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Molecular Characterization of the Genes Encoding the Tungsten-Containing Aldehyde Ferredoxin Oxidoreductase from *Pyrococcus furiosus* and Formaldehyde Ferredoxin Oxidoreductase from *Thermococcus litoralis*

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The hyperthermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis* contain the tungstoenzymes aldehyde ferredoxin oxidoreductase, a homodimer, and formaldehyde ferredoxin oxidoreductase, a homotetramer. Herein we report the cloning and sequencing of the *P. furiosus* gene *aor* (605 residues; M_r, 66,630) and the *T. litoralis* gene *for* (621 residues; M_r, 68,941).

Enzymes containing tungsten (W) are rare in biology, yet the hyperthermophilic archaea contain three distinct types, all of which catalyze aldehyde oxidation (6–9). The homodimeric aldehyde ferredoxin oxidoreductase (AOR) of *Pyrococcus furiosus* (maximum growth temperature [T_{max}], 105°C [3]) oxidizes a wide range of aliphatic and aromatic, nonphosphorylated aldehydes (7). Recent crystallographic analysis (refined at 2.3 Å [2]) demonstrated that the two subunits of AOR are bridged by a monomeric iron site and that each subunit contains a [4Fe-4S] cluster and a mononuclear W cofactor. The W site is coordinated by four dithiolene-sulfur ligands from two pterin molecules (2). In contrast to AOR, the homotetrameric formaldehyde ferredoxin oxidoreductase (FOR) of *Thermococcus litoralis* (T_{max}, 98°C [11]) oxidizes only C_1 to C_3 aldehydes, although it also contains one W atom and one [4Fe-4S] cluster per subunit (8). The third W-containing oxidoreductase (GAPOR) is monomeric and specifically oxidizes glyceraldehyde-3-phosphate (9). We have now cloned and sequenced the genes encoding *P. furiosus* AOR and *T. litoralis* FOR. The primary structure of a tungstoenzyme has not been previously reported.

***P. furiosus* AOR.** For *aor*, a 7.2-kbp *Eco*RI *P. furiosus* (DSM 3638 [3]) genomic fragment was cloned into pBluescript II KS$^+$ (pAOR 3-1), using probes derived from the amino-terminal sequence and standard procedures (Southern blotting and hybridization and colony lifts) (12). From pAOR 3-1, 4,951 bp were sequenced, beginning 90 bp upstream of a unique internal *Eco*RV site and ending at the downstream *Eco*RI site (Fig. 1). Sequencing was performed with a mixture of deletion clones constructed with appropriate restriction enzymes and by primer walking. The fragment contained the genetic information for the entire AOR polypeptide (Fig. 1 and 2) together with (i) a *P. furiosus* gene, termed *cmo* for cofactor modification, which may code for a cofactor-modifying protein; (ii) the first 169 amino acid residues of a *P. furiosus ahc* gene encoding an *S*-adenosylhomocysteine hydrolase; and (iii) a short open reading frame and a partially sequenced long open reading frame of unknown function (Fig. 1) (5).

The gene for AOR contained 605 codons which correspond to a protein with a molecular weight of 66,630 (compared with 85,000 by sodium dodecyl sulfate (SDS)-gel analysis [7]) or 136,066 for the homodimer, including cofactors (Fig. 2). The [4Fe-4S] cluster in each AOR subunit is liganded by the cysteinyl residues at positions 288, 290, 295, and 494 (Fig. 2). The remote Cys-494 residue forms a hydrogen bond to a ring nitrogen of one pterin, thus linking the two metal centers at their smallest distance (2). The two pterins are coordinated in part by two homologous DXXGL groups (positions 338 to 342 and 489 to 494), Arg-76, Arg-182 (which forms a salt bridge to the phosphate moiety of one of the pterins), Thr-344, and Leu-495 (Fig. 2). Other relevant residues indicated in Fig. 2 include (i) Asn-93 and Ala-183 next to Arg-182, the carbonyl moieties of which coordinate a magnesium ion (which bridges the pterin phosphates) and Lys-450; (ii) Glu-313 and His-488, whose side chains are in the vicinity of the substrate binding site and might participate in electron transfer reactions; and (iii) Glu-332 and His-382, which are part of separate EXXH motifs and coordinate the monomeric iron site (2).

