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REVIEW

Unsaturated Organic Acids as Terminal Electron Acceptors for Reductase Chains of Anaerobic Bacteria

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Abstract—This paper summarizes the current knowledge of unsaturated organic acids in their role as terminal electron acceptors for reductase chains of anaerobic bacteria. The mechanisms and enzyme systems involved in the reduction of fumarate by *Escherichia coli, Wolinella succinogenes*, and some species of the genus *Shewanella* are considered. Particular attention is given to reduction of the double bond of the unnatural compound methacrylate by the δ-proteobacterium *Geobacter sulfurreducens* AM-1. Soluble periplasmic flavocytochromes *c*, found in bacteria of the genera *Shewanella* and *Geobacter*, are involved in the hydrogenation of fumarate (in *Shewanella* species) and methacrylate (in *G. sulfurreducens* AM-1). In *E. coli* and *W. succinogenes*, fumarate is reduced in cytosol by membrane-bound fumarate reductases. The prospects for research into organic acid reduction at double bonds in bacteria are discussed.

Key words: anaerobic respiration, reduction of double bonds, *Wolinella succinogenes, Shewanella, Geobacter sulfurreducens*, fumarate reductase, methacrylate reductase, flavocytochrome *c*.

Many anaerobic bacteria produce ATP through either substrate phosphorylation or phosphorylation coupled with electron transport to certain organic and inorganic compounds, which serve as terminal electron acceptors. As a rule, the redox potential E_o' of such compounds is more negative than that of oxygen, due to which the energy yield of anaerobic respiration is lower (no more than 1 molecule of ATP is synthesized per two electrons transferred via the electron transport chain) than in the case of aerobic respiration [1].

Hydrogenation of the double bonds of organic acids is a form of anaerobic respiration that is widespread among various taxonomic groups of strictly and facultatively anaerobic bacteria (see Table 1; [2–13]). However, the number of unsaturated compounds that can be used by bacteria as terminal electron acceptors is small.

The use of fumarate as a terminal electron acceptor in anaerobic bacteria is well known [14–18]. There are also a number of studies concerned with reduction of the synthetic compound methacrylate by the anaerobic δ-proteobacterium *Geobacter sulfurreducens* AM-1 [13, 19, 20]. However, little is known about the enzymes involved in the reduction of the phenylpropionic acid derivatives acrylate and pentenoate.

The enoate reductase found in bacteria of the genus *Clostridium* [21] catalyzes the reduction of various α,β-unsaturated carboxylic acids (enoates), with NADH and reduced viologen dyes acting as electron acceptors. However, there is no direct evidence that the reaction catalyzed by enoate reductase, which represents an iron–sulfur flavoprotein, is coupled with energy conservation.

The aim of this paper is to review recent data on the use of unsaturated organic acids by anaerobic bacteria as terminal electron acceptors.

FUMARATE AS AN ELECTRON ACCEPTOR: CLASSIC MEMBRANE-BOUND FUMARATE REDUCTASES

E. coli and *W. succinogenes* are able to grow due to fumarate reduction with hydrogen or formate according to the reactions

$$H_2 + \text{fumarate} \longrightarrow \text{succinate}, \quad (1)$$

$$\text{Formate} + \text{fumarate} + H^+ \longrightarrow CO_2 + \text{succinate}. \quad (2)$$

It is known that anaerobic bacteria reduce compounds with double bonds by using them as electron acceptors with the aid of electron transport chains [14, 15, 22]. Such redox chains contain at least three components: (1) dehydrogenase specific to an electron donor, (2) menaquinone (or demethylmenaquinone), and (3) fumarate reductase [22–25]. Dehydrogenases catalyze the reduction of bacterial menaquinone into the respective hydroquinone, with certain substrates serving as electron donors. Fumarate reductase (more specifically, menaquinol–fumarate oxidoreductase) reoxidizes hydroquinone into quinone and reduces fumarate [14, 15, 24]. The hydrogenase and formate dehydrogenase of the reductase chain of *W. succino-*

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Table 1. Unsaturated organic compounds used by bacteria as terminal electron acceptors

Bacteria	Donors	Acceptors	Ref.
Escherichia coli	Lactate, glycerol, formate, hydrogen, malate	Fumarate	[2]
Shewanella frigidimarina	Formate	Fumarate	[3]
Shewanella oneidensis	Lactate, succinate	Fumarate	[4]
Bacillus macerans	Glycerol	Fumarate	[5]
Wolinella succinogenes	Formate, hydrogen	Fumarate	[6]
Desulfuromonas acetoxidans	Acetate, ethanol, propanol, butanol, butanol + succinate, pyruvate	Fumarate	[7]
Desulfovibrio multispirans	Lactate	Fumarate	[8]
Geobacter sulfurreducens PCA^T	Acetate	Fumarate	[9]
Acetobacterium woodii	Hydrogen	Ferulate Caffeate	[10]
Syntrophospora bryantii	Butyrate	Pentenoate	[11]
Desulfovibrio acrylicus	Lactate, fumarate, succinate, malate, pyruvate, ethanol, propanol, glycerol, glycine, serine, alanine, cysteine, formate, hydrogen	Acrylate	[12]
Geobacter sulfurreducens AM-1	Acetate, formate, hydrogen	Methacrylate	[13]

