Isolation and Analysis of the Gene Encoding the Pyruvate-Ferredoxin Oxidoreductase of Desulfovibrio africanus, Production of the Recombinant Enzyme in Escherichia coli, and Effect of Carboxy-Terminal Deletions on Its Stability

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Previous studies have shown that the pyruvate-ferredoxin oxidoreductase (POR) of the sulfate-reducing bacterium Desulfovibrio africanus is a homodimer that contains one thiamine pyrophosphate and three [4Fe-4S]++ centers/subunit. Interestingly, the enzyme isolated from a strictly anaerobic bacterium is highly stable in the presence of oxygen, in contrast to the other PORs characterized in anaerobic organisms (L. Pieulle, B. Guigliarelli, M. Asso, F. Dole, A. Bernadac, and E. C. Hatchikian, Biochim. Biophys. Acta 1250:49–59, 1995). We report here the determination of the nucleotide sequence of the por gene encoding the D. africanus POR. The amino acid sequence deduced from this nucleotide sequence corresponds to the first primary structure of a homodimeric POR from strictly anaerobic bacteria. The subunit of the D. africanus POR contains two ferredoxin-type [4Fe-4S] cluster binding motifs (CX2CX2CX3CP) and four additional highly conserved cysteines belonging to a nontypical motif. These 12 cysteine residues may coordinate the three Fe-S centers present in D. africanus POR. The thiamine pyrophosphate binding domain is located in the C-terminal part of the protein close to the four conserved cysteine residues. The D. africanus enzyme sequence appears homologous to the other POR sequences. However, the enzyme differs from all other PORs by a C-terminal extension of about 60 residues of its polypeptide chain. The two cysteine residues located in this additional region may be involved in the formation of a disulfide bridge associated with the activation process of the catalytic activity. The por gene has been expressed, for the first time, in anaerobically grown Escherichia coli behind the isopropyl-β-D-thiogalactopyranoside-inducible tac promoter, resulting in the production of POR in its active form. The recombinant enzyme is stable toward oxygen during several days, and initial characterization of the recombinant POR showed that its activity increased in the presence of dithioerythritol. These properties indicate that the recombinant POR behaves like the native D. africanus enzyme. The study of carboxy-terminal deletion mutants strongly suggests that deletions in the C-terminal region of D. africanus enzyme can have dramatic effects on the stability of the enzyme toward oxygen.

Oxidative decarboxylation of pyruvate is a key reaction of intermediary metabolism. It is catalyzed by two types of thiamine pyrophosphate (TPP)-dependent enzymatic system. In most eukaryotes and also in many bacteria, the oxidation of pyruvate is catalyzed by pyruvate dehydrogenase (PDH), a multienzyme complex with a molecular mass of greater than 1 MDa (47). This complex is composed of three different enzymes, contains lipoate and flavin as prosthetic groups, and transfers electrons to acceptors with a more negative redox potential than that of NAD, in most cases to ferredoxin. The reaction catalyzed by the pyruvate oxidoreductase is summarized by the following equation: CH3-CO-COOH + CoASH ⇌ CH3-CO-S-CoA + CO2 + 2H+ + 2e− (E0 = −540 mV) (25), where CoA is coenzyme A.

In strictly anaerobic organisms and in anaerobic protozoa, pyruvate oxidoreductase is involved in the phosphorolytic reaction allowing energy conservation by substrate-level phosphorylation (42, 48). On the other hand, in many nitrogen-fixing microorganisms, it is the source of low-potential electrons for nitrogenase (5). In these organisms, the electrons are typically transferred to flavodoxin (see references in reference 5). Furthermore, the role of pyruvate oxidoreductase in the formation of pyruvate from acetyl-CoA and CO2 has been clearly established (41).