***T. litoralis* FOR.** For *for*, a 4,516-bp *Bam*HI-*Bgl*II genomic *T. litoralis* (DSM 5473 [11]) fragment was identified and cloned into the *Bam*HI site of pBluescript II KS$^-$ (pTli8a1) and sequenced. It contained the entire gene, which encodes the FOR subunit (Fig. 1 and 2), together with (i) the N-terminal part of a *T. litoralis* pyruvate kinase gene (*pki*), (ii) a gene coding for a putative iron-sulfur protein homologous to two different activating enzymes of the two *Escherichia coli* pyruvate formate lyases, and (iii) a short ORFZ (Fig. 1) of unknown function (5). The *for* gene contained 621 codons which corresponded to a protein with a molecular weight of 68,941 (compared with 69,000 by SDS-gel analysis [8]) or 281,264 for the homotetramer, including cofactors (Fig. 2).

Comparison of AOR and FOR sequences. The alignment of both sequences is shown in Fig. 2. The two enzymes share 231 identical residues (38% identity; 59% similarity). However, a much higher sequence similarity was observed for residues 1 to

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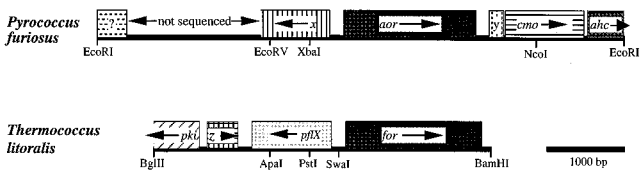

FIG. 1. Graphic representation of *P. furiosus* and *T. litoralis* genomic fragments. Gene arrangement and some relevant restriction sites are indicated. The insert size of the *P. furiosus Eco*RI fragment is approximately 7.2 kbp, and the *T. litoralis Bam*HI-*Bgl*II fragment size is 4,516 bp. Gene designations are as follows: *x*, long unknown ORFX; *y*, short unknown ORFY; *pflX*, unknown putative FeS-containing protein which is homologous to the activase of pyruvate formate lyases; *z*, short unknown ORFZ. ?, no sequence similarity and no meaningful reading frame found.

210 (48% identity), which coincide with domain I of AOR (Fig. 2) (2), while the similarity in domains II and III was about 33%. The four cysteinyl residues that coordinate the [4Fe-4S] cluster in AOR are conserved in the FOR sequence, as are the magnesium-coordinating Asn-93 and Ala-183 residues and most, but not all, of the pterin-coordinating residues. These include Glu-313 and His-488, which are near the substrate binding site adjacent to the W atom. The second DXXGL motif was not present in FOR, although it is possible that Asp-489 in AOR, which is a bidentate ligand to one of the pterins, is replaced in FOR by Glu-489. At this point it is not clear which particular sequence differences reflect the different substrate specificities of the two enzymes (Fig. 2). FOR lacked all three of the EXXH motifs found in AOR. These include residues Glu-332 and His-382, which bind the mononuclear iron site, suggesting that this type of subunit interaction is not present in FOR.

Sequence comparisons of AOR and FOR with other proteins. All molybdenum (Mo)-containing enzymes (except nitrogenase) also contain a pterin cofactor and, typically, one or more iron-sulfur centers (13). However, the primary structures of AOR and FOR showed no similarity to any molybdoenzyme