Note: The table summarizes the most thoroughly studied bacteria able to utilize unsaturated organic compounds as terminal electron acceptors.

genes (Fig. 1) face the periplasmic space with their hydrophilic subunits, whereas the fumarate reductase of this bacterium faces the cytoplasm [15, 16, 24]. Fumarate reductase, which contains FAD, iron–sulfur proteins, and, in some cases, heme *b*, is located on the inner side of the cytoplasmic membrane. It is an enzyme complex (quinol:fumarate reductase (QFR)) composed of three or four nonidentical subunits [16, 23–31]. QFR, together with succinate:quinone oxidoreductase (SQR, complex II), is involved in aerobic metabolism as one of the key enzymes of the tricarboxylic acid (TCA) cycle and of the aerobic respiration chain and relates to succinate:quinone oxidoreductases (EC 1.3.5.1).

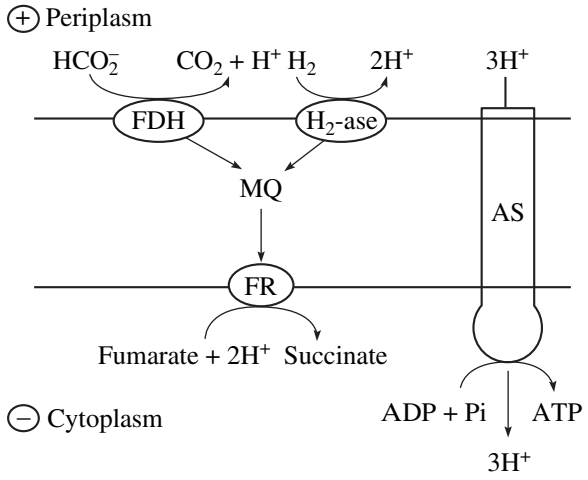


Fig. 1. Composition and organization of the electron transport chain of *W. succinogenes*: a putative mechanism of $\Delta\mu_{H^+}$ generation driven by transmembrane electron transfer [42, 43]. The following designations are used: FDH, formate dehydrogenase; H_2-ase, hydrogenase; FR, fumarate reductase; MQ, menaquinone; and AS, ATP synthase.

In the ε-proteobacterium *W. succinogenes*, each subunit of fumarate reductase (menaquinol-fumarate oxidoreductase) FrdCAB is composed of one molecule (see Table 2) [16, 24, 32]. The large hydrophilic subunit FrdA (79 kDa) has a substrate-binding center and FAD, the latter being covalently bound to the histidine residue of the polypeptide chain. The hydrophobic subunit FrdC (25 kDa) anchors fumarate reductase in the membrane. This subunit represents cytochrome *b*, with two heme groups possessing different redox potentials, specifically, –220 to –200 mV and –20 to –10 mV [16, 26]. The heme with the higher redox potential is involved in electron transport from reduced quinone to the hydrophilic subunits of fumarate reductase. The small hydrophilic subunit FrdB (31 kDa) is located between the large hydrophilic and the hydrophobic subunits of fumarate reductase and mediates electron transfer between cytochrome *b* (–20–10 mV) and FAD [27]. This subunit has an iron–sulfur center [2Fe-2S]. The location of the other two iron–sulfur centers ([3Fe-4S] and [4Fe-4S]) is unknown. The *frdCAB* operon, which encodes the fumarate reductase subunits, was sequenced by Simon *et al.* [33]. The enzyme was obtained in crystal form by Lancaster *et al.* [34, 35].

The *fccA* gene, which encodes the protein FccA found in *W. succinogenes*, is cotranscribed with two other genes: *fccB* and *fccC*. Some authors have suggested that this protein is a soluble fumarate reductase [36], although there is no direct supporting evidence.