Pyruvate-ferredoxin (flavodoxin) oxidoreductases (PORs) have been isolated from a variety of anaerobic bacteria, archaea, and eucaryotes. Three types of POR can be distinguished on the basis of molecular structure. The enzymes isolated from various mesophilic organisms are commonly homodimeric enzymes with sizes of around 240 kDa (5, 9, 27, 34, 44, 46, 48). The POR from the aerobic archaeon Halobacterium halobium is a heterotetramer (α2β2) composed of two different subunits with molecular masses of 67.7 and 34.4 kDa (20, 35). More recently, a new group of PORs containing four different subunits per molecule has been characterized. This type of enzyme is present in the anaerobic archaea Pyrococcus furiosus (2), Archaeoglobus fulgidus (22), and Methanosarcinaarkeri (4) as well as in the bacteria Thermotoga maritima (3) and Helicobacter pylori (18). The molecular masses of the four
different subunits are around 45, 33, 24, and 13 kDa. The genes encoding the PORs from several mesophiles, two hyperthermophiles, and *H. halobacterium* have been cloned and sequenced (21). The multiple alignment of the amino acid sequences has revealed that the multisubunit hyperthermophilic and the homodimeric PORs are phylogenetically related. It has been suggested that the evolution of all of the various PORs, whether they comprise one, two, or four subunits, can be explained by the rearrangement of four ancestral genes (21). The mechanism of pyruvate oxidation by PDH has been firmly established, but this is not the case with the PORs. For PORs, two different catalytic mechanisms have been proposed for the first step of the reaction, involving either a TPR radical-base reaction or a non-radical-base reaction. For example, no free radical was detected with the clostridial (29, 46) and *T. maritima* PORs (38). In contrast, an electron paramagnetic resonance signal assigned to the hydroxyethyl-TPP radical appears on addition of pyruvate to *H. halobacterium* and *P. furiosus* PORs (6, 38). The second step of the mechanism including electron transfer from the acetyl moiety to CoA is unknown, in contrast to the PDH complex, which functions with lipic acid. Moreover, the exact role and, in many cases, the actual number of [4Fe-4S] centers present in PORs are a matter of debate (3, 21). The uncertainty about the number of iron and labile sulfide atoms could be related to the high sensitivity of the PORs isolated from anaerobic microorganisms to oxygen (3, 46, 48).

Bacteria of the genus *Desulfovibrio* exhibit a unique strictly anaerobic mode of growth based on the reduction of sulfate as the terminal electron acceptor. In the presence of sulfate, pyruvate is a key intermediate during oxidation of organic substrates such as lactate, fumarate, malate, and alanine into acetate (32, 39). In addition, pyruvate is a well-known fermentative substrate for sulfate reducers. Differences in pyruvate metabolism in the absence of sulfate have been described for various species of these microorganisms; however, acetate is always present in the end products (40). The presence of pyruvate oxidoreductase activity in extracts of *Desulfovibrio* has been reported previously (13, 31, 33). The POR is a key enzyme of the energy metabolism of these bacteria, allowing ATP generation via the phosphoroclastic reaction. Recently, the POR from *Desulfovibrio africanus* has been isolated and characterized (34). *D. africanus* enzyme is a homodimeric protein with a reported mass of 256 kDa which differs from the PORs isolated from other anaerobic organisms in its high stability to oxygen (34). Moreover, the presence of three [4Fe-4S]$^{3+/2+}$ centers per subunit has been proved unambiguously (34), and this value is higher than for other PORs, which have at most two [4Fe-4S] clusters per subunit (2–5, 9, 20, 22, 27, 44, 46, 48). The midpoint potentials of the three centers are $-390$, $-515$, and $-540$ mV (34). The activity of *D. africanus* POR, isolated in aerobic conditions, can be increased approximately fivefold in the presence of sulphydryl compounds such as dithioerythritol (DTE). This property distinguishes this enzyme from all the other PORs.

So far, the known bacterial homodimeric POR primary structures are confined to several non-*D. africanus* genes coding for PORs of the nitrogen-fixing operons of bacteria. In the present communication, we report for the first time the primary structure of a homodimeric POR from a strictly anaerobic bacterium, *D. africanus*. This result has permitted a comparison of the sequence of this type of enzyme with related sequences, and we propose the identification of the 12 cysteine involved in the chelation of the three [4Fe-4S] centers per subunit. We also report for the first time the production of a pyruvate oxidoreductase, *D. africanus* POR, in *Escherichia coli*. Moreover, we have prepared two carboxy-terminal deletion mutants of POR to gain a better understanding of the role of this region.

**MATERIALS AND METHODS**

*Bacterial strains and plasmids. D. africanus* (NCIB 8401) was grown at 37°C in a basic lactate-sulfate medium (23). *E. coli* MO5Blue (F$^{-}$endA1 hsdR17 (rK$^{-}$mK$^{-}$)) supE44 thi-1 gyrA96 relA1 (F lacZAM15 proAB ThI1 Tcr) (recA1) was used for phagemid propagation (Amersham). *E. coli* TGI (supE44 thi-1 dΔ36 proAB Ftr-dΔ36 proAB lacI135 lacZAM15) was used for POR expression. PCR products to be sequenced were directly cloned in phagemid PMO5Blue-T (Amersham). Ampicillin was used at the concentration of 100 μg ml$^{-1}$.

**Trypptic digestions of the D. africanus POR.** Internal amino acid sequences of *D. africanus* POR were obtained by using tryptic peptides of the protein as reported previously (28).