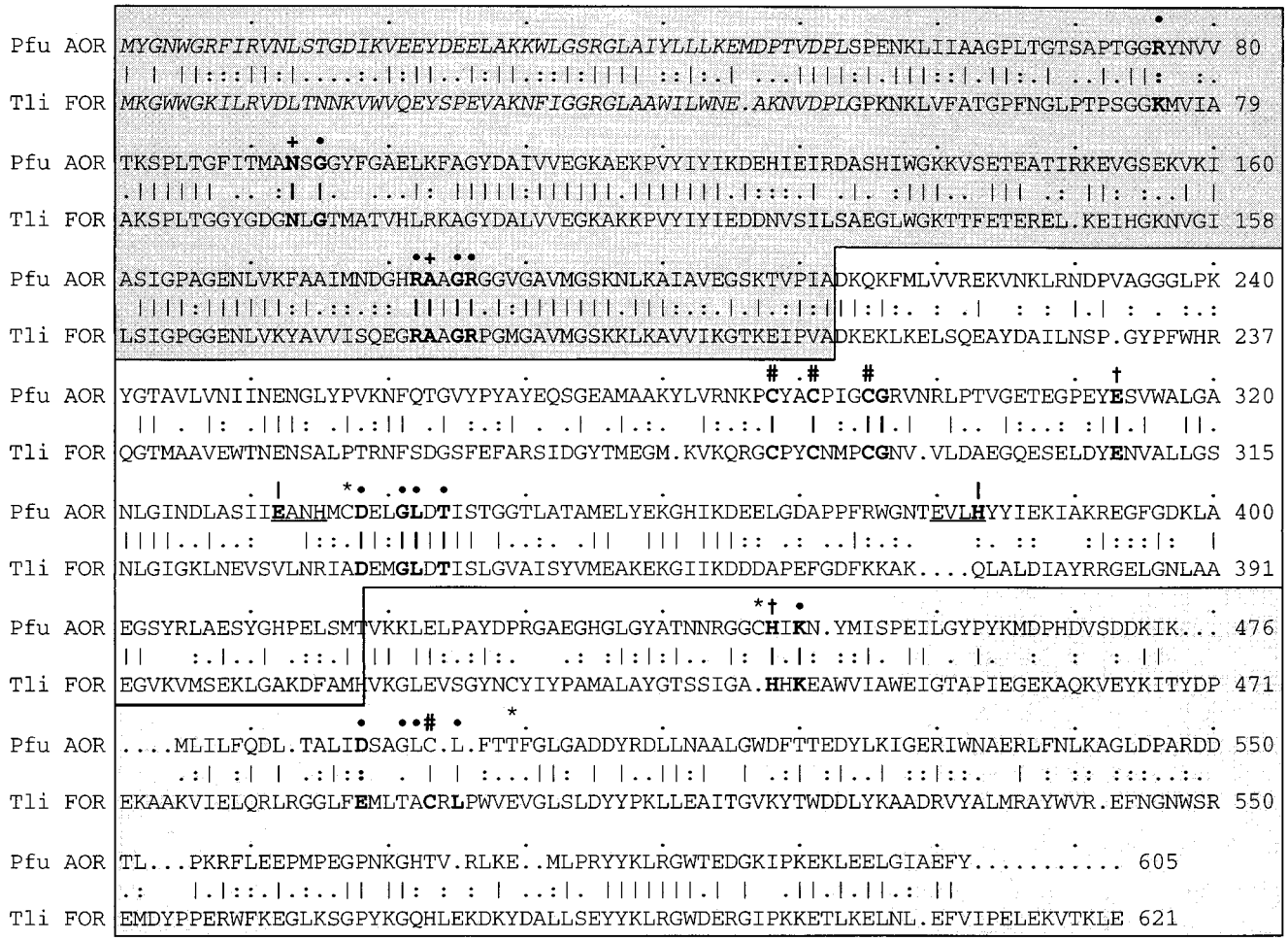

FIG. 2. Pairwise alignment of the AOR and FOR amino acid sequences. The boxes indicate the three domains of AOR (2): I (dark grey, upper), II (white, middle), and III (light grey, lower). N-terminal amino acid sequences determined from the purified proteins (7, 8) are indicated by italics. Specific residues (where numbers refer to the AOR sequence) or groups of residues are denoted as follows: cofactor-coordinating residues in AOR (boldface type); EXXH conserved motifs of diiron oxo clusters (underlined); cysteine residues that coordinate the [4Fe-4S] cluster (three of them are located in a conserved Cys-cluster motif [CXXCXXXCG]) (#); pterin-coordinating residues, including two symmetric DXXGLC/DX motifs (●). The fourth ligand of the FeS cluster (Cys-494) and Arg-74 bridge the FeS cluster and the tungstopterin; the Glu-332 and the His-383 residues (indicated by thick vertical lines) in both subunits provide the ligands of the non-heme iron bridging the two subunits. The carbonyl moieties from the peptide backbone of the Asn-93 and Ala-183 residues (+) provide two of the six ligands to the magnesium ion; the side chains of Glu-313 and His-448 (†) are in the vicinity of the substrate binding site. The function of the remaining cysteine residues (*; two in AOR and one in FOR) is not known.

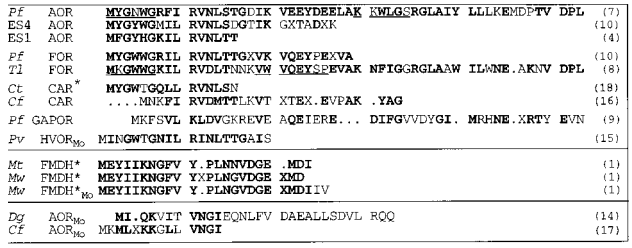

FIG. 3. N-terminal amino acid sequences of related tungsto- and molybdoenzymes. References (in parentheses) are shown. *, large subunit; Mo, the enzyme contains Mo. The N-terminal sequences of the small subunit of *C. thermoaceticum* CAR and the large and small subunits of *Methanobacterium wolfei* formylmethanofuran dehydrogenase (FMDH) show no similarity to the sequences of these enzymes. Identical positions or conservative exchanges are indicated by boldface type. The amino acid residues used in the design of oligonucleotides for the generation of probes for cloning of the *aor* and *for* genes are underlined.