Table 2. Terminal bacterial reductases of unsaturated organic acids

Bacteria	Terminal reductases	Cellular location	Molecular mass	Redox centers	Accession numbers of genes or operons	Accession numbers of protein crystal structures
Escherichia coli	Fumarate reductase FrdABCD [23, 30]	Inner surface of cytoplasmic membrane	FrdA (66 kDa)	FAD	J01611 [37–39]	1KF6, 1KFY [40, 41]
			FrdB (27 kDa)	2Fe-2S, 4Fe-4S, 3Fe-4S		
			FrdC (15 kDa)			
			FrdD (13 kDa)			
Wolinella succinogenes	Fumarate reductase FrdCAB [16, 24, 32]	Inner surface of cytoplasmic membrane	FrdA (79 kDa)	FAD	AJ000662 [33]	1E7P [34, 35]
			FrdC (25 kDa)	2 hemes *b*		
			FrdB (31 kDa)	2Fe-2S, 4Fe-4S, 3Fe-4S		
Shewanella frigidimarina	Flavocytochrome c_3–fumarate reductase Ifc_3 [51]	Periplasm	63.9 kDa	FAD 4 hemes *c*	AJ236923 [51, 59]	1QO8 [60]
	Flavocytochrome c_3–fumarate reductase Fcc_3 [3, 52]	Periplasm	63.8 kDa	FAD 4 hemes *c*	AJ132010 [52]	1QJD [55, 56]
Shewanella oneidensis	Flavocytochrome *c*– fumarate reductase Fcc_3 [36, 61]	Periplasm	65 kDa	FAD 4 hemes *c*	AE015541 [36, 49, 62]	1D4C, 1D4D, 1D4E [63]
Geobacter sulfurreducens AM-1	Methacrylate reductase [20]	Periplasm	50 kDa	FAD	–	–
Acetobacterium woodii	Phenylpropenoate reductase [79]	Soluble fraction	–	–	–	–

Note: The table summarizes data on the crystal structure of enzymes and the nucleotide sequences of their genes available in the Entrez database of the National Center of Biological Information at www.ncbi.nlm.nih.gov.

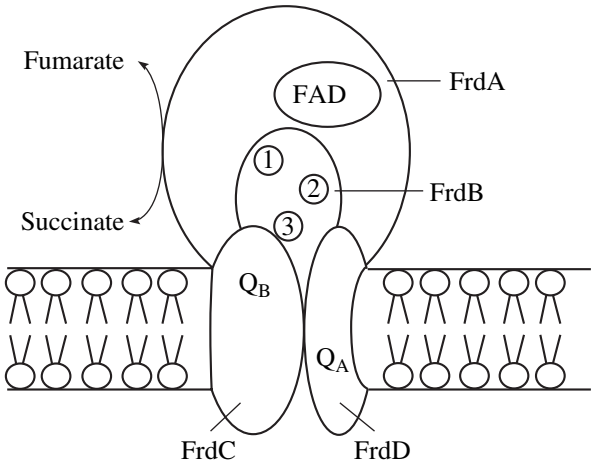


Fig. 2. Topographic model for the fumarate reductase of *E. coli* [17, 23, 30]. FrdA is the large hydrophilic subunit, FrdB is the small hydrophilic subunit, and FrdC and FrdD are hydrophobic subunits. 1, 2, and 3 are redox centers and Q_A and Q_B are quinone-binding centers.

The fumarate reductase of the γ-proteobacterium *E. coli*, unlike that of *W. succinogenes*, is a tetramer (see Fig. 2 and Table 2). The molecule of this enzyme contains two structural domains: a hydrophilic catalytic domain and a hydrophobic membrane-bound domain [23, 30]. Each of these domains is composed of two subunits. The FrdC (15 kDa) and FrdD (13 kDa) subunits of the hydrophobic membrane-bound domain are involved in binding catalytic subunits to the inner surface of the cytoplasmic membrane and in electron transfer [17, 31]. There is also evidence that these subunits induce conformational changes in the catalytic domain that stabilize the enzyme molecules [23]. FrdA (66 kDa), the large subunit of the catalytic domain, contains FAD and an active enzyme center [32]. The FAD molecule is bound covalently at position 8α of the N(3)-histidine residue of the polypeptide chain and noncovalently to two FAD-binding domains [32]. The potential E_o^I of the 8α-histidyl-FAD/$FADH_2$ pair is equal to –55 mM; i.e., it is considerably higher than that of free FAD (–219 mV) [17]. The higher redox potential of the bound FAD facilitates its reduction with menaquinol and succinate. The large hydrophilic FrdA subunit covers the small subunit FrdB (27 kDa) (Fig. 2) [23], which contains three different iron–sulfur centers formed by eleven cysteine residues. Center 1 [2Fe-2S] has E_m^{oI} = – 79–(–20) mV, center 2 [4Fe-4S] has E_m^{oI} = –320 mV, and center 3 [3Fe-4S] has E_m^{oI} = –70 mV [17, 32]. It is believed that center 3 is closely bound to one of the quinone-binding centers found in fumarate reductase [31], thereby participating in the interaction of quinone with the hydrophobic subunits. Attempts to detect cytochrome *b* in the fumarate reductase of *E. coli* have not been successful [23].

