Catalytic and Molecular Properties of the Quinohemoprotein Tetrahydrofurfuryl Alcohol Dehydrogenase from *Ralstonia eutropha* Strain Bo

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The quinohemoprotein tetrahydrofurfuryl alcohol dehydrogenase (THFA-DH) from *Ralstonia eutropha* strain Bo was investigated for its catalytic properties. The apparent k_{cat}/K_m and K_i values for several substrates were determined using ferricyanide as an artificial electron acceptor. The highest catalytic efficiency was obtained with *n*-pentanol exhibiting a k_{cat}/K_m value of 788×10^4 M^{-1} s^{-1}. The enzyme showed substrate inhibition kinetics for most of the alcohols and aldehydes investigated. A stereoselective oxidation of chiral alcohols with a varying enantiomeric preference was observed. Initial rate studies using ethanol and acetaldehyde as substrates revealed that a ping-pong mechanism can be assumed for in vitro catalysis of THFA-DH. The gene encoding THFA-DH from *R. eutropha* strain Bo (*tfaA*) has been cloned and sequenced. The derived amino acid sequence showed an identity of up to 67% to the sequence of various quinoprotein and quinohemoprotein dehydrogenases. A comparison of the deduced sequence with the N-terminal amino acid sequence previously determined by Edman degradation analysis suggested the presence of a signal sequence of 27 residues. The primary structure of TfaA indicated that the protein has a tertiary structure quite similar to those of other quinoprotein dehydrogenases.

The xenobiotic cyclic ether tetrahydrofurfuryl alcohol (THFA) is used as a versatile solvent in industry and is released in large amounts during the synthesis of furan resins. We have recently isolated a bacterium able to use THFA (at concentrations of up to 200 mM) as its sole source of carbon and energy which was identified as *Ralstonia eutropha* and designated strain Bo (56). Enzymatic studies revealed that the degradation of THFA is initiated by an oxidation of the alcohol via the aldehyde to the corresponding carboxylic acid (Fig. 1). In vitro, both steps are catalyzed by an inducible tetrahydrofurfuryl alcohol dehydrogenase (THFA-DH). In contrast to various NAD(P)-dependent alcohol dehydrogenases (ADHs) and aldehyde dehydrogenases described for *R. eutropha* (23, 31, 42), THFA-DH could be identified as a type I quinohemoprotein ADH containing pyrroloquinoline quinone (PQQ) and a heme c as cofactors (56).

NAD(P)-independent ADHs containing PQQ as a prosthetic group are found in many aerobic bacteria (3, 10–13, 20, 21, 25, 40, 48, 53, 54). According to their structure and cofactor content, quinoprotein alcohol dehydrogenases are divided into two main types (24). Class I is represented by enzymes containing PQQ and Ca^{2+}-like methanol dehydrogenase from methylotrophic bacteria (8), whereas class II includes proteins containing an additional heme c designated quinohemoprotein ADHs. Quinohemoprotein ADHs type I are monomeric enzyme of about 72 kDa with an equimolar content of PQQ, Ca^{2+}, and heme c (9, 48, 56). Quinohemoprotein ADHs type II are multimeric proteins of at least three subunits found in bacteria of the genera *Acetobacter* and *Gluconobacter* (1, 34).

These enzymes contain one PQQ molecule and one heme c in the α-subunit and several heme c binding motifs in the other subunits. It became obvious from the structural data that the type I dehydrogenases resemble the α-subunit of type II enzymes (22, 45).

It is known that quinoprotein ADHs can be used for biotechnological applications, e.g., as biosensors or for the stereoselective oxidation of chiral alcohols (14, 17, 28, 39, 51). The broad substrate spectrum makes them useful for the production of different pure enantiomers (44). In the present paper, we report some of the catalytic features of THFA-DH from *R. eutropha* strain Bo. Furthermore, the complete amino acid sequence of THFA-DH derived from the isolated gene was determined and analyzed.

MATERIALS AND METHODS

Chemicals. The chemicals used in the present study were at least of analytical reagent grade and were obtained from Sigma, Merck, or Fluka. Chromatographic materials were obtained from Pharmacia.

Bacterial strains, plasmids, and growth conditions. Maintenance of strains, growth of mass cultures, and cell harvest of strain Bo (DSM 11098) was done as reported before (56). *Escherichia coli* XL-1 Blue used for cloning purposes was grown in Luria-Bertani medium or on agar plates containing antibiotics as described by Sambrook et al. (38). The plasmid pUC19 was used for construction of a genomic library of *R. eutropha* strain Bo. PCR products were ligated into the pGEM-T Easy vector (Promega).

Enzyme assay. The standard assay for determination of THFA-DH activity was performed as described previously with ferricyanide as an artificial electron acceptor (56) except that the Tris HCl buffer was replaced by 75 mM morpholinepropanesulfonic acid (MOPS)-NaOH, pH 8.2.

The kinetic constants were determined by fitting the obtained data to the model of Michaelis-Menten with or without substrate inhibition. The data determined for substrates exhibiting inhibition kinetics were analyzed by equation 1.

$$v = \frac{V + S}{S + K_m + \frac{S^2}{K_i}} \tag{1}$$

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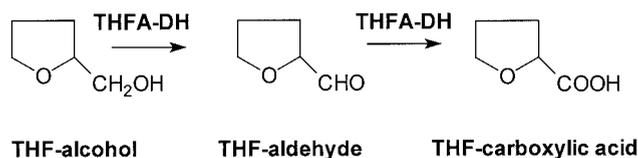
FIG. 1. Oxidation of THFA to the corresponding carboxylic acid catalyzed by THFA-DH from *R. eutropha* strain Bo.

Data analysis was done with the SigmaPlot program (Jandel Scientific). Analysis of the reaction mechanism was performed with a modified enzyme assay using 20 mM MOPS-NaOH, pH 8.0, containing 5 mM CaCl$_2$. Semicarbazide (2 mM) was added to the reaction mixture if ethanol was used as a substrate. The observed endogenous ferricyanide reduction was subtracted from the substrate-dependent activity.

