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Enzymes Involved in Anaerobic Polyethylene Glycol Degradation by *Pelobacter venetianus* and *Bacteroides* Strain PG1

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In extracts of polyethylene glycol (PEG)-grown cells of the strictly anaerobically fermenting bacterium *Pelobacter venetianus*, two different enzyme activities were detected, a diol dehydratase and a PEG-degrading enzyme which was characterized as a PEG acetaldehyde lyase. Both enzymes were oxygen sensitive and depended on a reductant, such as titanium citrate or sulfhydryl compounds, for optimal activity. The diol dehydratase was inhibited by various corrinoids (adenosylcobalamin, cyanocobalamin, hydroxocobalamin, and methylcobalamin) by up to 37% at a concentration of 100 μM. Changes in ionic strength and the K^+ ion concentration had only limited effects on this enzyme activity; glycerol inhibited the enzyme by 95%. The PEG-degrading enzyme activity was stimulated by the same corrinoids by up to 80%, exhibited optimal activity in 0.75 M potassium phosphate buffer or in the presence of 4 M KCl, and was only slightly affected by glycerol. Both enzymes were located in the cytoplasmic space. Also, another PEG-degrading bacterium, *Bacteroides* strain PG1, contained a PEG acetaldehyde lyase activity analogous to the corresponding enzyme of *P. venetianus* but no diol dehydratase. Our results confirm that corrinoid-influenced PEG degradation analogous to a diol dehydratase reaction is a common strategy among several different strictly anaerobic PEG-degrading bacteria.

Polyethylene glycol (PEG) is a synthetic water-soluble polyether with the common structural formula $H(OCH_2CH_2)_nOH$. PEGs with molecular weights of 106 to 40,000 are used for a broad variety of commercial purposes (2).

The central problem of PEG degradation is cleavage of an aliphatic ether linkage. In a mixed culture of aerobic *Flavobacterium* and *Pseudomonas* species, PEG is dehydrogenated to the aldehyde and further to the carboxylic acid derivative before the ether linkage is cleaved, by a still enigmatic oxidative reaction, to yield glyoxylate as a cleavage product (8, 9). Three different dehydrogenating enzymes are involved in this process and have been partly purified and characterized (7, 10).

Degradation of PEG in the absence of molecular oxygen was reported for a nitrate-reducing bacterium, *Alcaligenes faecalis* var. *denitrificans* (5), and for homoacetogenic and propionate-forming bacteria (17). Studies with *Desulfovibrio desulfuricans* and *Bacteroides* strain PG1, obtained indications that long-chain PEGs are cleaved by either extracellular hydrolysis or "hydrogenation" (3, 4), but the reported activities were extremely low (4). A different way of PEG degradation was suggested for *Pelobacter venetianus*. This bacterium ferments ethylene glycol (EG [$HO-CH_2-CH_2-OH$]) and PEGs with molecular weights of up to 40,000 stoichiometrically to acetate and ethanol (13).

EG can be converted to acetaldehyde through a diol dehydratase reaction by coenzyme B_{12}-dependent exchange of a hydroxyl and a hydrogen residue and elimination of water (16). Since acetaldehyde was also found as the first intermediate in PEG degradation by *P. venetianus*, a diol dehydratase-analogous reaction mechanism was suggested, but the enzymes responsible have not been demonstrated (15).

The present study was initiated to check for the enzymes involved in PEG degradation in cell extracts of *P. venetianus*. Of primary interest was the question of whether the diol dehydratase can also cleave the ether bond of PEG or whether the latter is carried out by a different PEG-degrading enzyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. venetianus* GraPEG1, DSM 2394, was grown in saltwater mineral medium as described previously (13). *Bacteroides* strain PG1 was kindly provided by James M. Tiedje, Michigan State University, East Lansing, and cultivated in freshwater mineral medium at 30°C. Both mineral media contained 30 mM sodium bicarbonate as a buffer, 1 mM sodium sulfide as a reductant, trace element solution SL 10 (18), and a vitamin solution (12). Freshwater medium contained 0.5 g of NaCl and 0.4 g of $MgCl_2 \cdot 6H_2O$ per liter, and saltwater medium contained 20.0 g of NaCl and 3.0 g of $MgCl_2 \cdot 6H_2O$ per liter. The pH was 7.1 to 7.3.

Chemical determinations and chemicals. Protein was determined as described by Bradford (1). All of the chemicals used were of analytical or reagent grade and were obtained from Merck (Darmstadt, Germany), Sigma (Munich, Germany), Fluka (Neu-Ulm, Germany), Serva (Heidelberg, Germany), and Boehringer (Mannheim, Germany). DiEG ($HO-CH_2-CH_2-O-CH_2-CH_2-OH$) and TriEG [$H(O-CH_2-CH_2)_3OH$] of high purity were purchased from Hoechst (Gendorf, Germany), and alcohol dehydrogenase (from *Saccharomyces cerevisiae*) was obtained from Boehringer.