**Specific amplification of the por gene.** From the N-terminal amino acid sequences of two tryptic peptides (B and D [Fig. 1]), two pairs of degenerate oligonucleotides, one each from the coding and anticoding strands, were designed: 5'-TAT GTN GGN ATH TAY GAY AT-3' ($b^+$) plus 5'-ATR TCR TAD ATN CCN ACR TA-3' ($b^-$) and 5'-GAY GAY AYC CCN ATG GC-3' ($d^+$) plus 5'-GCC ATC ATN GGN GTR TCR TC-3' ($d^-$). All meaningful combinations of oligonucleotides from the different peptides were chosen for PCR amplification. PCR amplification was performed by using chromosomal DNA prepared as previously described (26). PCR products were carried out as reported previously (37), using a mixture of Taq and Pwo DNA polymerases (Boehringer Mannheim) with addition of 5% dimethyl sulfoxide because the *D. africanus* DNA is $G+C$ rich (7).

**Cloning and sequencing of the POR products.** The PMO5Blue-T vector (Amersham) was used for cloning the POR products. The presence of insert was checked by PCR directly on the transformants, using T7 and U-19 universal primers. Insert in the PMO5Blue-T vector was then sequenced by the exonuclease III, using an Exonese deletion kit (Biolabs). The sequencing reaction was performed by PCR amplification according to the Applied Biotecnology protocol.

**Amplification of the por gene 5′ and 3′ ends by inverse PCR.** Amplification of flanking sequences by inverse PCR was carried out as described previously (30). PCR was performed with using two outward-facing oligonucleotides (N1 and N2 for the 3′ end of the por gene; C1 and C2 for the 3′ end of the por gene [Fig. 1]). The inverse PCR products were directly sequenced by using 100 ng of PCR products.

The complete nucleotide sequence of the por gene was carefully checked by PCR amplification followed by direct sequencing of the products on both strands in order to eliminate all errors produced by *Taq* DNA polymerase. Sequences of the oligonucleotides used (p1 to p13) are shown in Fig. 1.

**Construction of plasmids pLP1, pLP231, and pLP251.** The por sequence was amplified by PCR using oligonucleotides pK and pF as primers. To minimize the occurrence of mutations during amplification, we used *Pwo* DNA polymerase (Boehringer Mannheim) and performed 25 cycles. The sequence of pK (5′-TGG TGA CCC AAG GAG CAT CAT ATG GGA AAG AAG-3′) is composed of a *Kpn I* restriction site (underlined) followed by the putative ribosome binding site of the por gene (boldface) and then 18 nucleotides corresponding to the beginning of the por sequence including the ATG (boldface). The sequence of pF (5′-TGA TAC CCT GTA CTG CTC GGA GAC GGA-3′) contains a BamHI cleavage site (underlined) followed by two nucleotides and a triplet complementary to the stop codon (boldface) and by nucleotides complementary to the very 3′ end of the por coding sequence. The *Kpm/BamHI* fragment was introduced into the polylinker cloning sites of plasmid pBluescript II (12) to give plasmid pLP1.

We prepared two deletion mutants of *D. africanus* PORs lacking the carboxy-terminal 51 amino acids, POR (Ala1182-Lys1322), or 21 amino acids, POR (Cys1122-Lys1322). In this way, the por nucleotide sequence was deleted by PCR amplification using pK and p221B or p251B as primers. The sequences of p221B (5′-TCT GTA TCA CTA GCA GTC ACC GAG GGC GAT ATG GGC-3′) and p251B (5′-TGA TCC CTA CTA GCT GGC GAG ATG TTC GGC GGC GAG GGC-3′) contain a BamHI cleavage site (underlined) followed by two stop codons complementary sequence (boldface) and by nucleotides complementary to the very 3′ end of the por coding sequence deleted of the last 63 and 153 nucleotides, respectively. The two *Kpm/BamHI* fragments obtained were introduced into a polylinker cloning sites of plasmid pBluescript II to give plasmids pLP231 and pLP251.