whose complete sequence is available. In spite of the absence of the primary structure of other tungstoenzymes, a comparison of amino-terminal sequences (Fig. 3) showed that the AORs of *P. furiosus*, *Pyrococcus* sp. strain ES-4 (10), and *Thermococcus* sp. strain ES-1 (4) and the FORs of *P. furiosus* (10) and *T. litoralis* (8) are homologous to the W-containing GAPOR from *P. furiosus* (9), the W-containing carboxylic acid reductases (CARs) from *Clostridium thermoaceticum* and *Clostridium formicoaceticum* (16, 18), and surprisingly, the Mo-containing (2R)-hydroxycarboxylate viologen oxidoreductase (HVOR) from *Proteus vulgaris* (15). This high degree of similarity suggests that domain I of AOR, which provides a structural backbone for the tungstodipterin site (2) and is highly conserved in FOR, might also be conserved in these other oxidoreductases. In contrast, no significant similarity in amino-terminal sequence was found with W-containing formylmethanofuran dehydrogenase, or its Mo-isozyme, from methanogens (1) (sequence information for the only other W-containing enzyme known, formate dehydrogenase from acetogens, is not available [19]). It is therefore concluded that AOR, FOR, GAPOR, CAR, and HVOR form a family of homologous enzymes, all of which catalyze the oxidation of aldehydes (Fig. 3). *C. thermoaceticum* also possesses a second aldehyde-oxidizing enzyme (also termed AOR) that contains Mo (17), and this has a subunit size and an amino-terminal sequence which are similar to those of the Mo-containing, aldehyde-oxidizing AOR of *Desulfovibrio gigas* (Fig. 3) (14). Hence, it can be concluded that there are at least two phylogenetically separate enzyme families of aldehyde-oxidizing enzymes present in various microorganisms, W-based AOR-CAR and Mo-based AOR.

Nucleotide sequence accession numbers. The sequences for *P. furiosus aor* and *T. litoralis for* were deposited in the EMBL data bank under accession numbers X79777 and X83963, respectively.

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