The *frd* operon of the fumarate reductase of *E. coli* includes four genes, which are cotranscribed in the form of polycistronic RNA in the following order: *frdA, frdB, frdC*, and *frdD* [37–39]. Cole *et al.* [37, 38] determined the nucleotide sequence of this fumarate reductase and Iverson *et al.* [40, 41] determined its crystal structure.

In *W. succinogenes*, reactions (1) and (2) generate a potential of about 180 mM on the cell membrane of this bacterium, with the potential being positive outside it [42]. According to the chemiosmotic theory of Mitchell, ATP is synthesized by the ATPase of the cytoplasmic membrane of this bacterium at the expense of the electrochemical potential $\Delta\mu_{H^+}$. It is suggested that this potential is produced by a transmembrane transfer of electrons without a translocation of protons across the membrane in a vector manner (Fig. 1) [14, 42, 43]. Consequently, electrons are transferred from a donor substrate to the acceptor fumarate in a vector manner, but the proton gradient is produced in a scalar manner. In *E. coli*, the proton gradient is produced similarly [44].

Fumarate is transported across the membrane to the substrate-binding center of fumarate reductase. Under anaerobic conditions, the bacterium *E. coli* has three carriers for C_4-dicarboxylic acids [45], each of which performs a homologous and heterologous antiport of dicarboxylic acids (for instance, fumarate and succinate), with three H^+ being produced [45, 46].

SOLUBLE FUMARATE REDUCTASES OF BACTERIA FROM THE GENUS *SHEWANELLA*

In 1999, γ-proteobacteria of the genus *Shewanella* were substantially reclassified on the basis of polyphasic taxonomy [47, 48]. Two strains of the iron-reducing bacterium *S. putrefaciens* were reclassified into strains of two different species: *S. oneidensis* MR-1 [47] and *S. frigidimarina* NCIMB 400 [48]. *S. oneidensis* was the first iron-reducing bacterium for which the complete genome was decoded [49]. An analysis of this genome showed the presence of 39 genes encoding cytochromes *c* (for comparison, *E. coli* has only seven cytochrome *c* genes) [49]. The fumarate reductases of both bacteria (Table 2) are soluble [3, 50].

The fumarate reductase of the bacterium *S. frigidimarina* NCIMB 400 is synthesized as two isoenzymes, Ifc_3 and Fcc_3, which catalyze only a direct reaction [51, 52]. The soluble flavocytochrome c_3-fumarate reductase Fcc_3 is located in the periplasmic space and has a molecular mass of 63.8 kDa [3, 52]. Other physicochemical parameters, kinetic characteristics, and structural properties [3], as well the nucleotide sequence of the flavocytochrome c_3 gene [52], have also been thoroughly studied.

The soluble periplasmic fumarate reductase of *S. frigidimarina* NCIMB 400 possesses low hydrophobicity and differs considerably from the membrane-bound fumarate reductases of other bacteria [3, 52, 53]. First,

it is a monomer. Second, this fumarate reductase contains noncovalently bound FAD (1 mole per mole protein, $E_o^I = -152$ mV). Third, instead of iron–sulfur centers, the periplasmic fumarate reductase contains four different hemes *c* with redox potentials of –238, –196, –146, and –102 mV. Fourth, flavocytochrome c_3 is synthesized with an N- terminal secretory signal sequence of 25 amino acid residues, which is responsible for the periplasmic location of this enzyme.

The mature protein contains 571 amino acid residues and has two domains. The N-terminal tetraheme cytochrome domain contains 117 residues, and the C-terminal flavoprotein domain contains 454 residues. The latter domain is involved in fumarate reduction and has many features in common with the flavoprotein subunits of membrane-bound fumarate reductases and succinate dehydrogenases of other organisms [52].

Based on spectroscopic and kinetic studies of amino acid sequences and redox potentials, Pealing *et al.* suggested that the four hemes of flavocytochrome *c* bound through His–His bridges are structurally and functionally organized into two pairs [54]. This circumstance makes the enzyme capable of operating as a two-electron transferase. The known crystal structure of the enzyme [55, 56] admits the residence of a malate-like molecule in the active center of fumarate reductase. The mechanism of fumarate reduction can be conceived as follows: The fumarate molecule is polarized and inverted, which facilitates the transfer of hydride and, hence, a proton from the reduced flavin. In the oxidized state, the active center of the enzyme is immersed between the flavin-containing and clamp domains. A displacement of the cytochrome and clamp domains may result in release of the product.