**Production of D. africanus recombinant POR and deletion mutants in E. coli.** *D. africanus* POR was produced by growing 500 ml of *E. coli* TGI carrying plasmid pLP1 in 2× YT medium containing 100 μg ml$^{-1}$ ampicillin in anaerobic conditions. When the culture reached 0.5 $A_{590}$ unit, production of the POR enzyme was induced for 4 h with 0.2, 0.4, and 1 mM IPTG (isopropyl-$β$-D-thiogalactopyranoside). The cells were subsequently harvested by centrifugation, and the pellet was washed with 20 mM Tris-Cl (pH 8.5) degraded degenerate. The pellet was then gently resuspended in 4 ml of degassed buffer (50 mM Tris-Cl [pH 8.5]) containing the protease inhibitors benzamidane (1 mM) and Pefabloc (1 mM) (Boehringer Mannheim). Finally, the cells were passed once through a French pressure cell at 100 MPa, and cell debris was removed by centrifugation at 30,000 × g for 20 min. The crude extract from *E. coli* TGI/pLP1

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(0.4 mM IPTG) was then centrifuged at 120,000 $\times g$ for 90 min to obtain the soluble protein extract.

Deletion mutants of POR were produced as described above by growing 250 ml of *E. coli* TG1 carrying plasmid pLPD21 or pLPD51. Preparation of crude extracts from TG1/pJF119EH, TG1/pLP1, TG1/pLPD21, and TG1/pLPD51 was carried out as reported for TG1/pLP1 but also in an anaerobic glove box under N$_2$. In this case, all buffers used were degased and equilibrated in the glove box. The cells were suspended in 4 ml of 20 mM Tris-HCl buffer (pH 8.5) containing benzamide (1 mM) and Pefabloc (1 mM) and transferred to a French pressure cell equilibrated with N$_2$ in the glove box. The cell suspension was passed once through the French pressure cell at 100 MPa outside the glove box, and the extract was collected under N$_2$.

FIG. 1. Nucleotide and deduced amino acid sequences of the POR from *D. africanus*. The putative Shine-Dalgarno sequence is in boldface, and the inverted repeats for putative transcription termination are marked by arrows. Triangles mark cysteine residues; circles mark the TPP binding motif. Sequences confirmed by protein chemical work (N-terminal [N-term.] and peptides A through D) are in boldface and bracketed. * Translation stop codon.
Gel electrophoresis, enzyme assays, and immunological techniques. Analytical polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE were performed as reported previously (14). Measurement of POR activity in the extracts and detection of recombinant D. africanus POR by the Western blot technique were done as described previously (34). The POR activity was located in the gels by the method of Jin et al. (19). For anaerobic manipulations, POR activity was measured by injecting the crude extracts prepared anaerobically into assay cuvettes previously equilibrated in the glove box. Spectrophotometric measurements were carried out outside the glove box.

Nucleotide sequence accession number. Nucleotide sequence data have been submitted to the EMBL/GenBank data library and assigned accession no. D81580.

RESULTS

Sequencing of the por gene. D. africanus POR was hydrolyzed with trypsin to obtain internal peptide sequences of the protein. Of the numerous peptides obtained by trypsin digestion, four (peptides A, B, C and D [Fig. 1]) were sequenced. The amino-terminal sequence of the enzyme has been also determined (Fig. 1). Of a set of meaningful pairwise combinations of oligonucleotides (b1, b2, c1, and c2) derived from the amino termini of two tryptic peptides (B and D [Fig. 1]), the combination b1+c2 has permitted PCR amplification of a specific DNA fragment with a size of around 2.1 kb. This DNA fragment was cloned and sequenced. The sequence of peptide C and the end of the sequence of peptide B were easily recognized in the deduced amino acid sequence, indicating unambiguously that the nucleotide sequence was identical to that determined by amino acid sequencing, showing agreement with the number present in the POR amino acid sequence. In this way, a value of 18 cysteine residues was obtained instead of 16 reported previously (34), which is in agreement with the number present in the POR amino acid sequence. Two cysteine-rich clusters with the sequence -Cys-Xaa2-Cys-Xaa2-Cys-Xaa3-Cys-Pro- were found in the region of the ATG codon, this segment may correspond to a noncoding region of D. africanus DNA. The proposed initiator codon was preceded by a strong putative Shine-Dalgarno sequence (AAG GAG) located 6 bp upstream from the ATG. Downstream from the por gene, a sequence able to form stable stem-loop structures with a weak energy (ΔG of about −10 kcal/mol) is present (Fig. 1).

