell free extracts of the methylotrophs by centrifugation (140,000*g*, 1 h) and subsequent purification using ion-exchange chromatography and gel filtration [27]. The protein is easily crystallized yielding crystals of different shapes.

Substrate specificity and electron acceptors. QMDHs oxidize methanol yielding formaldehyde with K_m values in the range of micromolar concentrations. In methylotrophs and methanotrophs, the formaldehyde is then metabolized by the serine and ribulose monophosphate pathways. Other primary alcohols (from C_2 to C_8) are also oxidized by QMDHs of most methylotrophic bacteria yielding corresponding aldehydes; some of them are also capable of oxidizing secondary alcohols. These enzymes are acidic or basic proteins (p*I* value varies from 3.7 to 9.4).

QMDHs oxidize alcohols in the presence of natural or artificial electron acceptors. In the case of the natural acceptors, QMDHs exhibit maximal activity in the pH 5-6 range. With artificial acceptors, the maximal activity is observed at pH 8-9.

QMDH structure. QMDHs from some methylotrophs are best studied. For the enzymes from *Methylophilus* W3A1 [28-32], *Methylobacterium extorquens* [19, 33-35], and *Paracoccus denitrificans* [36] the structures have been determined by X-ray analysis at 2.6 to 1.2 Å resolution.

The enzymes of these methylotrophs are heterotetramers ($\alpha_2\beta_2$) consisting of two α-subunits (60-66 kDa) and two β-subunits (8.5-10 kDa) with total molecular weight of about 140 kDa. The pseudo axis of one αβ-dimer is perpendicular to the pseudo axis of the other αβ-dimer (Fig. 1b).

The large subunit is composed of eight topologically identical antiparallel β-sheets twisted in the shape of the letter W from polypeptide fragments A, B, C, and D (W-motif or blade) (Fig. 1c). The W-blades are arranged radially around the pseudo axis going through the center of the large subunit, forming an 8-bladed propeller-like super-barrel (Fig. 1d) [37]. Such packing of the polypeptide chain is very compact, and it has been found for a number of enzymes. A similar 8-bladed β-propeller structure was described for the nitrite reductase from *Thiosphera pantotropha* containing heme as the prosthetic group [38]. Besides, 4- and 7-bladed propeller proteins containing PQQ as the prosthetic group have been described. More detailed information is presented in the review by Jawad and Paol [39]. The blades of the propeller interact with each other by the stacking of W residues, as well as by van der Waals contacts between the residues of A, W, and G, this providing high stability to the super-barrel.

The β-subunit is an extended structure having no hydrophobic nucleus. There is an α-helix (seven turns) at its C-terminus, and several open turns with a disulfide bridge and a proline-enriched region at the N-terminus. The β-subunit has the shape of the letter J and is connected with the external surface of the W_1-W_4 blades of the α-subunit predominantly by ionic and hydrophobic interactions. Conditions for reversible dissociation of the enzyme into α- and β-subunits have not been found. Thus, the role of the small subunit has not been determined. It takes a small area in the proximity to the active site, being not involved into the contacts between α-subunits, and together with the α-subunit forms a common domain. It presumably takes part in the stabilization of the enzyme spatial structure, in electrostatic interactions with the electron carrier cytochrome c_L, or influences indirectly the catalytic function of QMDH. However, other quinoproteins lack this subunit, which may indicate that it has a specific function.

At the distance of 2-3 Å from the PQQ molecule, there is a disulfide group formed by two cysteine residues located in proximity to each other. This group must be formed during the binding of PQQ to the active site of the enzyme and presumably retains the coenzyme in the active site. Besides, PQQ interacts with the polypeptide chain by hydrogen bonds and ionic interactions.

The active site of QMDH contains a Ca^{2+} ion. It interacts with oxygen ligands and the N6 atom of the PQQ ring, which are located at the distance of 2.4-2.8 Å from the metal ion (Fig. 2a). The removal of Ca^{2+} ions results in the lost of the enzyme activity. In contrast, the addition of Ca^{2+} ions restores the activity of the enzyme [40-43].

The distance between the active sites of two αβ-subunits of the molecule is 45 Å, so they are not supposed to interact.

Natural electron acceptors: cytochrome *c* group. Electrons are transferred from an oxidizing substrate to the PQQ moiety, and protons are released into the cytoplasm. The reduced PQQ moiety donates electrons to cytochrome *c* one after the other, yielding the semiquinone form of PQQ [44-52]. Perhaps the disulfide bond that is located close to the PQQ moiety and prevents access of solvent to the active site of the enzyme participates in the stabilization of the semiquinone PQQ form and in subsequent electron transfer to cytochrome *c*.

The natural electron acceptors are acidic cytochromes c_L, c_{551i}, and c_{553i} [44-53]. Presumably, QMDH first interacts with cytochrome c_L by forming ion pairs between a number of K residues of the enzyme and carboxyl groups of the cytochrome.

Paracoccus denitrificans contains constitutive cytochrome c_L, but synthesis of cytochromes c_{551i} and c_{553i} is induced while growing on methanol. The dissociation constant of the complex of QMDH with cytochrome c_{551i} is 375 μM. The molecular weight of cytochrome c_{551i} is 17.5 kDa, and the polypeptide chain contains 155 amino acid residues, 27 of which are acidic and only eight basic. The p*I* value of the protein is 3.5. The molecule contains five α-helixes, the three central ones surrounding the heme group. Two areas of the heme group are partially exposed to the solvent. Other cytochromes of this group presumably have similar structures.

During the formation of the complex, a rearrangement of the protein is possible the results in advantageous orientation for electron transfer [54]. This is consistent with the high energy of the rearrangement. From the temperature dependence of the electron transfer between QMDH and cytochrome c_L, the rearrangement energy of the transfer was estimated according Marcus theory [54] and found to be 1.9 eV. The distance between the redox centers in the QMDH–cytochrome c_L complex is supposed to be 14-15 Å.