The major physiological function of flavocytochrome c_3 is to reduce fumarate [3, 57]. According to the hypothesis of Pealing *et al.*, the physiological donor of electrons for fumarate reductase in *S. frigidimarina* is menaquinone ($E_m^{oI} = -74$ mV) [54]. If this is the case, a modifying factor must change the redox potentials of the hemes or menaquinone, since the redox potential of menaquinone is considerably less negative than that of any of the hemes.

The reduction of flavocytochrome *c* by formate, the absence of cytochrome *b*, and the closeness of the redox potentials of the formate–CO_2 pair and the flavocytochrome hemes has made it possible to suggest the absence of an intermediate carrier between formate dehydrogenase and flavocytochrome *c* [3].

The 63.9-kDa periplasmic tetraheme flavocytochrome *c* (Ifc_3) is synthesized under anaerobic conditions when the only terminal electron acceptor is iron citrate or pyrophosphate but not fumarate [51]. Ifc_3 exhibits fumarate reductase activity only *in vitro*. The homogeneous preparation of Ifc_3 contains noncovalently bound FAD and four hemes *c* connected through His–His bridges. The redox potentials of the hemes are –73, –141, – 174, and –259 mV [51]. Based on voltammetric studies, Butt *et al.* suggested two pathways of electron transfer to the active center of Ifc_3 [58]. The heme *c* with the lowest potential acts as an electronic relay in one of these pathways, modulating the reduction rate of fumarate. The nucleotide sequence of the Ifc_3 gene is known [51, 59]. The crystal structure of Ifc_3 has been studied in an open conformation [60]. Ifc_3 is an asymmetric octaheme diflavin homodimer with a molecular mass of 130 kDa in its native form.

A fractionation study of *S. oneidensis* cells using four different methods has shown that the soluble fraction of these cells contains more than 98% of fumarate reductase activity. In some experiments, the highest fumarate reductase activity was detected in the periplasmic fraction [50].

Mutant studies have shown that the bacterium *S. oneidensis* contains a soluble 65-kDa fumarate reductase, which was found to be a tetraheme flavocytochrome *c* [61]. This low-potential tetraheme flavocytochrome *c*, Fcc_3, has been isolated from *S. oneidensis* cells [36]. The synthesis of Fcc_3 was found to be controlled by anaerobiosis and the presence of fumarate. Fcc_3 was shown to be 59% homologous to the fumarate reductase of *S. frigidimarina* [36]. The nucleotide sequence of the *fccA* gene encoding Fcc_3 was determined from a genome analysis and with the aid of transposon mutant strains [49, 62]. Leys *et al.* described the crystal structure of the fumarate reductase of *S. oneidensis* and its mechanism of action [63]. All the redox centers of this enzyme show Van der Waals interactions, thereby being capable of providing for the efficient transfer of electrons from hemes to fumarate via FAD [63]. Fcc_3 is the only fumarate reductase of *S. oneidensis* with physiological importance among several potential fumarate reductases revealed from the amino acid homology of the genome sequence [49, 61, 62].

In *S. oneidensis*, the tetraheme cytochrome *c*, CymA, is involved in fumarate reduction [64]. The 21-kDa protein CymA is detected in both cytoplasmic and soluble cell fractions. The CymA protein isolated from *S. frigidimarina* cells has a molecular mass of 20 kDa and contains four hemes with redox potentials of +10, –108, –146, and –229 mV [65]. The CymA proteins of both bacteria are homologous to the TorC, NapC, and NirT periplasmic electron carriers of other bacteria. This circumstance suggests that the CymA proteins of both *Shewanella* species are involved in electron transfer from reduced menaquinone to several terminal reductases, including fumarate reductase, located in the periplasm and/or in the outer membrane [65, 66].

The 35-kDa outer-membrane protein (Omp35) of *S. oneidensis* is necessary for the normal growth of this bacterium on fumarate, nitrate, and Fe(III) [67]. It is believed that Omp35 is a porin that is regulated anaerobically by a post- transcriptional mechanism. Thus, even proteins that are not components of redox systems can influence the anaerobic respiration phenotype.