The calculated molecular mass of the putative translation product was 133,649 Da. This value is close to the value obtained for the POR by SDS-PAGE (133 kDa); however, the molecular mass of the native POR determined by analytical ultracentrifugation was estimated to be close to 256 kDa (34). More recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (16) of the D. africanus POR indicated that it had a molecular mass of 133,718 Da, in good agreement with that calculated from the amino acid sequence deduced from the por gene sequence (133,649 Da). The amino-terminal sequence and the four peptides (A to D) were identical to those determined by amino acid sequencing, showing unambiguously that the correct gene had been identified (Fig. 1). The calculated amino acid composition of the D. africanus POR was likewise in good agreement with the composition based on chemical amino acid analysis when recalculated on the basis of the molecular mass deduced from the nucleotide sequence. In this way, a value of 18 cysteine residues was obtained instead of 16 reported previously (34), which is in agreement with the number present in the POR amino acid sequence. Two cysteine-rich clusters with the sequence -Cys-Xaa2-Cys-Xaa2-Cys-Xaa3-Cys-Pro- were found in the region between amino acid positions 689 and 755 (Fig. 1). In addition, the C-terminal part of the protein contained a third cysteine motif (-Cys-Xaa2-Cys-) including cysteine residues 812 and 815.

Comparisons of the D. afric anus POR sequence with related sequences. The deduced amino acid sequence of D. afric anus por gene was compared with four POR sequences corresponding to the three types of POR (Fig. 2). The four subunits (α, β,
The amino acid sequences of the D. africanus (Da) POR, K. pneumoniae (Kp) and T. vaginalis (Tva) POR A subunits, P. furiosus (Pf) POR b subunits, and H. halobium (Hh) POR b subunit were aligned in a single sequence (agdb and ab) and compared with the single A subunit of the mesophilic PORs. Moreover, for the H. halobium enzyme, the alignment was started at position 211 of the large a subunit as described previously (21). The sequence of D. africanus POR was very similar to the sequences of other homodimeric PORs. In fact, the enzyme was 55 and 46% identical to K. pneumoniae and T. vaginalis PORs, respectively. However, the D. africanus enzyme differed from all other homodimeric enzymes by an extension of about 60 residues of its polypeptide chain (Fig. 2) (17). Interestingly, this additional region, located at the C-terminal extremity, contains two cysteine residues (Fig. 2). Like the other homodimeric PORs, the D. africanus enzyme was similar to the abgd PORs, whereas it exhibits only a few motifs in common with H. halobium a2b2 POR. In fact, the identity scores were 22 and only 10% between the sequence of D. africanus POR and those of P. furiosus and H. halobium, respectively. The multiple alignment presented in Fig. 2 showed that D. africanus POR contains the 12 cysteines highly conserved in all PORs except the H. halobium enzyme (21). The latter contains only 4 of these 12 conserved residues. The region of the D. africanus enzyme that includes the -Gly-Asp-Gly- tripeptide is a highly conserved cysteine residue implicated in the binding of the iron-sulfur centers. The alignment was obtained with the CLUSTAL W multiple sequence alignment program (version 1.5). GenBank accession numbers: D. africanus POR, Y09702; K. pneumoniae POR, X13109 (1); T. vaginalis POR subunit A, U16822 (17); P. furiosus POR X85250 (21); H. halobium POR, X64521 (35).
subunit was detected when cells were grown in the presence of IPTG, respectively; lane 3, extract from TG1/pJF119EH (70 μg), without and with 0.4 mM IPTG, respectively; lane 3, extract from TG1/pJF119EH (70 μg); lane 4, pure POR (2 μg); lanes 5 to 8, POR activity without DTE stained with triphenyl tetrazolium; lane 5, pure POR (20 μg); lanes 7 and 8, extract from TG1/pLP1 (200 μg) with and with 0.4 mM IPTG, respectively. Lanes 9 to 11, pure POR (250 ng), extract from TG1/pJF119EH (23 μg), and extract from TG1/pLP1 (9.5 μg), respectively (Western blotting, detection with D. africanus POR polyclonal antibodies).

stretch from residue 958 downstream in all homodimeric PORs (identity score close to 80%) (Fig. 2). Moreover, the corresponding area of the b subunit of P. furiosus and D. africanus correspond to the b subunit of H. halobium and β subunit of P. furiosus represents a stretch homologous to the D. africanus motif.

**Production of D. africanus POR in E. coli.** The por coding sequence was amplified by PCR, and the putative ribosome binding sequence of the por gene (AAGGAG) has been conserved because it was very close to the consensus ribosome binding sequence in E. coli. The amplified fragment was then cloned downstream from the IPTG-inducible tac promoter of plasmid pJF119EH to give plasmid pLP1. Strain TG1 harboring plasmid pLP1 was grown anaerobically in rich medium in the presence of IPTG. When crude extracts from TG1/pLP1 were analyzed by SDS-PAGE, a protein band migrating in the same position as the native D. africanus POR was detected (Fig. 3A, lanes 3 to 6). This protein band exhibits an apparent molecular mass of 133 kDa, consistent with the size predicted by the gene sequence. Moreover, this band was absent in E. coli harboring plasmid pJF119EH without the insert (Fig. 3A, lane 2). No significant variation of the production of the POR subunit was detected when cells were grown in the presence of three concentrations of IPTG (0.2, 0.4, and 1 mM) (Fig. 3A, lanes 3 to 5). The protein band detected by SDS-PAGE of the crude extract from TG1/pLP1 was recognized by D. africanus POR polyclonal antibodies (Fig. 3A, lane 8).