Artificial electron acceptors. Some artificial electron acceptors can take electrons from the reduced enzyme. These include 1,6-dichlorophenolindophenol (ε_{600} = 21,990 $M^{-1}\cdot cm^{-1}$), Wurster's blue (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, ε_{560} = 11,600 $M^{-1}\cdot cm^{-1}$), and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfuric acid) (ε_{630} = 35,000 $M^{-1}\cdot cm^{-1}$). The artificial electron acceptors cannot be directly reduced by the enzyme. Their reduction requires the presence of phenazine etho-

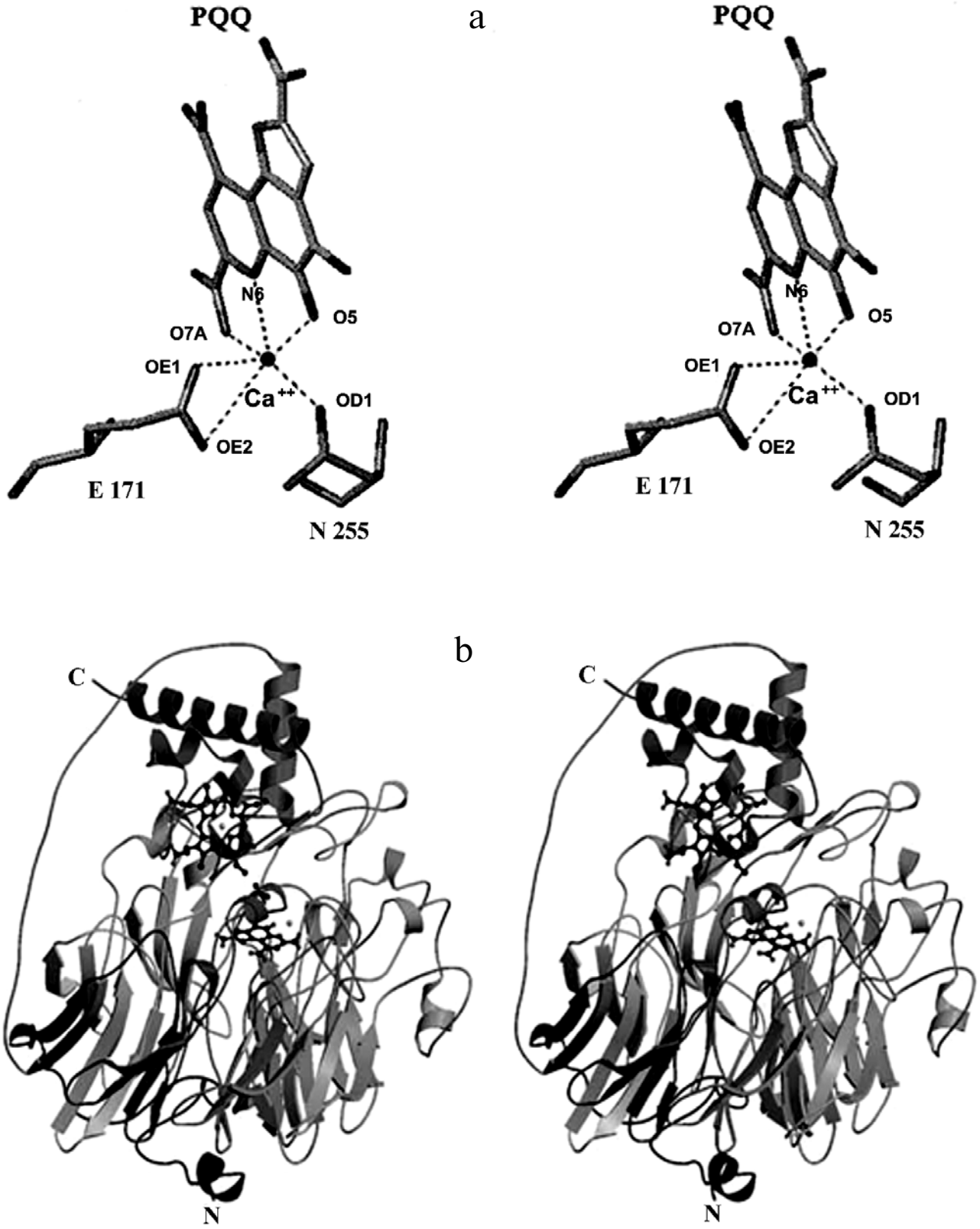

Fig. 2. Spatial models of the active site of quinoprotein methanol dehydrogenase (QMDH) (a) and the molecule of quinohemeprotein alcohol dehydrogenase (QHADH) (b). a) Ca^{2+} is coordinated with the carboxyl group of E171, N6, and the carbonyl oxygen at C5 of the PQQ ring. The ring structure formed between C103 and C104 of the polypeptide chain is not shown [28]; b) spatial model of QHADH [69]; terminal amino acid residues are designated as C and N.

sulfate or phenazine methosulfate (PMS), which function similarly. Oxidized PMS inhibits the enzyme. Methanol and natural endogenous reductants protect the enzyme from inactivation.

From the large group of electron-rich aromatic nitroso compounds, some artificial electron acceptors have been selected [55] that are likely to be successfully employed for measuring the activity of QMDH.

Artificial electron acceptors require the presence of an activator (ammonium salts, methylamine, or glycine ethyl ester). The kinetics and mechanism of action of these activators on QMDH have been studied in detail [56]; they cannot be replaced with high molecular weight aliphatic or aromatic amines. However, in the case of QMDH from *Rh. acidophila*, amines with different carbohydrate chain length can be used instead of methylamine.