The enantioselectivity of THFA-DH during the conversion of racemic substrates was estimated as previously described (44) (in equation 2, *A* and *B* are the different enantiomers).

$$E = \frac{V_{\max}A}{K_m A} \bigg/ \frac{V_{\max}B}{K_m B} \qquad (2)$$

Purification of THFA-DH. Crude extracts were prepared as previously described (56) by suspending cell paste of *R. eutropha* strain Bo grown on THFA (1 g/ml) in 50 mM Tris HCl, pH 8.0, containing 5 mM CaCl$_2$, 0.5 mM dithiothreitol, and 1 mM EDTA. THFA-DH was purified from cell extracts of *R. eutropha* strain Bo as described previously (56).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis was done according to the method of Laemmli (27) with 12.5% polyacrylamide gels. Electrophoresis was carried out under a constant current of 25 mA per gel and maximum voltage.

Standard DNA techniques. DNA was treated by standard methods (38). Genomic DNA was isolated from cells of *R. eutropha* strain Bo grown in mineral salts medium with 0.2% fructose (wt/vol) as described by Marmur (33). Plasmid DNA was isolated from *E. coli* with the Qiagen plasmid purification kits. The molecular masses of DNA fragments were estimated by using restricted λ DNA or pGEM vector as molecular mass markers (Promega). Restriction enzymes purchased from MBI Fermentas or Roche were used according to the manufacturer's instructions. DNA fragments were isolated from agarose gels using Qiaex gel extraction kits (Qiagen). The standard PCR was performed by using *Taq* DNA polymerase (Roche) in an appropriate buffer, an annealing temperature optimized for the primer combination used, and 30 cycles. Hybridization experiments were carried out using the DIG DNA labelling and detection kit (Roche) according to the manufacturer's instructions.

DNA sequences were determined by primer walking and the dideoxynucleotide chain-termination method with an ABI 377 Sequencer, version 4.0 (Applied Biosystems), a Cycle Sequencing kit (Pharmacia), and universal, reverse, or sequence-specific primers. All primers were obtained from a commercial supplier (Gibco or Metabion).

Cloning of the gene encoding THFA-DH. Degenerated primers were designed from the N-terminal amino acid sequence of THFA-DH (primer 1, 5′AAYGA RGCIGGIACICCIAAYTGG3′) and from highly conserved sequence regions of other quinohemoprotein ADHs comprising the region between nucleotides 1087 and 1109 (primer 2, 5′AARAAICCITTYTTIGGIGCRTG3′) and the region between nucleotides 1903 and 1919 (primer 3, 5′CCRTGRCARAARACRCA 3′) (quinohemoprotein ethanol dehydrogenase [QH-EDH]; *Comamonas testosteroni* numbering). The obtained specific PCR products were cloned in the pGEM-T Easy vector and sequenced. A genomic library of *R. eutropha* strain Bo was constructed by partial digestion of genomic DNA with *Sau*3A. DNA fragments of 2.5 to 6 kb were isolated from agarose gels and cloned in the *Bam*HI-restricted pUC19 vector (MBI Fermentas). After transformation in *E. coli* XL-1 Blue, about 6,300 clones were obtained, and their isolated plasmids were stored as a mixture at −20°C.

Computer-aided analysis of gene sequences. DNA sequences were analyzed using computer programs DNASIS (version 5.00) and Clone Manager (version 4.0) (Scientific & Educational Software). The sequences were aligned with the program Fasta3 of the European Molecular Biology Laboratory outstation, Hinxton (http://www2.ebi.ac.uk/fasta3/), or BlastX at the homepage of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov /blast/). The multiple alignment of various sequences available at EMBL and GenBank databases was done with the program ClustalW at the European

TABLE 1. Kinetic parameters for several substrates of THFA-DH from *R. eutropha* strain Bo[a]

Substrate	Parameter		
	K_m (mM)	$k_{cat}/K_m \times 10^4$ (M^{-1} s^{-1})	K_i (mM)
Primary alcohols			
Ethanol[b]	3.1	3	—
n-Propanol	0.036	248	ND
n-Butanol	0.015	594	14.7
n-Pentanol	0.012	788	3.2
n-Hexanol	0.014	495	1.4
n-Heptanol	0.016	413	1.9
Aldehydes			
Formaldehyde[b]	1.93	2.3	—
Acetaldehyde[b]	0.075	120	—
Propionaldehyde	0.057	159	8.2
Butyraldehyde	0.033	244	0.95
Pentanal	0.061	121	0.33
Secondary alcohols			
2-Propanol	1.9	0.6	ND
2-Butanol[b]	1.12	3.1	—
2-Pentanol	0.12	99	8.6
2-Hexanol	0.08	167	1.6
2-Heptanol	0.058	204	1.0
2-Octanol	0.076	129	1.3
***O*-heterocyclic compounds**			
THF-2-alcohol	0.039	138	2.2
THF-3-alcohol	0.056	98	ND
Furfurylalcohol	0.025	185	1.9
Furfuraldehyde	0.147	16	11.0
Tetrahydropyran-2-alcohol	0.011	386	1.5
Diols			
1,2-Butandiol[b]	1.03	5	—
1,3-Butandiol[b]	0.49	15	—
1,4-Butandiol[b]	0.25	30	—
PEG 6000[b]	0.55	7	—

[a] Activities were determined with homogeneous THFA-DH (18.8 nM) in 75 mM MOPS-NaOH, pH 8.2, and 5 mM CaCl$_2$ under standard assay conditions with appropriate substrate concentrations. The apparent kinetic constants were determined by nonlinear curve fittings based on Michaelis-Menten kinetics. ND, not determined; —, no substrate inhibition detected under the conditions used.
[b] The curves were fitted based on the model of Michaelis and Menten. All other curves were fitted based on the model of substrate inhibition as described in Materials and Methods.

Molecular Biology Laboratory service homepage (http://www2.ebi.ac.uk/clustalw/). The theoretical molecular mass and isoelectric point were estimated with program tools located at http://www-biol.univ-mrs.fr/cgi-bin/abim/.