A POR specific activity of 0.09 U/mg was estimated in TG1/pLP1 crude extracts whatever the IPTG concentration used, and this activity was increased approximately fivefold (specific activity, 0.4 U/mg) in the presence of DTE. Moreover, the POR activity directly revealed in the gels and Western blots confirmed the presence of the D. africanus recombinant POR in the E. coli crude extracts (Fig. 3B, lanes 2 to 4 and 9 to 11). The enzyme activity detected in the gels is not dependent on the presence of DTE (Fig. 3B, lanes 5 to 7). Therefore, the activation process of the recombinant POR by sulfhydryl compounds seems to be similar to the activation process previously described for the native enzyme (34). Moreover, after 6 days, 90% of the POR activity was conserved at 4°C, indicating that the recombinant POR was stable by exposure to air for several days.

A weak endogenous methyl viologen reductase activity was detected in the extracts of E. coli/TG1 and E. coli/TG1/pJF119EH only in the presence of DTE. This activity, which was not pyruvate or CoA dependent, was found to be 250-fold lower than the activity measured in the cells harboring plasmid pLP1. This endogenous activity present in the E. coli extracts can be correlated with the presence of a second band of methyl viologen reductase activity in the gels, detected only in the presence of DTE (Fig. 3B, lanes 1 to 3 and 6 to 8).

When the TG1/pLP1 cells were grown without IPTG, a POR specific activity of 0.06 U/mg was found in the presence of DTE. This result indicates that the total POR activity of the cells was increased about sevenfold after induction by IPTG. This result has been confirmed by POR activities detected directly in the gels (Fig. 3B, lanes 1 and 8).

The POR specific activity of the soluble protein extract prepared from TG1/pLP1 (0.4 mM IPTG) was found to be 0.35 U/mg, which is comparable to the specific activity of the native enzyme in D. africainus extract (0.7 U/mg) (34).

**Effect of carboxy-terminal deletions on the recombinant POR properties.** Two deletion mutants of D. africanus POR have been constructed to identify the role of its C-terminal extension, which contains two cysteines (Cys1195 and Cys1212) (Fig. 1). Thus, two truncated forms of POR lacking one cysteine residue (Cys1212) were produced in E. coli. The protein band observed in TG1/pLP1 crude extracts whatever the IPTG concentration used, and this activity was increased approximately fivefold (specific activity, 0.4 U/mg) in the presence of DTE. Moreover, the POR activity directly revealed in the gels and Western blots confirmed the presence of the D. africanus recombinant POR in the E. coli crude extracts (Fig. 3B, lanes 2 to 4 and 9 to 11). The enzyme activity detected in the gels is not dependent on the presence of DTE (Fig. 3B, lanes 5 to 7). Therefore, the activation process of the recombinant POR by sulfhydryl compounds seems to be similar to the activation process previously described for the native enzyme (34). Moreover, after 6 days, 90% of the POR activity was conserved at 4°C, indicating that the recombinant POR was stable by exposure to air for several days.

When crude extracts from TG1/pLP51 and TG1/pLP21 were analyzed by SDS-PAGE, a protein band recognized by polyclonal antibodies against D. africanus POR was detected (Fig. 4, lanes 4 and 6). The same electrophoretic pattern was observed when the extracts were prepared in aerobic or anaerobic conditions. The protein band observed in TG1/pLP51 extract exhibited a slightly lower mobility than the native enzyme correlating with the deletion of the C-terminal extension of 51 amino acids (Fig. 4, lanes 1 and 4). Moreover, the intensities of the protein bands show a decrease in the amounts of truncated PORs, specially in the case of POR21, compared to the amount of the recombinant POR (Fig. 4, lanes 2, 4, and 6). These results strongly suggest that the two truncated forms of POR are more unstable than the recombinant enzyme in E. coli. The presence of a weak amount of the truncated form, POR21, could be the result of an increased proteolysis due to incorrectly folded enzyme. The immunodetection of a numer-
ous protein bands in the crude extracts from TG1/pLPΔ21 favors this hypothesis (data not shown).