The reduction of artificial electron acceptors (in contrast to cytochrome *c*) proceeds at pH 9.0-9.5, since the active form is not the ammonium ion but the ammonia molecule. The role of ammonia in the oxidation of α-alcohols by QMDH has been studied using quantum mechanical calculations [56].

Mechanism of methanol oxidation by QMDH. Although the details of the mechanism of action of QMDH are still unclear, some common features are understandable. The enzyme catalyzes the reaction by the ping-pong mechanism. The PQQ moiety is reduced by the substrate yielding a product. The subsequent sequential electron transfer to cytochrome *c* results in the oxidation of the $PQQH_2$ moiety through the stage of semiquinone free radical, yielding the quinone. The rate-limiting step of the process is the transformation of the oxidized complex into the reduced enzyme, and it is this stage that requires the presence of ammonia.

In the literature, two mechanisms of oxidation of alcohols by QMDH are considered: the mechanism of addition–elimination and the hydride mechanism [19, 57-64]. Both mechanisms have been studied using theoretical quantum mechanical calculations. In recent years [8, 62, 63] the acid-base catalysis of hydride transfer has been considered to be the most probable, where amino acid residue N303 is a base and the Ca^{2+} ion serves as an acid (Fig. 3a), although some theoreticians have another opinion [64].

In the reductive stage of the reaction, a large isotopic effect is observed that corresponds to the elimination of the hydrogen [64]. This is the stage that is stimulated by NH_3, although the detailed mechanism is still unclear.

Calcium ion plays a structural role by maintaining the PQQ moiety in the active conformation. Due to the coordination bonds with the carbonyl oxygen at the C5 atom of PQQ, Ca^{2+} acts as a Lewis acid. Thus, it increases the electrophilicity of C5 for the attack by the hydride. Also, calcium ion forms coordination bonds with the oxygen of the substrate, thus facilitating its oxidation. The important role of Ca^{2+} in the catalysis is supported by the data obtained with methanol dehydrogenases in which Ca^{2+} is replaced by Sr^{2+} or Ba^{2+} ions [40-43].

Soluble quinoprotein ethanol dehydrogenases and quinohemeprotein alcohol dehydrogenases. There are several types of PQQ-containing alcohol dehydrogenases that differ from QMDH [14-17, 64-68]. However, they all contain a calcium ion, and the reductive part of the catalytic mechanism of these enzymes seems to have much in common.

A representative of one such type is soluble alcohol dehydrogenase from *Pseudomonas*. This enzyme consists of two α-subunits that are almost identical to the α-subunits of QMDH in molecular weight and propeller structure, but it contains no β-subunits. The enzyme poorly oxidizes methanol, but well oxidizes ethanol (the K_m value for ethanol is 12 μM). For this reason it was named quinoprotein ethanol dehydrogenase (QEDH) [68]. It is also capable of catalyzing oxidation of other primary and secondary alcohols.

Soluble QEDH has been found in different strains of *Pseudomonas*. In the presence of cytochrome c_L, the pH optimum for QEDH activity is close to 7.0, but in the presence of artificial electron acceptors the activity is maximal at more alkaline pH values, and the presence of ammonia or a primary amine is necessary for activation. QEDH has an 8-blade structure, and its EPR spectrum is analogous to that of QMDH [66, 67]. QEDH contains a disulfide structure that is similar to the analogous structure in QMDH. This ring structure is supposed to be involved in electron transport from the PQQ moiety to cytochrome c_{550} [68]. In spite of significant differences in amino acid sequence in the region of the loops, high conservatism is observed in the region of the narrow funnel leading to the active site, in the active site region including the W residue and the disulfide ring from both sides of the PQQ plane, in the area of the active site basis (D303 in QMDH), and in the area of the ligand surrounding the calcium ion.

In contrast to QMDH, the enzyme contains a second Ca^{2+} ion away from the active site near the N-terminus of the molecule. The active site of this enzyme has a higher capacity than QMDH, so it oxidizes substrates with higher molecular weights. It could be that the dimensions of the active site cavity are provided by the stabilization of the spatial enzyme structure by the second Ca^{2+} ion. In this connection, the β-subunit of QMDH must stabilize the spatial structure of the enzyme with small capacity of the active site that is accessible mainly for methanol. This assumption is consistent with data on the absence of a β-subunit in quinoprotein glucose dehydrogenase and soluble quinohemeprotein alcohol dehydrogenase.

Quinohemeprotein alcohol dehydrogenase (QHADH) is a soluble monomeric protein of 60 kDa. Cytochrome *c*