Nucleotide sequence accession number. The nucleotide sequence of *tfaA* is available from GenBank under accession number AF277373.

RESULTS

Catalytic properties of THFA-DH. (i) Kinetic parameters. We previously reported that THFA-DH from *R. eutropha* strain Bo exhibited a broad substrate spectrum (56). To obtain further information about the catalytic properties of THFA-DH, we performed initial rate measurements in more detail. In addition to the substrates previously reported to be converted by THFA-DH, the following compounds were also oxidized to a significant extent (values in parentheses are V_{max} values in units per milligram [THFA (43.6)]: *n*-heptanol (53.8), 2-propanol (9.5), tetrahydropyran-2-alcohol (32), propionaldehyde

TABLE 2. Enantioselective oxidation of chiral alcohols by homogeneous THFA-DH from *R. eutropha* strain Bo[a]

Substrate	K_m[b] (mM)	V_{max} (U/mg)	$K_{cat}/K_m \times 10^4$ (M^{-1} s^{-1})	K_i (mM)	E
($\pm$)-2-Butanol	1.12	28.2	3.1	—	
R-($-$)-2-Butanol	1.14	17.5	1.9	—	
S-($+$)-2-Butanol	0.59	32.0	6.6	—	3.5
($\pm$)-2-Heptanol	0.058	96.8	204.2	1.03	
R-($-$)-2-Heptanol	1.16	22.1	2.3	0.82	
S-($+$)-2-Heptanol	0.046	131.6	350.0	0.93	150.2
($\pm$)-2-Octanol	0.076	80.4	129.4	1.34	
R-($-$)-2-Octanol	0.269	18.2	8.3	6.62	
S-($+$)-2-Octanol	0.051	101.3	243.0	0.98	29.4
($\pm$)-Solketal	ND				
R-($-$)-Solketal	0.11	3.1	3.4	—	
S-($+$)-Solketal	0.74	5.6	0.9	—	0.27

[a] The kinetic parameters were determined using ferricyanide as an artificial electron acceptor and under the conditions described in Table 1, footnote a. The enantiomeric ratios are expressed in E values as described in Materials and Methods. —, no substrate inhibition detected under the conditions used.
[b] The curves were fitted to the model of Michaelis and Menten with or without substrate inhibition. ND, not determined.

(73.8), 1,3-propandiol (61.2), 1,2-pentandiol (44.6), 1,5-pentandiol (73.4), and 1,7-heptandiol (73.7). Although THFA-DH was isolated from *R. eutropha* strain Bo enriched and grown on THFA as a substrate, the purified enzyme showed the highest catalytic efficiency under in vitro conditions with *n*-pentanol, whereas THFA was converted with a comparable low rate (Table 1). Ethanol was the only primary alcohol investigated which was a poor substrate of THFA-DH. No dye-linked THFA-DH activity was detectable in crude extracts of *R. eutropha* strain Bo grown on ethanol, 2-propanol, and 1,5-pentandiol. However, the purified enzyme showed detectable activities with these substrates in the in vitro enzyme assay (see above).

Initial rate measurements of in vitro substrate conversion of THFA-DH showed that in most cases the obtained data can be explained by a strong substrate inhibition, although some substrates were not inhibitory (Table 1 and 2) and these curves fitted the kinetic model without substrate inhibition.
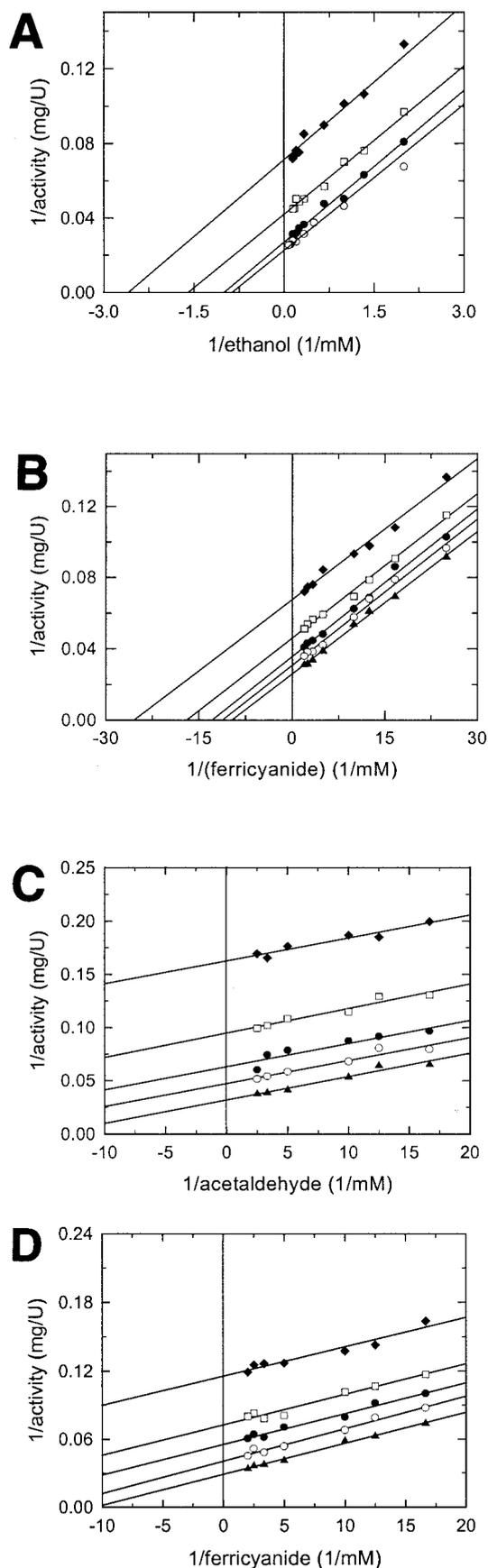
(ii) Kinetic mechanism. Due to the observed lack of substrate inhibition, the kinetic mechanism of THFA-DH was investigated with ethanol or acetaldehyde as a substrate and ferricyanide as an electron acceptor. Analysis was performed by varying the concentration of ethanol or acetaldehyde while maintaining the concentration of ferricyanide at different values in the range of its apparent K_m value and vice versa. The double-reciprocal plots obtained from the data of initial rate measurements are depicted in Fig. 2. The fact that the plot lines are parallel suggested that a ping-pong kinetic mechanism should be assumed for the THFA-DH catalyzed reaction. Because *n*-pentanol as the best substrate of THFA-DH had an inhibitory effect, analysis of the kinetic mechanism was more difficult. However, at low substrate concentrations, the lines of double-reciprocal plots were parallel (data not shown), thus indicating that this substrate is also converted by a ping-pong mechanism.