TG1/pLPΔ51 and TG1/pLPΔ21 crude extracts prepared anaerobically display a POR activity corresponding to 58 and 3.4% of the recombinant POR activity, respectively, in accordance with the intensity of the protein bands on SDS-PAGE. In contrast to the recombinant enzyme, the crude extracts from TG1/pLPΔ51 and TG1/pLPΔ21 exhibited the same activity in the presence or in the absence of DTE, and losses of 80 and 100%, respectively, of their activities were observed after exposure to air for 3 h (TG1/pLPΔ51 extract) or 2 h (TG1/pLPΔ21 extract). After overnight incubation at 4°C, the activity measured with TG1/pLPΔ51 extract was close to the endogenous methyl viologen activity (1.7 × 10^{-3} U/mg) of TG1/pJF119EH extracts. When the cells harboring plasmid TG1/pLPΔ51 or TG1/pLPΔ21 were passed once through the French pressure cell under air, no POR activity was detectable in the spectrophotometric assay.

**DISCUSSION**

In this report, we have described the sequence, cloning, and expression in *E. coli* of the gene encoding the 133-kDa subunit for the homodimeric POR from the sulfate-reducing bacterium *D. africanus*. We have also investigated the effect of carboxy-terminal deletions on the stability of the recombinant enzyme. The homodimeric POR primary structures known to date are confined to several eubacterial and eukaryotic POR genes (1, 17). These two cysteine-rich clusters are highly conserved (Fig. 2). More recently, the sequences of the genes encoding subunits A of PORs of two intestinal parasites, *Giardia lamblia* and *Entamoeba histolytica*, have been entered in the database. The *D. africanus* POR primary structure sequence is thus the first sequence of a homodimeric POR from a strictly anaerobic bacterium.

The sequence of the *D. africanus* enzyme was very similar to the sequences of all homodimeric PORs reported in the database. This striking sequence similarity of *D. africanus* POR, eubacterial *nifH* products, and eukaryotic PORs is indicative of the close evolutionary relationship of these three classes of homodimeric enzymes. However, comparison of all homodimeric POR sequences reveals that the enzymes of the amitochondriate organisms are more closely related to the *D. africanus* enzyme than to *nifH* gene products. Moreover, the POR amino acid sequences of *Clostridium* species, in view of their biochemical and functional relatedness to the POR of *Desulfovibrio* species, are likely to be homologous to the *D. africanus* sequence and therefore to all other homodimeric POR sequences.

As already described for the homodimeric PORs, several domains of the single large A subunit of *D. africanus* POR were homologous to the four subunits of the hyperthermophilic PORs (Fig. 2) (21). Moreover, the multiple alignment including the three types of POR has shown that there may exist in the C-terminal part of the subunit A, at least, two functional domains (17). The very highly conserved region containing a tripeptide Gly-Asp-Gly represents the putative TPP binding site found in many decarboxylases (15). But in contrast to *H. halobium* POR, the *D. africanus* enzyme lacks the additional common features present in almost all decarboxylases. In particular, the highly conserved sequence -Asn- located approximately 30 residues after the -Gly-Asp-Gly- is not present in the latter enzyme. However, the high degree of conservation of this region in the three types of POR, including the *H. halobium* enzyme, which globally shows only weak similarities with homodimeric POR sequences, represents a good basis for the assignment of this region to the putative TPP binding site of *D. africanus* enzyme.

The multiple alignment showed also that of the 18 cysteines present in *D. africanus* enzyme, 12 were highly conserved in all of the PORs except that of *H. halobium*, which contains only 4 of the 12 conserved cysteines. Two cysteine-rich clusters with the sequence -Cys-Xaa,-Cys-Xaa,-Cys-Xaa,-Cys-Pro- characteristic of the binding sites of ferredoxin-type [4Fe-4S] centers were found in the subunit of *D. africanus* POR as well as in all homodimeric POR sequences (1, 17). These two cysteine-rich clusters were also found in the 6 small subunits of *T. maritima* and *F. furiosus* enzymes (21). Therefore, the eight cysteinyl residues of these two motifs are assumed to bind to two ferredoxin-type iron-sulfur clusters in *D. africanus* POR. It may be postulated that the four other cysteine residues (Cys812, -815, -840, and -1071) highly conserved in all known POR primary structures coordinate the third iron-sulfur cluster present in the *D. africanus* enzyme (34). In particular, the involvement of Cys812 and Cys815 in formation of the third cluster appeared highly probable because they are arranged in the peptide motif -Cys-Xaa,-Cys- known to chelate [4Fe-4S] clusters in other iron-sulfur proteins (11, 44). Moreover, the regions around Cys812, Cys815, and Cys840 as well as the regions flanking the cysteine-rich clusters are highly conserved (Fig. 2).