Enzyme assays. Cells for enzyme measurements were cultivated with 10 mM TriEG as the substrate in 1-liter infusion bottles and harvested in the late logarithmic growth phase at an optical density at 578 nm of 0.3 by centrifugation

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at 1,500 × *g* for 20 min in 125-ml infusion bottles under an O_2-free N_2 atmosphere. Cell pellets were washed once and suspended in 3 ml of 50 mM potassium phosphate buffer (pH 8.0) reduced with 2.5 mM titanium citrate (19). Cell extracts were prepared anoxically by disruption in a French pressure cell (140 MPa) and centrifuged at 4,000 × *g* to remove cell debris. The membrane fraction was separated from the cytoplasmic fraction by centrifugation at 200,000 × *g* for 1 h.

All enzyme assays were carried out under strictly anoxic conditions in rubber-sealed 1-ml cuvettes with oxygen-free buffers and reagent solutions with a 100-40 spectrophotometer (Hitachi, Tokyo, Japan). Diol dehydratase activities were determined as NADH-dependent reduction of the acetaldehyde formed at a wavelength of 340 nm (modified as described in reference 16). The reaction mixture contained, in 1 ml of potassium phosphate buffer (0.5 M, pH 8.0, prereduced with 2.5 mM titanium citrate), NADH (0.2 mM), alcohol dehydrogenase (30 μg with a specific activity of 400 μmol · min^{-1} · mg of $protein^{-1}$), and cell extract (30 to 50 μg of protein). The reaction was started by addition of the substrate (100 mM EG). The PEG-degrading enzyme was assayed in the same manner with 100 mM DiEG as the substrate.

RESULTS

Enzymes of *P. venetianus*. EG- and PEG-degrading enzyme activities of *P. venetianus* were determined through acetaldehyde formation from EG or DiEG and higher PEGs in cell extracts. In the diol dehydratase assay (16), only EG was degraded with very high activity. After modification of the test conditions (reductant, pH, and ionic strength of the potassium phosphate buffer), acetaldehyde formation from DiEG or higher PEGs was also demonstrated in cell extracts. This was designated PEG-degrading enzyme activity.

Both enzymes depended on the presence of a low-potential reductant. With titanium citrate ($E^{0\prime}$ = −480 mV) as the reductant, enzyme activities were about 20% higher than with dithiothreitol ($E^{0\prime}$ = −330 mV). The diol dehydratase had an optimum pH of 8.0, and the PEG-degrading enzyme had an optimum pH between 7.0 and 8.0. The PEG-degrading enzyme exhibited optimal activity in 0.75 M potassium phosphate buffer buffer (45% increase compared with the enzyme activity in 0.05 M buffer) or at 4 M KCl. In cell extracts, both enzymes lost 10 to 30% of their activity per hour under optimal conditions. Moreover, diol dehydratase and the PEG-degrading enzyme were inactivated during the enzyme reaction itself. The reaction rate with EG was constant for about 10 min, and the PEG degradation reaction was constant for only 1 to 2 min.

Corrinoids had different effects on the enzyme activities (Tables 1 and 2). Whereas diol dehydratase was inhibited by all of the corrinoids tested, the PEG-degrading enzyme was stimulated by all of the corrinoids. The greatest stimulation (80%) was measured with 10 μM hydroxocobalamin. Diol dehydratase was inhibited by 95% by 100 mM glycerol, whereas PEG degradation was inhibited by only 8%. Both enzymes were localized in the cytoplasmic fraction; no activity was detected in culture supernatants, periplasm preparations obtained after lysozyme-EDTA treatment in hypertonic buffer, or the membrane fraction. The K_m for EG was 5.0 mM, and that for DiEG was 6.5 mM.

P. venetianus grew with various PEGs, methoxyethanol, and ethoxyethanol, and also enzyme activities were determined with all of the degradable substrates tested (data not shown). No growth was found with 10 mM EG, but diol dehydratase showed an extremely high specific activity with EG. The PEG-degrading enzyme activity measured with TriEG as the substrate under optimal test conditions (0.68 μmol · min^{-1} · mg of $protein^{-1}$) accounted for 68% of the physiological activity of cells growing exponentially with TriEG (doubling time, 4.25 h; growth yield, 5.0 g · mol^{-1}).