(iii) Enantioselectivity. It was previously reported that the quinohemoprotein ADHs isolated from *C. testosteroni*, *Acetobacter pasteurianus*, and *Acetobacter aceti* exhibited an enantiomeric preference if racemic mixtures of chiral alcohols were used as substrates (15, 17, 29, 30, 43, 44). Thus, we examined if THFA-DH from *R. eutropha* strain Bo also showed a preference for one enantiomer of different chiral alcohols. Initial

rate measurements performed with secondary aliphatic alcohols indicated that the enzyme had a high preference for the oxidation of the S-($+$)-enantiomers (Table 2). Although the catalytic efficiency for conversion of the cyclic ether solketal was low, THFA-DH unequivocally preferred the R-($-$)-enantiomer (Table 2).

Cloning and sequencing of the gene encoding THFA-DH. To obtain more information on the primary structure of THFA-DH from *R. eutropha* strain Bo, the encoding gene was cloned and sequenced. Two DNA fragments of 0.95 and 1.8 kb were obtained by PCR and identified by sequencing to encode parts of THFA-DH comprising the N-terminal sequence previously determined by Edman degradation (56). These fragments were used as a probe to screen the genomic library of *R. eutropha* strain Bo. Three positive clones were obtained carrying plasmids pRE21-17, pRE28-15, and pRE18-87, which contained 6.1-, 6.3-, and 6.0-kb fragments of genomic DNA from strain Bo inserted into the pUC19 vector.

Sequencing of the inserts of pRE21-17, pRE28-15, and pRE18-87 led to the identification of a large open reading frame designated as *tfaA*. This open reading frame started with the translation start codon ATG (nucleotides 104 to 106) and ended with a TGA termination codon (nucleotides 2198 to 2200). A putative ribosome binding site (AGGAG) is located six nucleotides upstream of the translation start codon (data not shown). The protein encoded by *tfaA* is composed of 698 amino acids. The N-terminal amino acid sequence of THFA-DH previously determined by Edman degradation (56) corresponded exactly to the deduced sequence from Ala-28 to Ala-57 (Fig. 3). These data indicated that THFA-DH contains a leader peptide which is cleaved off during maturation of the protein (Fig. 3). Analysis of the N-terminal amino acid sequence deduced from *tfaA* by the computer program PSORT (36) or SignalP (version 1.1) suggested a signal peptide for translocation of the protein into the periplasm with a possible cleavage site between Gly-27 and Ala-28 (Fig. 3). The presence of a leader peptide was also supported by sequence alignments with other quinohemoprotein ADHs, all known to contain a signal sequence (Fig. 3). A theoretical molecular mass of 72,689 Da was calculated for TfaA from the deduced amino acid sequence (without leader peptide, PQQ, and heme cofactor). These data confirmed our previous molecular mass de-

termination of about 73 kDa (56). Furthermore, the calculated isoelectric point of 9.22 corresponded to 9.1, previously determined by isoelectric focusing (56). The G+C content of the *tfaA* gene was 67.5 mol%, close to the 65 mol% determined for the genomic DNA of *R. eutropha* strain Bo (U. Lechner, personal communication). These results together with the homology studies described below strongly suggested that we have cloned the DNA fragment encoding THFA-DH from *R. eutropha* strain Bo. Southern blot analysis of genomic DNA from strain Bo showed that the *tfaA* gene was present as a single copy (data not shown).
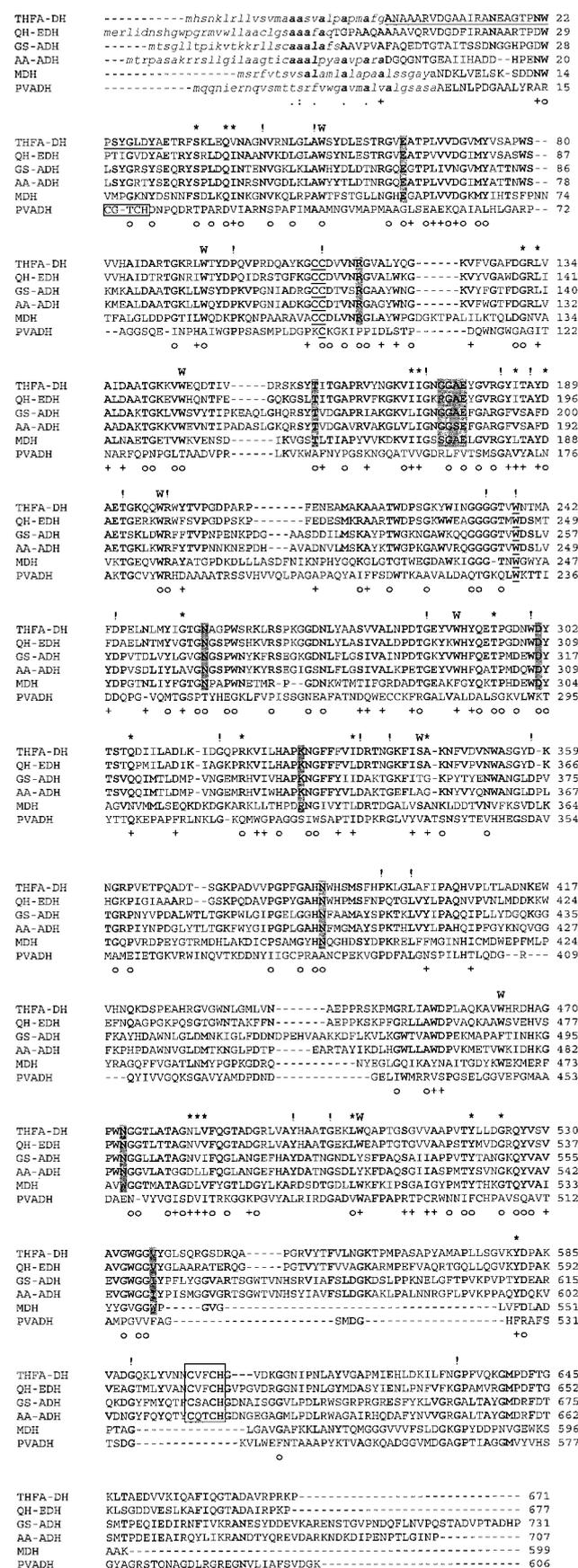
Analysis of the sequence located upstream and downstream of the *tfaA* gene (each, 0.8 kb) did not reveal an open reading frame which might encode a protein involved in THFA degradation or PQQ biosynthesis (data not shown).