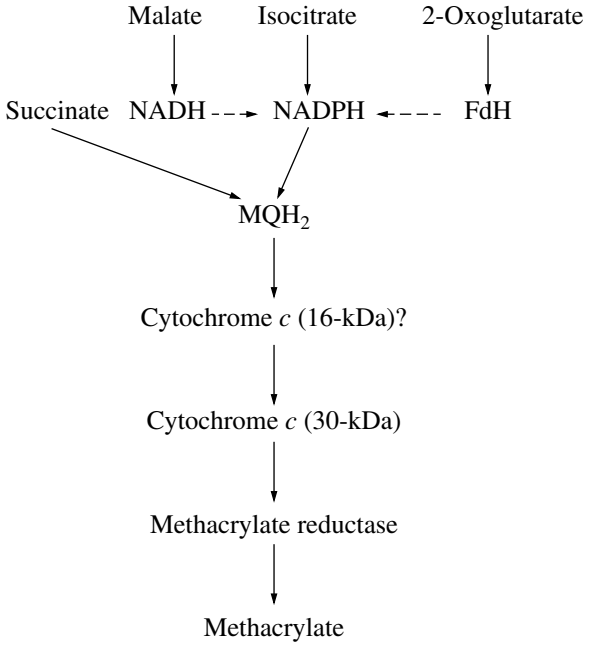


Fig. 3. Putative scheme of electron transfer from acetate to methacrylate in the bacterium *G. sulfurreducens* AM-1 [20].

METHACRYLATE AS A TERMINAL ELECTRON ACCEPTOR IN *GEOBACTER SULFURREDUCENS*

The interest of researchers in the acetate-oxidizing iron-reducing bacteria of the genus *Geobacter* (a group of δ-proteobacteria) is due to the part they play in bioremediation of radioactive metals and electricity generation [9, 68, 69]. These bacteria reduce Fe(III) to Fe(II) and are involved in humus decomposition, which suggests that they have an essential role in the global cycles of metals and carbon. Both iron reduction and humus decomposition require cytochrome *c*. Intense decade-long investigations of the type strain *G. sulfurreducens* PCA led to the decoding of the genome of this bacterium [70], which was found to contain a great number of genes encoding electron- transport proteins, including 111 cytochromes *c*. Among them, 73 of the cytochromes *c* contain 2 or more hemes and 1 cytochrome *c* contains as many as 27 hemes.

During study of the decomposition of methacrylate wastes, Galushko *et al.* isolated another *G. sulfurreducens* strain, which was designated AM-1, in a pure culture [13]. This strain can utilize fumarate, malate, methacrylate, sulfur, and Fe(III) as electron acceptors and acetate, formate, and molecular hydrogen as electron donors. The reduction of the unnatural synthetic compound methacrylate, coupled with the oxidation of another organic compound, acetate, has attracted particular interest. The reduction proceeds according to the reaction

$$H_3C{-}COO^- + 3H_2C{=}\underset{\displaystyle CH_3}{\underset{|}{C}}{-}COO^- + 4H_2O \longrightarrow 3H_3C{-}\underset{\displaystyle CH_3}{\underset{|}{CH}}{-}COO^- + 2HCO_3^- \quad (3)$$

In the bacteria *Desulfuromonas acetoxidans* and *G. sulfurreducens* PCA^T, which oxidize acetate coupled to fumarate reduction, fumarate serves not only as a terminal electron acceptor but also as an intermediate product of acetate dehydrogenation [7, 9]. In contrast, strain AM-1 is the only known organism that completely oxidizes one organic substance (acetate) through the reduction of another organic substance (methacrylate) [13].

The catabolism of acetate in *G. sulfurreducens* AM-1 has been studied using cells grown on acetate and methacrylate [20]. In similar studies of *G. sulfurreducens* PCA^T, cells have been grown either on acetate and fumarate or in a syntrophic coculture with *W. succinogenes* on acetate and nitrite [71]. Both strains of *G. sulfurreducens* oxidize acetate in the TCA cycle [20, 71]. The strain *G. sulfurreducens* PCA^T oxidizes acetate, with fumarate acting as the electron acceptor, via a truncated TCA cycle lacking succinate dehydrogenase but containing fumarate reductase as the key enzyme of electron transfer [71]. In strain AM-1 and the syntrophic association of strain PCA^T with *W. succinogenes*, acetate is dehydrogenated via the complete citric acid cycle. The operation of the TCA cycle in both the AM-1 and the PCA^T strains gives rise to reducing equivalents (NADH, NADPH, and FdH) and reduced menaquinone (MQH_2).

Transfer of electrons and protons to the terminal acceptor methacrylate via the TCA cycle may occur according to the following putative scheme (Fig. 3). NADPH, which is formed in the TCA cycle or results from operation of Fd^{red}–$NADP^+$ and NADH–$NADP^+$ transhydrogenases, transfers electrons and protons from the cytoplasm to menaquinone MQ-8 (this menaquinone, with 8 isoprenoid residues in the side chain, was found in the membrane fraction of strain AM-1) [19, 72]. The menaquinone, either directly or via an intermediate carrier, reduces the 30-kDa periplasmic tetraheme cytochrome *c* [20]. The periplasmic cytochrome *c*–methacrylate oxidoreductase (methacrylate reductase) accepts electrons from the aforementioned cytochrome *c* and reduces methacrylate to isobutyrate.