Enzymes of *Bacteroides* strain PG1. Formation of acetalde-

TABLE 1. Effects of corrinoids on the activity of the diol dehydratase of *P. venetianus*[a]

Corrinoid and concn (μM)	Sp act (μmol · min^{-1} · mg of $protein^{-1}$)		Change in sp act (%)
	Before cofactor addition	After cofactor addition	
Adenosylcobalamin			
1	6.77	6.77	0
10	5.80	5.32	−8
100	7.25	6.29	−13
Cyanocobalamin			
1	5.80	5.80	0
10	5.31	4.84	−9
100	4.35	2.71	−37
Hydroxocobalamin			
1	5.32	5.32	0
10	5.32	4.83	−9
100	4.35	2.90	−33
Methylcobalamin			
1	5.80	5.80	0
10	4.84	4.64	−4

[a] Enzyme reactions were started by addition of 100 mM EG as the substrate, and the reaction rates were compared before and after corrinoid addition. All of the values shown are mean results of at least two independent assays.

TABLE 2. Effects of corrinoids on the activity of the PEG-degrading enzyme of *P. venetianus*[a]

Corrinoid and concn (μM)	Sp act (μmol · min^{-1} · mg of $protein^{-1}$)		Change in sp act (%)
	Before cofactor addition	After cofactor addition	
Adenosylcobalamin			
1	0.197	0.197	0
10	0.148	0.222	+50
100	0.130	0.145	+12
Cyanocobalamin			
1	0.483	0.483	0
10	0.290	0.338	+17
100	0.193	0.218	+13
Hydroxocobalamin			
1	0.411	0.435	+6
10	0.242	0.435	+80
100	0.242	0.363	+50
Methylcobalamin			
1	0.178	0.178	0
10	0.169	0.241	+42

[a] Enzyme reactions were started by addition of 100 mM DiEG as the substrate, and the reaction rates were compared before and after corrinoid addition. All of the values shown are mean results of at least two independent assays.

hyde from DiEG and higher PEGs was also observed with cell extracts of *Bacteroides* strain PG1 (4) under conditions optimized for the *P. venetianus* PEG-degrading enzyme. No diol dehydratase activity was detected with either EG or 1,2-propanediol as the substrate. The PEG-degrading enzyme had an optimum pH of 8.0, and the highest activity was measured in 0.5 M potassium phosphate buffer. All of the corrinoids tested inhibited PEG degradation by up to 83%. Addition of 100 mM glycerol decreased the enzyme activity by 40%.

Bacteroides strain PG1 degraded all of the PEGs from DiEG to PEG 40000, but growth with PEG 20000 and PEG 40000 was very weak. No growth was found with EG, 1,2-propanediol, methoxyethanol, ethoxyethanol, or tetraethylene glycol dimethylether. The activity of the PEG-degrading enzyme was determined with all of the substrates which were degraded in growth experiments as well and was comparably high, even with the long-chain polymers.

DISCUSSION

Enzymes of *P. venetianus*. In the present study, the activities of enzymes involved in PEG degradation by *P. venetianus* were demonstrated in cell extracts for the first time. Two different enzyme activities that react with either EG or DiEG and higher PEGs were detected. The properties of the two enzymes are compared in Table 3.

Diol dehydratase and the PEG-degrading enzyme were highly oxygen sensitive, depended on a low redox potential for reaction, and were both found in the cytoplasm. Whereas the pH optima of the enzymes were about the same, the PEG-degrading enzyme exhibited optimal activity at a much higher ionic strength and K^+ ion concentration and was much less susceptible to glycerol inhibition than the diol dehydratase was.

The diol dehydratase was inhibited by all of the corrinoids tested, while the PEG-degrading enzyme was stimulated considerably by the same corrinoids. Also, the DiEG lyase involved in PEG degradation by an *Acinetobacter* strain was reported to be stimulated up to 26% by cyanocobalamin (11). In contrast, the PEG-degrading enzyme of a homoacetogenic bacterium was inhibited by all of these corrinoids (14).

All of these different properties indicate that diol dehydration and the PEG-degrading reaction are catalyzed by two different enzyme proteins. Unfortunately, despite numerous attempts, we could not demonstrate this difference by separation of the two enzyme activities by electrophoresis in a native polyacrylamide gel because both enzyme activities were destroyed during the separation procedure.