Protein sequence comparison. The amino acid sequence deduced from *tfaA* was compared to sequences deposited in the EMBL or GenBank database with the Fasta3 or BlastX program, respectively. This database analysis revealed a similarity of the deduced protein (TfaA) to various quinoprotein dehydrogenases. An identity of 43% was obtained to the large subunit of the type II quinohemoprotein ADHs isolated from different *Acetobacter* species and about 32% identity was determined to the α-subunit of different methanol dehydrogenases (Fig. 3). However, the highest similarity, with 67% identity, was determined to the type I QH-EDH from *C. testosteroni* (Fig. 3). Interestingly, the identity to polyvinyl alcohol dehydrogenase (PVADH) from *Pseudomonas* sp. VM15C, the second sequenced type I enzyme, was only 24%.

The C-terminal part of TfaA (about 88 amino acids) should be responsible for binding the heme cofactor. However, this region exhibited an identity of only 13% to the corresponding sequence of the α-subunit of type II quinohemoprotein ADH from acetic acid bacteria (Fig. 3) (22, 46, 47). Furthermore, there was no significant similarity to the β-subunit of type II quinohemoprotein ADHs known to contain heme-binding sites. On the other hand, the C-terminal part of TfaA showed an identity of 30 to 38% to various cytochromes c (references 26 and 40 and data not shown). The multiple sequence alignment summarizes the conserved amino acid residues of the analyzed quinoprotein and quinohemoprotein ADHs (Fig. 3).

The sequence motif Cys-Xaa-Yaa-Cys-His reported to be involved in binding the heme cofactor (35) was discovered in the C-terminal part of TfaA and located between Cys624 and

FIG. 2. Analysis of the reaction mechanism of THFA-DH from *R. eutropha* strain Bo in the in vitro enzyme assay with ethanol or acetaldehyde as a substrate and ferricyanide as an electron acceptor. The initial rate measurements are depicted as Lineweaver-Burk plots. The activity was determined with 20 mM MOPS-NaOH, pH 8.0, containing 5 mM $CaCl_2$ and 18.8 nM THFA-DH. In case ethanol was used as a substrate, 2 mM semicarbazide was added to the reaction mixture. (A) Initial rates at varying ethanol concentrations, depending on the ferricyanide concentration used: 0.05 (◆), 0.1 (□), 0.25 (●), and 0.5 (○) mM. (B) Initial rates at varying ferricyanide concentrations, depending on the ethanol concentration used: 0.5 (◆), 1.0 (□), 2.0 (●), 3.0 (○), and 5.0 (▲) mM. (C) Initial rates at varying acetaldehyde concentrations, depending on the ferricyanide concentration used: 0.02 (◆), 0.04 (□), 0.06 (●), 0.1 (○), and 0.3 (▲) mM. (D) Initial rates at varying ferricyanide concentrations depending on the acetaldehyde concentration used: 0.02 (◆), 0.04 (□), 0.06 (●), 0.1 (○), and 0.3 (▲) mM.

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THFA-DH  -----------mhsnklrlllvsvmaaasvalpapmafgANAAARVDGAAIRANEAGTPNW  22
QH-EDH   merlidnshgwpgrmvwllaaclgsaaafaqTGPAAQAAAAVQRVDGDFIRANAARTPDW  29
GS-ADH   ------mtsglltpikvtkkrllcaaalafsAAVPVAFAQEDTGTAITSSDNGGHPGDW  28
AA-ADH   ---mtrpasakrrsllgilaagtcaaalpyaavparaDGQGNTGEAIIHADD--HPENW  20
MDH      ------------------msrfvtsvsalamlalapaalssgayaNDKLVELSK-SDDNW  14
PVADH    -----------mqqniernqvsmttsrfvwgavmalvalgsasaAELNLPDGAALYRAR  15

                     *   **    !          !W
THFA-DH  PSYGLDYAETRFSKLEQVNAGNVRNLGLAWSYDLESTRGVEATPLVVDGVMYVSAPWS--  80
QH-EDH   PTIGVDYAETRYSRLDQINAANVKDLGLAWSYNLESTRGVEATPVVVDGIMYVSASWS--  87
GS-ADH   LSYGRSYSEQRYSPLDQINTENVGKLKLAWHYDLDTNRGQEGTPLIVNGVMYATTNWS--  86
AA-ADH   LSYGRTYSEQRYSPLDQINRSNVGDKLKAWYYTLDTNRGQEATPLVVDGIMYATTNWS--  78
MDH      VMPGKNYDSNNFSDLKQINKGNVKQLRPAWTFSTGLLNGHEGAPLVVDGKMYIHTSFPNN  74
PVADH    CG-TCHDNPQDRTPARDVIARNSPAFIMAAMNGVMAPMAAGLSEAEKQAIALHLGARP--  72
             o    o      o o o+o   o    o        o o+ o++o+o oo