The periplasmic location of the methacrylate reductase of *G. sulfurreducens* (Table 2) was reported in [19, 73]. Study of purified methacrylate reductase showed that it is composed of one polypeptide chain with a molecular mass of approximately 50 kDa and contains 1 mole of noncovalently bound FAD but no iron or acid-labile sulfur. These data suggest that methacrylate reductase has no iron–sulfur centers [20].

The activity of purified methacrylate reductase depends on the presence of a flavin-containing protein

and 30-kDa tetraheme cytochrome *c*. The latter has also been isolated from the periplasmic fraction of *G. sulfurreducens* AM-1 [20]. The enzyme activity was found to be at a maximum when the flavin protein and cytochrome *c* are present in the reaction mixture in a molar proportion of 1 : 1. The protein and cytochrome *c* can easily be separated by chromatography on hydroxyapatite. The very low apparent K_M of methacrylate reductase for cytochrome *c* (0.12 μM) suggests that the flavin- containing protein and cytochrome *c* closely interact in the periplasm.

Along with the monomeric flavocytochromes *c* of *Shewanella* bacteria with fumarate reductase activity [3, 50, 52], the available publications describe a number of flavocytochromes *c* from other bacteria [74]. These flavocytochromes *c* are composed of two different subunits and have different enzyme activities [75, 76].

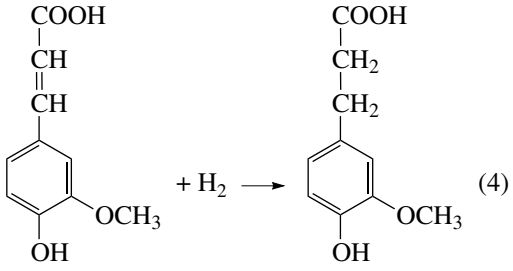
Purified methacrylate reductase can use not only methacrylate as the electron acceptor but also other unsaturated organic compounds, such as acrylate, crotonate, and pentenoate [20]. The enzyme shows the highest specificity for methacrylate and acrylate; however, unlike methacrylate, acrylate cannot be used by *G. sulfurreducens* AM-1 cells for growth. There is evidence that the bacterium *Desulfovibrio acrylicus*, which has been isolated from bottom sediments, also reduces acrylate; however, the relevant enzymes were not described [12]. The difference between the activity of *G. sulfurreducens* AM-1 cells and their methacrylate reductase toward acrylate may indicate that this reductase and its regulation have a sophisticated character.

A comparative analysis of the amino acid sequence of the N-terminus of methacrylate reductase has shown the similarity of their nucleotide-binding regions to some FAD-containing proteins of proteobacteria, namely, the flavocytochromes of *S. oneidensis* and *W. succinogenes* and the fumarate reductase of *S. frigidimarina.* The highest similarity was observed for two flavoproteins of *S. oneidensis*, AAN56611 (95% identity) and AAN54479 (90% identity), and the protein FccA of *W. succinogenes* (76% identity) [20]. It was found that the *fccA* gene of the 52-kDa protein FccA is cotranscribed with two other genes, *fccB* and *fccC*, encoding tetraheme cytochromes *c* [77]. This periplasmic protein does not have fumarate reductase activity and its function remained unknown until 2001, when Gross *et al.* [78] succeeded in obtaining mutant *W. succinogenes* strains deficient in the *fccABC* operon. Unlike the cell extract of the parent strain, the cell extracts of the mutant strains could not reduce methacrylate. These data suggest that the product of the *fccA* gene is necessary for the periplasmic methacrylate reductase activity of *W. succinogenes*, although this bacterium is unable to grow in the presence of methacrylate as the terminal electron acceptor.

What is the mechanism of methacrylate reduction by menaquinone? It is known that the 30-kDa cytochrome *c* donates electrons to methacrylate reductase and, hence, may potentially be a physiological acceptor of electrons from menaquinone. Experiments have shown, however, that reduced menadione and dimethylmenaquinone (menaquinone analogues) cannot reduce methacrylate in the presence of methacrylate reductase and the 30-kDa cytochrome *c* [20]. At the same time, there is evidence that another cytochrome *c* may be involved in electron transfer from reduced menaquinone to the 30-kDa cytochrome *c*. Indeed, the operon that encodes the 52-kDa flavin-containing FccA protein of *W. succinogenes* is homologous to the N-terminal amino acid sequence of methacrylate reductase and contains two ORFs of *fccB* and *fccC*, which encode tetraheme cytochromes *c*. The N-terminal hydrophobic helix of the FccB protein fixes the enzyme in the membrane of *W. succinogenes.* The amino acid sequence of the FccB protein, including that of the helix, is very similar to the sequences of some cytochromes *c* of the NirT/NapC family, in particular, the tetraheme cytochrome *c* of *S. oneidensis* encoded by the *cymA* gene. Electrophoretic study of a membrane fraction of *G. sulfurreducens* AM-1 grown on methacrylate showed the presence of a 16-kDa cytochrome *c* [72], which is probably involved in electron transfer between menaquinone and the 30-kDa tetraheme cytochrome *c* in the process of methacrylate respiration.