Our results confirm a mechanism of PEG degradation by *P. venetianus* which has been postulated earlier on the basis of physiological experiments (Fig. 1). In the first step, the terminal hydroxyl group of PEG is shifted to the subterminal carbon atom by the PEG-degrading enzyme, analogous to a coenzyme B_{12}-dependent diol dehydratase reaction (16). By this reaction, cleavage of the ether bond in PEG is prepared, because the hemiacetal thus formed is unstable and easily releases acetaldehyde. The product formed is a PEG molecule shorter by one EG unit. After degradation of the whole PEG chain, the remaining EG residue is cleaved by diol dehydratase to acetaldehyde and water. The observation that the PEG-degrading enzyme is stimulated by corrinoids corroborates this concept. This type of cleavage requires at least one unmasked terminal hydroxyl group. Results of our studies support this assumption. No growth or cleavage activity in cell extract was detected with tetraethylene glycol

TABLE 3. Comparison of diol dehydratase and the PEG-degrading enzyme of *P. venetianus*

Enzyme	Substrate range in enzyme assay	Sp act (μmol $min^{-1} \cdot$ mg of $protein^{-1}$)	pH optimum	Preferred K phosphate buffer concn (M)	Preferred KCl concn (M)	Influence of corrinoids	Inhibition by glycerol (%)	Apparent K_m (mM)	Localization
Diol dehydratase	EG	303 (EG)	8.0	0.25	1.0	Inhibition by all tested	95	5.0 (EG)	Cytoplasm
PEG acetaldehyde lyase	PEG,[a] methoxyethanol, ethoxyethanol	2.1 (DiEG), 1.3 (PEG 6000)	7.0–8.0	0.75	4.0	Stimulation by all tested	8	6.5 (DiEG)	Cytoplasm

[a] Up to an M_r of 40,000.

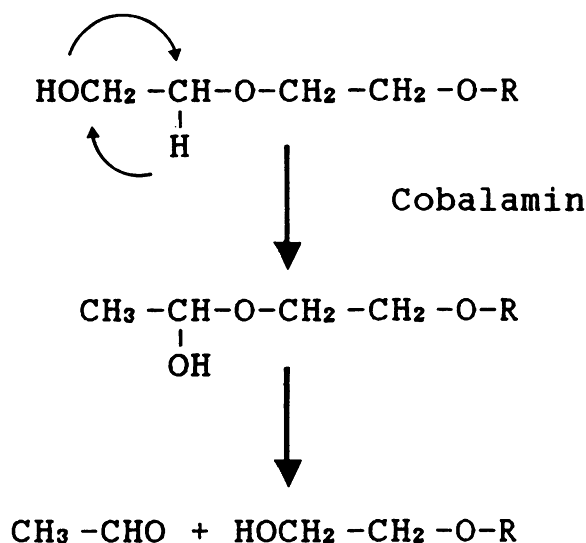

FIG. 1. Suggested mechanism of action of the PEG-degrading enzyme (PEG acetaldehyde lyase) of *P. venetianus*.

dimethylether, whereas methoxyethanol (ethylene glycol monomethylether) and ethoxyethanol (ethylene glycol monoethylether) were degraded and utilized. This PEG-degrading enzyme has to be characterized as a PEG acetaldehyde lyase.

Enzymes of *Bacteroides* strain PG1. The PEG-degrading enzymes of *Bacteroides* strain PG1 were investigated in cell extracts in the present study too. Similar to our findings with *P. venetianus*, PEG degradation depended on a high ionic strength of the assay buffer. In contrast to *P. venetianus*, the PEG-degrading enzyme of *Bacteroides* strain PG1 was inhibited by all of the corrinoids, and glycerol had a stronger influence on enzyme activity. In contrast to results reported in an earlier publication (4), we did not detect diol dehydratase activity in cell extracts of this bacterium. Moreover, in our assays the PEG-degrading enzyme was active with all of the substrates degraded by this bacterium.

These findings suggest that PEG degradation in both bacteria proceeds by the same corrinoid-dependent mechanism, although added corrinoids had a stimulatory effect in one case and an inhibitory effect in the other. Other researchers (4) have found dichlorophenolindophenol-dependent dehydrogenase activity in *Bacteroides* strain PG1 with EG and PEGs up to PEG 400. On the basis of these results, they assumed that long-chain PEGs are first degraded to oligomers by an unknown mechanism and that further degradation is initiated by an oxidative step. However, the dichlorophenolindophenol-dependent dehydrogenase activities observed were very low (4 to 10 nmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}) and could be interpreted as side activities of an unspecific alcohol dehydrogenase with EG and oligomeric PEGs. It should be mentioned that most commercial short-chain PEG preparations contain significant amounts of EG and acetaldehyde (6); for our PEG-degrading assays, we therefore used highly purified, acetaldehyde- and EG-free DiEG and TriEG preparations.

Our studies confirm that a diol dehydratase-analogous, corrinoid-dependent reaction is the only experimentally proven way of PEG degradation by strictly anaerobic bacteria. This type of degradation can proceed only from terminal hydroxyl groups of polymers inside the cytoplasmic space and cannot cleave long chains randomly into smaller fractions.

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