                   W               !                         *  *
THFA-DH  VVHAIDARTGKRLWTYDPQVPRDQAYKGCCDVVNRGVALYQG------KVFVGAPDGRLV  134
QH-EDH   VVHAIDRTGNRIWTYDPQIDRSTGFKGCCDVVNRGVALWKG------KVYVGAWDGRLI  141
GS-ADH   KMKALDAATGKLLWSYDPKVPGNIADRGCCDTVSRGAAYWNG------KVYFGTFDGRLI  140
AA-ADH   KMEALDAATGKLLWQYDPKVPGNIADKGCCDTVNRGAGYWNG------KVFWGTFDGRLI  132
MDH      TFALGLDDPGTILWQDKPKQNPAARAVACCDLVNRGLAYWPGDGKTPALILKTQLDGNVA  134
PVADH    --AGGSQE-INPHAIWGPPSASMPLDGPKCKGKIFPIDLSTP-------DQWNGWGAGIT  122
           +  +o         +  oo oo  + + o        +       oo o

               W                         **!        ! *  ! *
THFA-DH  AIDAATGKKVWEQDTIV-----DRSKSYTITGAPRVYNGKVIIGNGGAEYGVRGYITAYD  189
QH-EDH   ALDAATGKEVWHWDNTPE-----GQKGSLTITGAPRVFKGKVIIGKRGAEYGVRGYITAYD  196
GS-ADH   ALDAKTGKLVWSVYTIPKEAQLGHQRSYTVDGAPRIAKGKVLIGNGGAEFGARGFVSAFD  200
AA-ADH   AADAKTGKKVWEVNTIPADASLGKQRSYTVDGAVRVAKGLVLIGNGGAEFGARGFVSAFD  192
MDH      ALNAETGETVWKVENSD------IKVGSTLTIAAPYVVKDKVIIGSSGAELGVRGYLTAYD  188
PVADH    NARFQPNPGLTAADVPR------LKVKWAPNYPGSKNGQATVVGDRLFVTSMSGAVYALN  176
         + +        ++          o+ o +  o     o+ + o    o o o +++ +o

                    !   W!          !                  !       !
THFA-DH  AETGKQQWRWYTVPGDPARP-------FENEAMAKAAATWDPSGKYWINGGGGTVWNTMA  242
QH-EDH   AETGERKWRWFSVGDPSKP-------FEDESMKRAARTWDPSGKWWEAGGGGTMWDSMT  249
GS-ADH   AETSKLDWRFFTVPNPENKPDG---AASDDIIMSKAYPTWGKNGAWKQQGGGGTVWDSLV  257
AA-ADH   AETGKLKWRFYTVPNNKNEPDH---AVADNVLMSKAYKTWGPKGAWVNQGGGGTVWDSLV  249
MDH      VKTGEQVWRAYATGPDKDLLLASDFNIKNPHYGQKGLGTGTWEDGAWKIGGG-TNWGWYA  247
PVADH    AKTGCVYWRHDAAAATRSSVHVVQLPAGAPAQYAIFFSDWTKAAVALDAQTGKQLWKTTI  236
            oo +           o    +     o+ o    o    o o  o +

                 !      *             !    W  *   !
THFA-DH  FDPELNLMYIGTGNAGPWSRKLRSPKGGDNLYAASVVALNPDTGKYWHYQETPGDNWDY  302
QH-EDH   FDAELNTMYVGTGNGSPWSHKVRSPKGGDNLYLASIVALDPDTGKYKWHYQETPGDNWDY  309
GS-ADH   YDPVTDLVYLGVGNGSPWNYKFRSEGKGGDNLPLGSIVAINPDTGKYVWHFQETPMDEWDY  317
AA-ADH   YDPVSDLIYLAVGNGSPWNYKYRSEGIGSNLFLGSIVALKPETGEYVWHYQATPMDGWDY  309
MDH      YDPGTNLIYFGTGNPAPWNETMR-P--GDNKWTMTIFGRDADTGEAKFGYQKTPHDEWDY  304
PVADH    DDQPG-VQMTGSPTYHEGKLFVPISSGNEAFATNDQWECCKFRGALVALDALSGKVLWKT  295
         +          +      o +  o   o     oo ++  +  oo o oo +

                 *         !  *          *! !  !    W*        !
THFA-DH  TSTQDIILADLK-IDGQPRKVILHAPKNGFFVIDRTNGKFISA-KNFVDVNWASGYD-K  359
QH-EDH   TSTQPMILADIK-IAGKPRKVILHAPKNGFFVLDRTNGKFISA-KNFVPVNWASGYD-K  366
GS-ADH   TSVQQIMTLDMP-VNGEMRHVIVHAPKNGFFYIIDAKTGKFITG-KPYTYENWANGLDPY  375
AA-ADH   TSVQQIMTLDMP-VNGEMRHVIWHAPKNGFYVLDAKTGEFLAG-KNYVVQNWANGLDPL  367
MDH      AGVNVMMLSGLKDKDGKARKLLTHPDENGIVYTLDRTDGALVSANKLDDTVNVFKSVDLK  364
PVADH    YTTQKEPAPFRLNKLG-KQMWGPAGGSIWSAPTIDPKRGLVYVATSNSYTEVHHEGSDAV  354
            +     +       oo  ++  oo + +       + + o    o

                                        ! !
THFA-DH  NGRPVETPQADT--SGKPADVVPGPFGAHNWHSMSFHPKLGLAFIPAQHVPLTLADNKEW  417
QH-EDH   HGKPIGIAAARD--GSKPQDAVPGPYGAAHNWHPMSFHPQTGLVYLPAQNVPVNLMDDKKW  424
GS-ADH   TGRPNYVPDALWTLTGKPWLGIPGELGGHNFAAMAYSPKTKLVYIPAQQIPLLYDGQKGG  435
AA-ADH   TGRPIYNPDGLYTLTGKFWYGIPGPLGAHNFMGMAYSPKTHLVYLPAHQIPPGYKNQVGG  427
MDH      TGQPVRDPEYGTRMDHLAKDICPSAMGYHNQGHDSYDPKRELFFMGINHICMDWEPFMLP  424
PVADH    MAMEIETGKVRWINQVTKDDNYIIGCPRAANCPEKVGPDFALGNSPILHTLQDG--R---  409
           o  o        o                            +        +