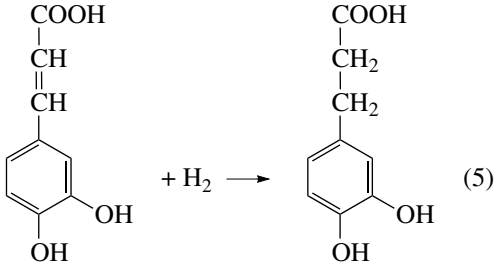
REDUCTION OF THE DOUBLE BOND IN THE SIDE CHAIN OF PHENYLPROPENOATE IN ACETOBACTERIUM WOODII

It is known that some acetogens are able to reduce the double bond in the side chain of cinnamate, forming hydroferulate from ferulate and hydrocaffeate from caffeate [10, 79]:



(4)

Ferulic acid — Hydroferulic acid



(5)

Caffeic acid — Hydrocaffeic acid

Hansen *et al.* showed that, in the bacterium *A. woodii* of the family *Clostridiaceae*, ATP formation is cou-

pled with caffeate reduction via the electron-transport chain [80]. Indeed, the concentration of ATP in a suspension of *A. woodii* cells incubated in a hydrogen atmosphere drastically increases in the presence of caffeate. The addition of a mixture of the ionophores valinomycin and nigericin diminishes the amount of ATP produced and augments the reduction rate of caffeate two times. The ionophore mixture probably eliminates the electrochemical proton gradient $\Delta\mu_{H^+}$, which comprises approximately 150 mV.

In order to elucidate whether caffeate reductase is a membrane-bound enzyme, Hansen *et al.* [80] subjected the cell extracts to fractionation. Unfortunately, none of the fractions were able to reduce caffeate with hydrogen, presumably because an intermediate electron carrier was lost in the process of fractionation. It should be noted that hydrogenase and caffeate reductase activities were detected for this bacterium in the soluble fraction (Table 2). The phenylpropenoate reductase activity of *A. woodii* was found to be NADH-dependent and inducible by ferulic acid [79]. The donor substrates for phenylpropenoate reductase can be either ferulate or caffeate. Thus, the roles of the NADH-dependent phenylpropenoate reductase and the soluble hydrogenase of *A. woodii* [81] in ATP formation remain unknown.

CONCLUSION

Most of the recent experimental data on the role of unsaturated organic acids as terminal electron acceptors of anaerobic bacteria refer to fumarate and methacrylate. Study of fumarate reduction in the ε-proteobacterium *W. succinogenes* was initiated by A. Kroeger about 30 years ago. Since that time, researchers have accumulated a great deal of knowledge about the fumarate reductases of *W. succinogenes* and other anaerobic bacteria (Table 2). Much is known about the crystal structure of these enzymes and the nucleotide sequence of the enzyme genes.

Soluble monomeric fumarate reductases belonging to the family of flavocytochromes *c* have been detected in the periplasm of the γ-proteobacteria *S. frigidimarina* and *S. oneidensis.* The periplasmic methacrylate reductase of the δ-proteobacterium *G. sulfurreducens* AM-1 probably belongs to the same family of flavocytochromes *c*. The cytochrome *c* hemes and the flavin-containing domain are located on different subunits and are not closely related [20]. Intense study of iron-reducing bacteria of the genera *Geobacter* and *Shewanella* [82] have shown that there is a correlation between the great number of cytochromes *c*, the presence of flavocytochromes *c* in the periplasm of these bacteria, and their capability for anaerobic respiration.

Of interest is the following fact: The bacteria *G. sulfurreducens* and *W. succinogenes* are able to syntrophically oxidize acetate in coculture [71] and both contain periplasmic enzymes with methacrylate reductase activity [20, 78]; however, the former bacterium is able to grow in the presence of methacrylate as the electron acceptor, whereas the latter bacterium is not.

The reduction of double bonds in anaerobic bacteria utilizing unsaturated organic compounds as terminal electron acceptors is far from being well understood. The nucleotide sequence of the gene encoding the methacrylate reductase of *G. sulfurreducens* is unknown. Furthermore, little is known about the enzymes involved in reduction of the side chains of phenylpropenoate and about the mechanisms of pentenoate and acrylate reduction in *Syntrophospora bryantii* and *Desulfovibrio acrylicus.* Relevant studies may provide deeper insight into the mechanism of double bond reduction in anaerobic bacteria.

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