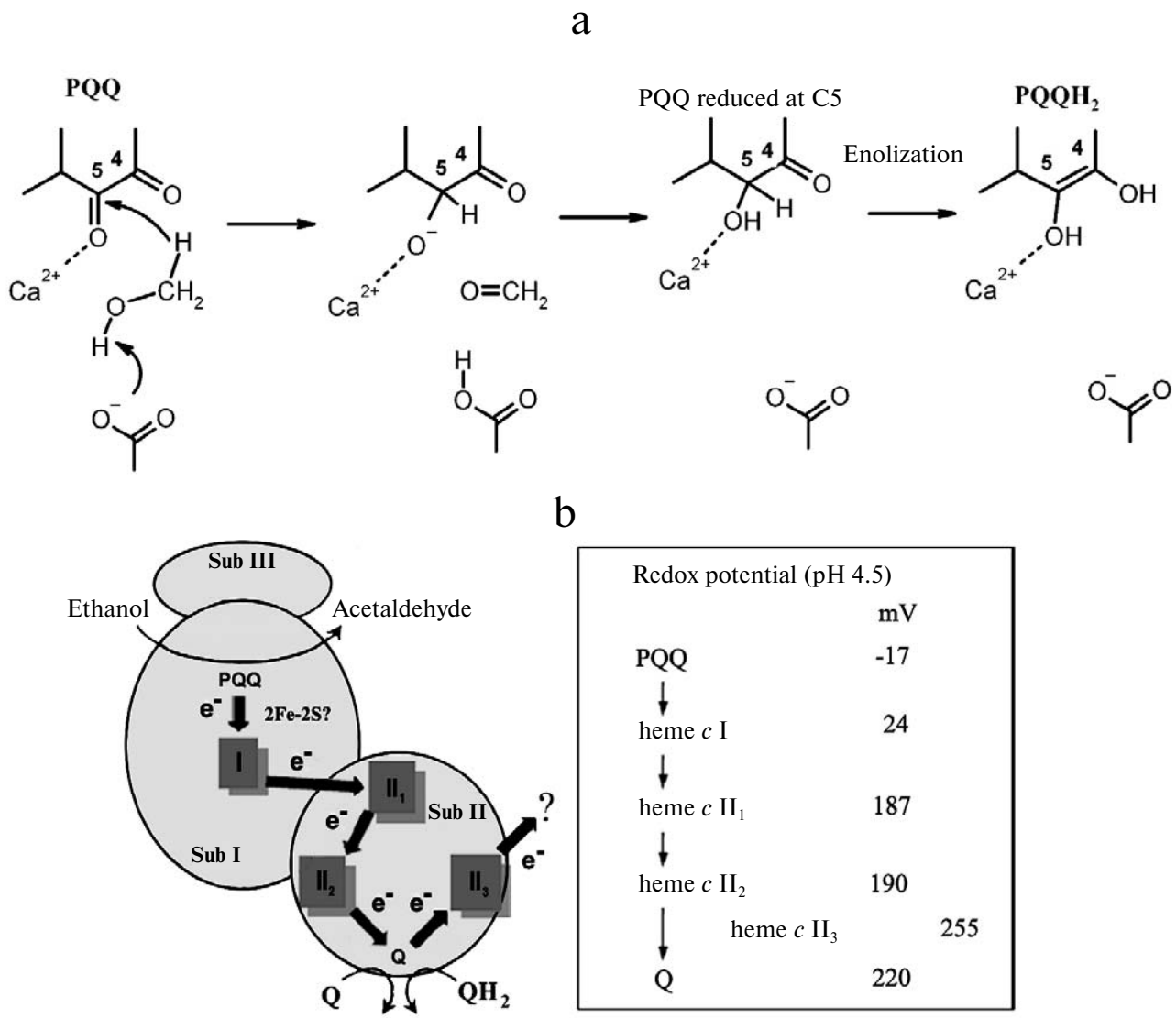

Fig. 3. Presumed structure of membrane-bound PQQ-dependent ethanol dehydrogenase from acetoacetic bacteria and the borohydride mechanism of alcohol oxidation by quinone-dependent dehydrogenases. a) Hydride mechanism of alcohol oxidation by PQQ-containing alcohol dehydrogenases [63]; b) presumed three-subunit structure of membrane-bound alcohol dehydrogenase: electrons are transferred from the oxidized substrate through several heme groups of two subunits to the ubiquinone following the redox potentials of the hemes [71].

is attached to the terminal part of the 8-blade subunit of QHADH (Fig. 2b) [69]. The active site of enzymes of this type includes a Ca^{2+} ion, a PQQ moiety, and amino acid residue side chains binding the PQQ and coordinating the calcium ion. The PQQ molecule is located between the indole ring of a W residue (W243 in QMDH and W245 in QHADH) and two sulfur atoms of the disulfide ring structure. The interaction of the PQQ moiety with the environment of the enzyme is similar to that in QMDH. The Ca^{2+} ion has virtually the same surrounding as in QMDH, and it must play a similar role in the two enzymes. The calcium ion presumably plays a structural role and serves as a Lewis acid in the catalysis, coordinating the oxygen atom at the C5 position of the quinone, and provides for the possibility of attack on the electrophilic C5 of the oxyanion or hydride ion of the substrate. The D308 residue is located similarly to D303 in QMDH and acts as a base, initiating the elimination of the proton from the alcohol substrate.

The electron from the oxidizing substrate is transferred to the PQQ moiety and then to the cytochrome *c* attached to the terminal structure of the molecule. The plane of the PQQ structure forms an angle of 70° with the plane of the cytochrome *c* heme group. This arrangement presumably facilitates electron transfer from the PQQ moiety to the iron ion. The distance between the centers of the PQQ and the heme group is 14-15 Å. From the cytochrome *c*, the electron is transferred to cytochrome

oxidase through azurin, which has a binding site in the QHADH molecule.

Membrane-bound quinohemeprotein alcohol dehydrogenase. Another type of quinohemeprotein alcohol dehydrogenases includes a large group of enzymes of acetoacetic bacteria (QHADH-1) (Fig. 4b). This type of enzyme usually consists of three subunits: I (72-80 kDa), II (44-50 kDa), and III (14-17 kDa) (Fig. 3b) [13, 70-73]. Subunit I (8-blade propeller-like protein) contains PQQ, a calcium ion, and cytochrome *c* that is attached to the terminus of the polypeptide chain. Subunit II contains three heme groups and firmly bound coenzyme Q. The distance between the heme groups in the complex is 9.4 and 8 Å. Subunit III contains no prosthetic groups. The enzymes of this group are bound to the membrane through subunit II. The role of subunit III has not been determined. It may be a chaperone facilitating the folding of subunits I and II. An electron is transferred from an oxidizing substrate to PQQ and then to the cytochrome *c* of subunit I. From this cytochrome, the electron is transferred to the cytochromes of subunit II and to coenzyme Q, then to the membrane coenzyme Q, and finally it reduces O_2 yielding H_2O in the active site of cytochrome oxidase.