                                                             W
THFA-DH  VHNQKDSPEAHRGVGWNLGMLVN-------AEPPRSKPMGRLIAWDPLAQKAVWHRDHAG  470
QH-EDH   EFNQAGPGCKPQSGTGWNTAKFFN-------AEPPKSKPFGRLLAWDPVAQKAAWSVEHVS  477
GS-ADH   FKAYHDAWNLGLDMNKIGLFDDNDPEHVAAKKDFLKVLKGWTVAWDPEKMAPAFTINHKG  495
AA-ADH   FKPHPDAWNVGLDMNTKNGLPDTF-----EARTAYIKDLHWGLLAWDPVKMETVWKIDHKG  482
MDH      YRAGQFFVGATLNMYPGPKGDRQ----------NYEGLGQIKAYNAITGDYKWEKMERF  473
PVADH    ---QYIVGQKSGAVYAMDPDND-------------GELIWMRRVSPGSELGGVEFGMAA  453
            oo   +o+++o  +o                 o+    ++ ++ oo  o ooo +

                    ***        !      !   *W          *  *
THFA-DH  PWNGGTLATAGNLVFQGTADGRLVAYHAATGEKLWQAPTGSGVVAAPVTYLLLDGRQYVSV  530
QH-EDH   PWNGGTLTTAGNVVFQGTADGRLVAYHAATGEKLWEAPTGTGVVAAPSTYMVDGRQYVSV  537
GS-ADH   PWNGGLLATAGNVIFQGLANGEFHAYDATNGNDLYSFPAQSAIIAPPVTYTANGKQYVAV  555
AA-ADH   PWNGGVLATGGDLLFQGLANGEFHAYDATNGSDLYKFDAQSGIIASPMTYSVNGKQYVAV  542
MDH      AVNGGTMATAGDLVFYGTLDGLYKARDSDTGDLLWKFKIPSGAIGYPMTYTHKGTQYVAI  531
PVADH    DAEN-VYVGISDVITRKGGKPGVYALRIRDGADVWAFPAPRTPCRWNNIFCHPAVSQAVT  512
            oo   o+o++++o  +o         o+        ++ ++ oo  o ooo +

                                                             *
THFA-DH  AVGWGGCYGLSQRGSDRQA-----PGRVYTFVLNGKTPMPASAPYAMAPLLSGVKYDPAK  585
QH-EDH   AVGWGGCYGLAARATEROQ-----PGTVYTFVVAGKARMPEFVAQRTGQLLQGVKYDPAK  592
GS-ADH   EVGWGGSYPFLYGGVARTSGWTVNHSRVIAFSLDGKDSLPPKNELGFTPVKPVPTYDEAR  615
AA-ADH   EVGWGGSYPISMGGVGRTSGWTVNHSYIAVFSLDGKAKLPALNNRGFLPVKPPAQYDQKV  602
MDH      YYGVGGLWP------GVG---------------------------------LVFDLAD  565
PVADH    AMPGVVFAG--------------------SMDG-------------------HFRAFS  531
            o oo                                            +o

                                                             *
THFA-DH  VADGQKLYVNNCVFCHG---VDKGGNIPNLAYVGAPMIEHLDKILFNGPFVQKGMPDFTG  645
QH-EDH   VEAGTMLYVANCVFCHDVPGVDRGGNIPNLGYMDASYIENLPNFVFKGPAMVRGMPDFTG  677
GS-ADH   QKDGYFMYQTPCSACHGDNAISGGVLPDLRWSGRPRGRESFYKLVGRGALTAYGMDRFDT  675
AA-ADH   VDNGYFPQYQTCQTCHGDNGEGAGMLPDLRWAGAIRHQDAFYNVVGRGALTAYGMDRFDT  662
MDH      PTAG--------------LGAVGAFKKLANYTQMGGGVVVFSLDGKGPYDDPNVGEWKS  596
PVADH    TSDG--------------KVLWEFNTAAAPYKTVAGKQADGGVMDGAGPTIAGGMVYVHS  577
                                           o