As recently proposed by Kletzin and Adams (21) and on the basis of our results (reference 34 and this work), it seems reasonable to conclude that all of the homodimeric PORs contain three iron-sulfur clusters per A or αβγδ subunit. However, the iron and sulfur contents as well as the results of electron paramagnetic resonance spectroscopic studies seem to indicate the presence of only one or two [4Fe-4S] centers per A or αβγδ subunit of all PORs (2, 3, 22, 27, 43, 45, 47) except that of *D. africanus* (34). The latter is the first example among anaerobic microorganisms whose primary structure data as well as biochemical and spectroscopic data are in agreement with the presence of three [4Fe-4S]1+2+ centers per subunit A. In the same way, the POR of the aerobic microorganism *H. halobium* contains only the four conserved cysteines belonging to the nontypical motif, and biochemical studies have demonstrated the presence of only one Fe-S cluster per ab subunit. Nevertheless, understanding of the role of the 12 highly conserved cysteinyl residues must await the determination of the three-dimensional structure of the *D. africanus* POR, which is now in progress.

The *D. africanus* POR gene has been expressed in *E. coli* under anaerobic conditions, and the biochemical properties of the recombinant POR strongly suggest that it is similar to the native enzyme (34). Therefore, production of *D. africanus* enzyme in its active form appeared to be feasible in *E. coli*, which...
implies that this bacterium can assemble the [4Fe–4S] clusters into the recombinant POR under anaerobic conditions.

Interestingly, Chen et al. (8) have reported the expression of the D. africanaus ferredoxin I gene in E. coli under aerobic conditions. This proved the ability of E. coli to incorporate [4Fe–4S] clusters into the recombinant apoferreredoxin under these conditions. In view of these results, we have tested the expression of D. africanaus POR gene in E. coli in aerobic conditions. In this case, the specific activity of the recombinant enzyme is 10-fold lower than that of the enzyme expressed in anaerobic conditions (data not shown). It may be postulated that in contrast to the monocluster D. africanaus ferredoxin I, which is a simpler protein than POR, the incorporation of the Fe-S clusters in the recombinant POR is more efficient in the anaerobically grown E. coli cells.

D. africanaus POR is distinguished from all other PORs by an additional sequence at the C-terminal extremity of the protein which contained two cysteinyl residues (Cys1195 and -1112) (Fig. 2). The catalytic activity of D. africanaus POR purified in aerobic conditions was stable to air and could be increased by addition of DTE, a reagent involved in the reductive cleavage of disulfide bridges (34). As the properties of the D. africanaus enzyme appear to be unique among all PORs so far characterized, it may be postulated that the two cysteines located in the additional region are involved in the formation of the disulfide bridge associated to the activation process of D. africanaus POR (34). These two cysteine residues are separated by 16 residues, which appears to be the correct amino acid spacing for the formation of a disulfide bridge (43). Moreover, the formation of the disulfide bridge might induce a change of conformation of the C-terminal part of the subunit, allowing protection of the enzyme toward oxygen.

The possible role of the additional C-terminal region in the protection of the enzyme toward oxygen has been studied by using carboxy-terminal deletions of D. africanaus POR and production in E. coli of the truncated proteins PORΔ51 and PORΔ21. As a result, the possible formation of disulfide bridge was suppressed in the two mutated enzymes. The carboxy-terminal deletions completely eliminated the POR catalytic activity when the crude extracts were prepared aerobically, whereas anaerobically prepared extracts expressed significant POR activity. These results indicate that deletions in the C-terminal region of D. africanaus POR can have dramatic effects on the stability of the enzyme toward oxygen. However, several hours are required to produce the complete loss of activity when the extracts obtained in N2 atmosphere are exposed to air, in contrast to the extracts prepared aerobically, which exhibited no activity at the end of the preparation. The inactivation of truncated PORs could be due to the degradation of [4Fe–4S] clusters of the enzyme by superoxide oxidation (10, 24, 36). The production of superoxide and other active oxygen species is significantly higher in the case of sudden exposure of the reduced cellular components to oxygen after disruption of the cells. In contrast, with the extracts prepared anaerobically, the reduced cellular components which were oxidized anaerobically under N2 prior to being exposed to oxygen generated only a small amount of active oxygen species.

Our results deserve further comment. The possible formation of a disulfide bridge between Cys1195 and -1112 was suppressed in deletion mutants of POR, and in contrast to the recombinant enzyme, no increase of activity was observed in the presence of DTE. This finding is in accordance with the hypothesis of the involvement of these cysteinyl residues in the formation of a disulfide bridge associated to the activation process of D. africanaus POR (34).

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**REFERENCES**


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