QHADH-1 exhibits wide substrate specificity (it oxidizes C_2-C_6 alcohols, formaldehyde, and secondary and tertiary alcohols) and is 10-fold more active than soluble QMDH. The enzyme is inactive without subunit II, but the reassociation of the complex restores the activity.

The enzyme reduces coenzyme Q with long carbohydrate chain (Q6 or Q10), ferricyanide, phenazine methosulfate, and dichlorophenolindophenol. Perhaps it can also reduce other electron acceptors as well [54].

Some species of *Gluconobacter* have another enzyme (QHADH-2) that is very similar in structure to QHADH-1, but lacking subunit III. Recently, a 2Fe-2S iron-sulfur cluster containing three C residues as the ligands has been revealed in subunit I of this enzyme [74]. The fourth ligand is probably an H or S residue. Subunit I of QHADH-1 may also contain such a cluster.

The electron is transferred from an oxidizing alcohol through the PQQ coenzyme and the 2Fe-2S cluster to the cytochrome of subunit I and further through the heme groups of subunit II to coenzyme Q (Fig. 3b).

Methylotrophic bacteria, methanotrophs, quinoprotein and quinohemeprotein alcohol dehydrogenases obtained from methylotrophs, methanotrophs, and acetic bacteria are of great practical importance. Recently, a thermotolerant QHADH was found [75] that can be used at higher temperatures. Methods for use of QMDH for quantitative electrochemical analysis of alcohols in different liquids and their incorporation in fuel cells have been described [76-78].

Acetic bacteria are used for the production of vinegar, and the biomass of some methylotrophs is used as nutrients for animals, fish, and insects.

FAD-DEPENDENT METHANOL OXIDASES

Enzymes that use FAD as a cofactor are involved in a wide range of biochemical reactions including 1- and 2-electron reactions, electron transfer, dehydrogenase reactions, signal transfer, and photosynthesis. This is explained by the appropriate value of the redox potential (from −0.03 to −0.39 V) that depends on the conformation of FAD (Fig. 4a) [79] and the surrounding amino acid residues of the flavoprotein. Due to this fact, the reduction of O_2 to H_2O_2 is considered to be irreversible (+0.25 V) [80-83].

There are four groups of flavin enzymes: hydrogenases and trans-hydrogenases (the reaction product is H_2O_2, slowly reacting with O_2), electron transferase (fast react with O_2, for example, flavodoxin), oxidases (fast react with O_2 yielding only H_2O_2), and monooxygenases (yield a product and H_2O_2). The intermediate with the oxygen in the C(4a) position was previously monitored spectrophotometrically only in the case of monooxygenases [84]. Recently, such an intermediate has also been revealed for one of the oxidases using fast kinetics methods [85]. For other enzyme groups, this intermediate has not been revealed, presumably due to a high rate of the last stage of O_2 reduction and low stability of the intermediate.

FAD-methanol oxidases (FMOs) (alcohol:dioxygen oxidoreductase, EC 1.1.3.13) belong to the superfamily of (methanol-glucose-cholesterol) oxidases [86]. There are several families of these enzymes. In this review we consider only oxidation reaction of OH-groups of different alcohols by FAD-containing oxidases of mainly methylotrophic organisms, the supposed structure of these enzymes, and their practical application.

FMO was first found in Basidiomycetes in 1965 [87-89]. The enzyme catalyzes the reaction $CH_3OH + O_2 = CH_2O + H_2O_2$. The formaldehyde produced is then metabolized in the cell by the serine or ribulose monophosphate pathways. Part of the formaldehyde is oxidized yielding formic acid with subsequent formation of H_2O and CO_2. Hydrogen peroxide is reduced by catalase to O_2 and H_2O.

Producers. Producers of FMO are organisms capable of growing on methanol as the single source of carbon and energy. They include some yeasts (*Gliocladium deliquescens*, *Paecilomyces variotii*, *Trichoderma lignorum*, *Candida* (*boidinii*, *methanolica*, *parapsilosis*, etc.), *Hansenula* (*capsulatus*, *glicozima*, *henricii*, *minuta*, *nonfermentans*, *phyloderma*, *polymorpha*, *wickerhamii*), *Pichia* (*haphophila*, *linderii*, *pastoris*, *pinus*, *trochophila*), *Torulopsis*, and some fungi [90-93]. Among them the yeast *Hansenula polymorpha*, *P. pastoris*, and *P. pinus* are the best studied producers in terms of physiology, accumulation of biomass, and production of FMO. While growing under deficiency in methanol, the content of FMO in the natural strains can reach 20-35%. Also, in