THFA-DH  KLTAEDVVKIQAFIQGTADAVRPRKP------------------------------- 671
QH-EDH   KLSGDDVESLKAFIQGTADAIRPKP-------------------------------- 677
GS-ADH   SMTPEQIEDIRNFIVKRANESYDDEVKARENSTGVPNDQFLNVPQSTADVPTADHP  731
AA-ADH   SMTPEIEAIRQYLIKRANDTYQREVDARKNDKDIPENPTLGINP----------- 707
MDH      AAK---------------------------------------------------- 599
PVADH    GYAGRSTQNAGDLRGREGNVLIAPSVDGK--------------------------- 606
```

His627 (Fig. 3). Additional residues involved in cofactor binding and β-propeller fold formation were found to be conserved in the sequence deduced for TfaA (Fig. 3) (19). Thus, we could assign the amino acids forming a sandwich structure with the PQQ cofactor. The two cysteine residues of TfaA, Cys109, and Cys110 (Fig. 3), probably form the disulfide ring which was shown to be located at the top of the PQQ cofactor and suggested to be involved in electron transfer during catalysis (4). In case of TfaA, Trp238 (Fig. 3) was identified as the amino acid located at the bottom of the active site interacting by its phenyl ring with the pyridine ring of PQQ (3, 19). The two residues responsible for binding of the Ca^{2+} ion and the active site base were also conserved in the sequence of TfaA (3, 19).

DISCUSSION

We report here some of the catalytic properties of the THFA-induced type I quinohemoprotein ADH THFA-DH from *R. eutropha* strain Bo. Furthermore, the complete primary structure of the protein was elucidated and analyzed. Thus, THFA-DH is the third type I quinohemoprotein ADH for which these data are available. Due to its basic properties, PVADH from *Pseudomonas* sp. strain VM15C should belong to the type I quinohemoprotein dehydrogenases (41). However, the sequence identity to the proteins from *C. testosteroni* and *R. eutropha* strain Bo was only about 25%. In contrast to all other quinohemoprotein ADHs described so far, PVADH was suggested to contain a heme-binding motif in the N-terminal region of the protein and should thus not be considered as a typical type I enzyme.

In general, quinohemoprotein dehydrogenases are not very specific with respect to their substrate spectrum. Furthermore, the catalytic efficiency by which different substrates are converted under in vitro conditions shows significant deviations among the enzymes. The more complex type II quinohemoprotein ADHs from acetic acid bacteria have a preference for primary alcohols as substrates, showing the highest level of activity with ethanol (1, 2), but they can also oxidize secondary alcohols, e.g., 2-butanol or solketal (29, 30). In contrast, the substrate spectrum of the type I enzymes is much broader, including primary and secondary alcohols, diols, polyols, and aldehydes. In case of QH-EDH from *C. testosteroni*, *n*-butanol

FIG. 3. Multiple sequence alignment of TfaA from *R. eutropha* strain Bo (THFA-DH) with various quinoprotein ADHs: QH-EDH from *C. testosteroni* (Q46444); GS-ADH, quinoprotein ADH from *Gluconobacter suboxydans* (O05542); AA-ADH, quinoprotein ADH from *A. aceti* (P18278); MDH, methanol dehydrogenase from *Methylobacterium extorquens* (P16027); PVADH from *Pseudomonas* sp. P77931. Signal sequences are depicted in lowercase letters. Amino acids which were conserved in all investigated sequences are indicated on top of the alignment by !, similar residues are marked by ∗, and residues which were conserved in most sequences are indicated by boldface letters. Amino acids identical in all sequences, excluding PVADH, are indicated at the bottom of the alignment by ○, and similar residues are shown by +. The heme c binding sites are boxed, and amino acids supposed to be involved in PQQ binding are indicated by shaded boxes. The disulfide ring-forming cysteine residues and the opposite tryptophan residue are underlined. The tryptophan residues corresponding to tryptophan-docking motifs are indicated by a boldface **W** above the sequences. The N-terminal sequence previously determined by Edman degradation is underlined.

or *n*-pentanol was found to be converted at the highest rate (44). Allyl alcohols and 1,2-propanediol, respectively, were preferred by ADH IIB or IIG of *P. putida* (48). The PVADH from *Pseudomonas* sp. VM15C was induced during growth on polyvinyl alcohol and could also oxidize short secondary alcohols. Interestingly, the enzyme was not able to convert primary alcohols (41). Polyethylene glycol (PEG) dehydrogenase isolated from *Rhodopseudomonas acidophila* converted several kinds of PEG and exhibited the maximum activity with *n*-propanol (55). The detailed initial rate measurements performed in the present study showed that primary alcohols (except ethanol) were converted by THFA-DH with the highest catalytic efficiency. The substrate inhibition observed for most alcohols and aldehydes tested is in agreement with the data obtained for QH-EDH from *C. testosteroni* (15).

Investigations of the enantioselectivity of THFA-DH from *R. eutropha* strain Bo during the oxidation of racemic mixtures of chiral alcohols showed that the enzyme exhibited a stereospecificity. Comparable studies with other quinohemoprotein ADHs were preferentially carried out using industrial relevant alcohols as substrates, e.g., solketal and glycidol (14–17, 29). QH-EDH from *C. testosteroni*, which showed the highest activity and *E* value for solketal, had the same preference for the *R*-(−)-alcohol as that observed for THFA-DH. Likewise, THFA-DH from strain Bo and QH-EDH from *C. testosteroni* had a preference for the *S*-(+)-enantiomer of aliphatic chiral alcohols (44). However, the *E* values determined for different chiral alcohols showed significant deviations.

As far as the catalytic mechanism of quinoprotein dehydrogenases was investigated, the results obtained always indicated that the substrates were converted by a ping-pong reaction mechanism (15, 30, 32, 37). Initial rate measurements performed with THFA-DH from *R. eutropha* strain Bo with ethanol or acetaldehyde as a substrate also revealed this catalytic mechanism. QH-EDH of *C. testosteroni* was analyzed in more detail (15). The data obtained indicated that catalysis takes place by a hexa-uni ping-pong mechanism where the aldehyde is released from the enzyme and uncompetitive substrate inhibition occurs in some cases. Due to the high similarity between both enzymes, one might assume that this model is also valid for THFA-DH from *R. eutropha* strain Bo.

The physiological function of quinoprotein dehydrogenases is to provide the respiratory chain with electrons, and the enzymes are usually located in the periplasm of gram-negative bacteria (19, 40). Due to the observed signal peptide probably responsible for translocation of the protein into the periplasm, THFA-DH seems also to be a periplasmic enzyme (5, 49).

R. eutropha strain Bo is able to synthesize PQQ; however, the sequence located up- and downstream from the *tfa*A gene (~800 bp) did not reveal any similarity to genes encoding enzymes involved in PQQ biosynthesis as was found for *Pseudomonas aeruginosa* (40). Furthermore, no similarity was obtained to genes which might be involved in the further oxidation of formed aldehydes, as was shown for *C. testosteroni* and *P. aeruginosa* (40, 45).

X-ray structures obtained for different methanol dehydrogenases led to the identification of amino acids responsible for cofactor binding and formation of an eight-bladed β-propeller fold maintaining the structure of the α-subunit (18, 19, 50, 52). Homology modelling of different PQQ-dependent dehydroge-

nases on the structure of methanol dehydrogenase led to the conclusion that the overall topology of these enzymes is similar (6, 7, 24). In the case of QH-EDH from *C. testosteroni*, the C-terminal part was modelled on cytochromes *c* for which structural information was available and revealed some structural similarity to these proteins (24). Our analysis of the deduced sequence of TfaA from strain Bo indicated that these topological features are probably conserved in this enzyme. Thus, QH-EDH from *C. testosteroni* and THFA-DH from *R. eutropha* strain Bo are closely related and might employ the same catalytic mechanism. The high level of similarity between these enzymes can be a powerful tool to elucidate the structural basis responsible for the observed catalytic deviations.

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