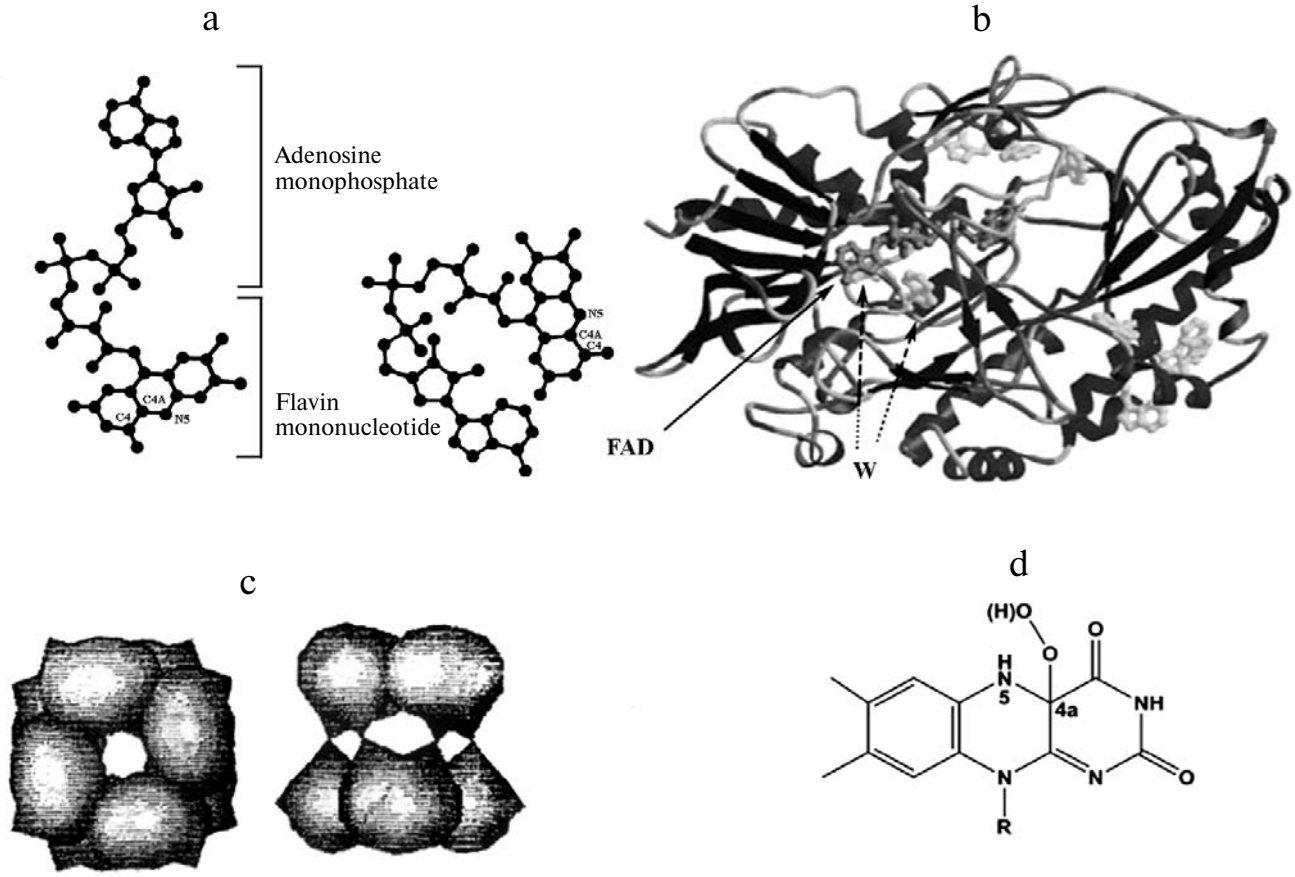

Fig. 4. Structure and function of FAD-containing alcohol oxidases (FAO). a) Two (unfolded and folded) possible conformations of FAD [81] within subunits of FAO; b) predicted structure of one of the FAO subunits [105]; c) two projections (of nine possible) of the packing of FAO subunits according to electron microscopy studies of two-dimensional crystals and single molecules of FAO [112, 125]; d) supposed structure of the FAD intermediate during the oxidation of alcohols by FAO.

some mutant strains the content of FMO constitutes 50% of total cell protein.

Localization and synthesis. In yeast, FMO is located in peroxisomes [93]. The peroxisomes have been studied in detail for the genera *Hansenula*, *Candida*, and *Pichia* [94-96]. Cells grown on glucose contain few peroxisomes, but their content sharply increases while growing on methanol or on methanol in the presence of glycerol. In this case, their content may constitute 80% of the cytoplasm volume.

Peroxisomes contain a crystalloid composed predominantly from FMO. They also contain catalase and dihydroxyacetone synthase. Other proteins are present in the peroxisomes in much lower amounts. Catalase is located at the periphery of the crystalloid and protects the cell from hydrogen peroxide. Since the core of the crystalloid is formed by FMO, other proteins located in the internal cavities of the crystalloid can move inside it and can be easily removed without changing its structure.

In contrast to other organelles of the cell, peroxisomes do not contain DNA, so all peroxisomal proteins are synthesized on the ribosomes in the cytoplasm. Immediately after the synthesis, the monomer of FMO interacts with pyruvate carboxylase (Pyc1p). Pyc1p in this case does not exhibit its catalytic function, but provides the monomeric FMO with a favorable conformation for FAD binding [97-104]. FAD binds to the protein noncovalently, yielding an enzymatically inactive monomer. During this process, according to spectrofluorimetric data, the structure of the monomer rearranges significantly [105]. This rearrangement presumably protects the protein from hydrolytic enzymes. The supposed spatial structure of the FMO monomer created based on the high similarity of the primary structures of the subunits of FMO and glucose oxidase and the spatial structure of glucose oxidase [85] is presented in Fig. 4b [105]. The LARF sequence at the C-terminus of the FMO subunit presumably facilitates its interaction with Pyc1p. The SKL sequence at the C-terminus of Pyc1p is important for the interaction with peroxisomal proteins. The monomer of FMO with bound FAD and Pyc1p interacts with the peroxisomal protein Pex5p that transports FMO into the

peroxisomes through the peroxisomal membrane by a mechanism that is not well understood. It is supposed that peroxisomal proteins 13-P, 14-P, and 15-P take part in this process. Inside the peroxisomes, FMO monomers form catalytically active octamers. This process is presumably assisted by chaperones, which may be proteins Pyc1p or Pex5p. The octamers of FMO form the core of the crystalline matrix of the peroxisomes. Catalase, dihydroxyacetone synthase, and some other proteins are synthesized in the cytoplasm and must be transported into the peroxisomes in the same way.

Methods of isolation. Yeast cells are grown in a reactor. The cell suspension is concentrated by centrifugation and washed twice with 0.02 M phosphate buffer, pH 6.25-8.0. The cells are broken and centrifuged (30 min, 5000*g*). FMO is isolated from the supernatant using classical methods: ammonium sulfate fractionation (40-60% saturation) and hydrophobic, ion-exchange, and gel-penetrating chromatography [106-109].

A convenient method for preparative purification of FMO is crystallization [110]. To purify FMO by this approach, the protein concentration must be rather high. This is achieved by breaking concentrated cell suspensions (no less than 75 g dry weight per liter), by concentrating the supernatant by ultrafiltration or ammonium sulfate fractionation with subsequent dialysis or ultrafiltration [111, 112]. Although crystallization is observed over a wide range of pH, the most preferable is pH 6.0-6.5 [111]. At ionic strength less than 0.01 M, some of the formed crystals dissolve. The time for crystallization is 18 h. The suspension of crystals is removed by centrifugation and stored in liquid nitrogen, dry ice, in a freezer at −80°C, or in the lyophilized form at temperatures from −10 to −20°C [112]. The yield of the electrophoretically pure enzyme is 60-80%. To stabilize the enzyme, sucrose (60%) or NaN_3 (0.02%) is added. Using the crystallization method, FMO has been successfully purified only from yeasts of the genus *Pichia*.

Properties of isolated FMO. Electrophoresis of purified native FMO in polyacrylamide gel without SDS gives nine protein bands. SDS-PAGE of the same preparation yields only one protein band corresponding to 72 kDa. This phenomenon was first described by Gruzman et al. [113], and later it was supported by others [112, 114-116]. Analytical centrifugation of the native FMO resulted in a single peak with sedimentation coefficient $S^0_{20,\hat{a}} = 19.2$ S and diffusion coefficient $D^0_{20,\hat{a}} = 3.1$ cm/sec, this corresponding to the molecular weight of FMO, approximately 580 kDa. Gel filtration of FMO preparation using gels with a low degree of cross-linking resulted in only one peak with molecular weight of 600 kDa. Thus, the enzyme consists of eight subunits of the same molecular weight and contains eight FAD molecules.

Two genes coding FMO were revealed in 1989 [117], but their products were closely studied much later [118-122]. It was found that methylotrophic yeast have genes encoding two different subunits, α and β [114]. These subunits have the same molecular weight but different number of acidic and basic amino acid residues, the p*I* values being 4.18 and 5.12 [114]. For this reason, native PAGE results in several protein bands, but in the presence of SDS, only one protein band is observed. In the yeast cell, α-subunits encoded by gene *MOD1* are synthesized only at low concentrations of methanol or in the presence of methanol with glycerol [120] or pectin and oxidizes methanol at low concentration of the alcohol. The β-subunits encoded by gene *MOD2* are synthesized only at high methanol concentrations (exceeding 3%). Glycerol and pectin do not influence the synthesis of β-subunits. Thus, α and β subunits of the octamer are active at different concentrations of the substrate.

Mutant forms have been obtained possessing only one gene, *MOD1* or *MOD2*. The α- and β-subunits produced by these mutant forms have been isolated. Native PAGE of both preparations resulted in single bands. However, after mixing the α- and β-subunits in the presence of high glycerol concentration (under these conditions, FMO dissociates into subunits) and subsequent dilution, PAGE of the resulting FMO preparation in the absence of SDS gave several protein bands. This supported the idea that the FMO molecule may be formed from different subunits. The physiological role of the nine possible isoenzymes of FMO is still unclear. Perhaps each of the subunits has some additional enzymatic function [122].

Urea and guanidine hydrochloride dissociate FMO into subunits, release FAD, completely inactivate the enzyme, and cause the enzyme to aggregate and precipitate. At high concentrations of glycerol, the active FMO octamer dissociates into separate subunits without loss of FAD. After the removal of glycerol by dilution, the active octamer reassembles. In contrast, the addition of 50% dimethylsulfoxide to FMO results in the loss of FAD. After the subsequent removal of dimethylsulfoxide, the monomers remain in the soluble form. Moreover, they are capable of interacting with Pyc1p [124], but they do not penetrate inside liposomes and exhibit no enzymatic activity.

The structures of the octamer in artificial crystals and in the crystalloid are virtually identical [125, 126]. The protein molecule is composed of two layers in which the subunits are located in the apexes of squares whose planes are arranged one above another with rotation of approximately 45°. According to electron microscopy data, the structure of the molecule does not depend on its subunit composition [112, 125] (Fig. 4c).

Each subunit contains noncovalently bound FAD. Therefore, each FMO molecule contains eight FAD molecules, and for this reason the enzyme in the oxidized state has yellow color. However, after the addition of sodium azide its color becomes bright red (absorption maxima at 367, 383, and 456 nm and a shoulder at 520 nm)

[127]. The FMO molecule binds 8 mol of sodium azide per mol FMO, i.e. sodium azide reacts with each of the subunits. At low concentrations, sodium azide does not inhibit the enzyme activity.

FMO is active in the pH range of 5-9 exhibiting maximal activity at pH 8.0-8.5. The enzyme activity does not depend on the nature of the buffer solution.

FMO of microorganisms catalyzes the oxidation of lower primary saturated and unsaturated alcohols to aldehydes with the following yield (percentage in reference to methanol): methanol, 100; ethanol, 85-92; propanol, 60-70; butanol, 30-40; pentanol, 5-10; mercaptoethanol, 20-25; formaldehyde, 12. The enzyme does not oxidize branched C–C chains and secondary alcohols [114].

Cyclopropanol is an irreversible inhibitor of FMO [128]. The stoichiometric binding of this compound to the oxidized enzyme decreases the absorption at 450 nm, which indicates its interaction with the FAD cofactor. The FAD molecule separated from the inhibited enzyme contains a linear aldehyde, but not cyclopropanal. According to the data of NMR, IR, and UV spectroscopy, the inactivation of the enzyme by cyclopropanol is due to covalent modification of the N5 atom of the isoalloxazine ring. The production of a linear aldehyde in the reaction of cyclopropanol with the isoalloxazine moiety of FMO (in contrast to the earlier work [129]) suggests that the reaction proceeds without participation of the radical.

Among numerous flavin enzymes, FMO is the only enzyme from which a modified FAD cofactor (mFAD) was separated [127]. The mFAD molecule differs from FAD in the position of one OH-group in the carbohydrate part of the flavin moiety of the coenzyme. Later, the presence of mFAD was supported in works of Russian [130-132] and Japanese authors [116-119]. Currently, it is considered that mFAD is a xylo-FAD [133] and it is formed in the cell not spontaneously, but during the biosynthetic processes. The content of mFAD in the purified FMO depends to a great extent on the procedure of flow cultivation and can vary from zero to several tens of percents. The role of mFAD in the enzyme has not been ascertained. The subunits containing mFAD may have some regulatory function or some additional enzymatic activity, for example, ATPase [123].

The thermostable FMO purified from the methylotrophic fungus *Thermoascus aurantiacus* NBRC 31693 differs from the classic enzyme in molecular properties, and besides methanol and ethanol, it oxidizes secondary and aromatic alcohols [133]. Recently, FMO has been isolated and purified from other organisms [134-136].

In spite of several attempts [137, 138] and easy crystallization of the enzyme [110-112, 139], X-ray analysis has not succeeded in determining the spatial structure of FMO. This may be accounted for microheterogeneity of the enzyme (combinations of different subunits in the enzyme molecule and unpredictable amount of mFAD in crystallized preparations of the enzyme).

Catalytic mechanism of FMO. Methanol is located in proximity of the N5 atom of the FAD coenzyme and the W residues. The first hydrogen atom is eliminated from the hydroxyl residue, and the second one from the HC-group of the alcohol. The FAD coenzyme is presumably reduced by a hydride ion with fast electron transfer to molecular oxygen [140-142]. Molecular oxygen interacts with the reduced isoalloxazine moiety of FAD (similarly to FAD-dependent hydroxylases [82] and oxidase [83]) presumably at the C4a atom resulting in the formation of C4a-hydroperoxy flavin intermediate (Fig. 4d), which is rather unstable. Perhaps it could be revealed by fast kinetics approaches at low temperature. The reaction products are formaldehyde and H_2O_2. Since 5,5′-dithiobis(2-nitrobenzoic acid), *p*-chloromercuribenzoate, and iodoacetate inactivate FMO [112, 113], this suggesting that SH-groups of cysteine residues of the enzyme are in some way involved in the oxidation of alcohols.

Practical application. FMO is widely used for the determination of ethanol concentration in beer, wine, and other alcohol beverages using different detectors (amperometric, potentiometric, gravimetric, optical, etc.) [143-153]. The application of FMO with peroxidase [154] greatly increases the possible use of this enzyme for determination of ethanol.

Under certain pH values and medium composition, some FMOs and methylotrophic yeast can be used for practical purposes at elevated temperatures [155].

The enzyme can also be used as a producer of H_2O_2 for bleaching tissues, as the oxygen scavenger in different devices, particularly to increase storage period of preserved food, as a catalyst while producing aldehydes from alcohols and chiral aldehydes from a racemic mixture of alcohols [156], and as a component for biofilters for purification of industrial wastewater [157].

FMO has two strong promoters [158], due to which methylotrophic yeasts are widely used for producing heterologous proteins. More than five hundred different proteins from different organisms have been cloned in methylotrophic yeast. Most of them were cloned in the yeasts *Candida boidinii*, *Hansenula polymorpha*, *Pichia methanolica*, and *Pichia pastoris*. Expression systems of methylotrophic yeast are rapidly responsive to genetic manipulations. Auxotrophic mutants facilitate expression of proteins and produce genetically stable hosts. Their stability is maintained by the introduction of the information into the chromosomal DNA. These yeast mutants are the best producers of heterologous proteins. They can be grown on inexpensive salt media under flow cultivation reaching high concentrations (~150 g dry substance per liter culture), releasing several grams of heterologous proteins into the culture liquid. Yeasts have low risk of contamination with endotoxins, oncogenes, and viral DNAs. Cloned proteins, in contrast to endogenous proteins, are usually released into the culture liquid, this significantly facilitating their purification. Currently, methylotrophic

yeast is considered to be the best biofactory for producing industrial amounts of desired peptides and proteins [159].

Some examples of cloned proteins are the following: galactose oxidase [160], herring antifreeze protein [161], lipase [162], human interleukin 2 [163], single-chain antibody fragment [164], pleurocidin [165], B virus protein [166], avidin [167], human antithrombin [168], human M carboxylase [169], lipase [170], human type II collagen [171], and isopenicillin N synthase [172]. More detailed information on the cloning and sequencing of heterologous proteins by methylotrophic yeast can be found in reviews [173-179].

Eukaryotic and prokaryotic unicellular microorganisms can use single-carbon substrates as the sole source of carbon and energy. However, key enzymes of these microorganisms significantly differ in the structure of both the polypeptide chain and cofactors. This suggests that these groups of enzymes and microorganisms originated independently, in spite of the great similarity in the mechanism of oxidation of the substrates.

Some changes in protein structure may result in the emergence of enzymes having similar structure but absolutely different properties. This results in the appearance of new microorganisms. A bright example is PQQ-containing enzymes. They are well studied in terms of reaction kinetics. The structures of these proteins are investigated by ultramicroscopy, EPR, NMR, and X-ray studies. Different theoretical methods have been used to specify the mechanism of action of these enzymes.

Low molecular weight alcohols play an important role in industry, medicine, and everyday human life. Thus, accumulation of information on enzymatic conversions of these alcohols and their application for chip production of heterologous and feeder proteins is of great practical significance.

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