The δ-Subunit of Pyruvate Ferredoxin Oxidoreductase from *Pyrococcus furiosus* Is a Redox-Active, Iron−Sulfur Protein: Evidence for an Ancestral Relationship with 8Fe-Type Ferredoxins†

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ABSTRACT: Pyruvate ferredoxin oxidoreductase (POR) from the hyperthermophilic archaeon *Pyrococcus furiosus* (Pf) catalyzes the final oxidative step in carbohydrate fermentation in which pyruvate is oxidized to acetyl-CoA and CO_2, coupled to the reduction of ferredoxin (Fd). POR is composed of two 'catalytic units' of molecular mass ∼120 kDa. Each unit consists of four subunits, $\alpha\beta\gamma\delta$, with masses of approximately 44, 36, 20, and 12 kDa, respectively, and contains at least two [4Fe-4S] clusters. The precise mechanism of catalysis and the role of the individual subunits are not known. The gene encoding the δ-subunit of Pf POR has been expressed in *E. coli*, and the protein was purified after reconstitution with iron and sulfide. The reconstituted δ-subunit (recPOR-δ) is monomeric with a mass of 11 879 ± 1.2 Da as determined by mass spectrometry, in agreement with that predicted from the gene sequence. Purified recPOR-δ contains 8 Fe mol/mol and remained intact when incubated at 85 °C for 2 h, as judged by its visible absorption properties. The reduced form of the protein exhibited an EPR spectrum characteristic of two, spin−spin interacting $[4Fe\text{-}4S]^{1+}$ clusters. When compared with the EPR properties of the reduced holoenzyme, the latter was shown to contain a third $[4Fe\text{-}4S]^{1+}$ cluster in addition to the two within the δ-subunit. The reduction potential of the two 4Fe clusters in isolated recPOR-δ (−403 ± 8 mV at pH 8.0 and 24 °C) decreased linearly with temperature (−1.55 mV/°C) up to 82 °C. RecPOR-δ replaced Pf Fd as an in vitro electron carrier for two oxidoreductases from Pf, POR and Fd:NADP oxidoreductase, and the POR holoenzyme displayed a higher apparent affinity for its own subunit (apparent $K_m = 1.0\ \mu M$ at 80 °C) than for Fd (apparent $K_m = 4.4\ \mu M$). The molecular and spectroscopic properties and amino acid sequence of the isolated δ-subunit suggest that it evolved from an 8Fe-type Fd by the addition of ∼40 residues at the N-terminus, and that this extension enabled it to interact with additional subunits within POR.

The hyperthermophilic archaeon *Pyrococcus furiosus* (Pf),[1] grows optimally at 100 °C by the fermentation of carbohydrates and peptides (*1*). The fermentation pathways involve four distinct cytoplasmic 2-keto acid oxidoreductases which have different substrate specificities (*2*). These enzymes contain thiamin pyrophosphate (TPP) and catalyze the oxidative decarboxylation of various 2-keto acids to their acyl (or aryl) coenzyme A (CoA) derivatives with ferredoxin (Fd) as the electron acceptor. Pyruvate Fd oxidoreductase (POR) catalyzes one of the final steps in carbohydrate fermentation, in which pyruvate is oxidized to acetyl-CoA and CO_2 (*3*). The other three types, termed 2-ketoglutarate Fd oxidoreductase (KGOR), isovalerate Fd oxidoreductase (VOR), and indolepyruvate Fd oxidoreductase (IOR), are thought to function in peptide fermentation, in which they oxidize 2-keto acids derived by transamination of glutamate and branched chain and aromatic amino acids, respectively (*4*−*6*).

Like the analogous enzymes in mesophilic organisms, all four of the Pf oxidoreductases appear to be composed of two 'catalytic units' of mass ∼120 kDa (*2*, *7*, *8*). In mesophilic enzymes, this unit is typically a single large subunit, but this is not the case for the Pf enzymes. In Pf IOR, this unit comprises two subunits of mass 66 and 23 kDa, while in POR, VOR, and KGOR it is four subunits, termed α, β, γ, and δ, with masses of 43, 35, 23, and 12 kDa, respectively (*2*). The genes for Pf POR and VOR have been cloned and sequenced (*9*). The two enzymes are encoded by three distinct and adjacent units, with the operons

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[1] Abbreviations: CoA, coenzyme A; Cpa, *Clostridium pasteuranium*; DTT, dithiothreitol; EPR, electron paramagnetic resonance; Fd, ferredoxin; FNOR, ferredoxin:NADP oxidoreductase; IBs, inclusion bodies; IOR, indolepyruvate ferredoxin oxidoreductase; IPTG, isopropyl β-D-thiogalactopyranoside; KGOR, 2-ketoglutarate ferredoxin oxidoreductase; Mth, *Methanosarcina thermophila*; PCR, polymerase chain reaction; Pf, *Pyrococcus furiosus*; POR, pyruvate ferredoxin oxidoreductase; POR-δ, δ-subunit of pyruvate ferredoxin oxidoreductase; recPOR-δ, reconstituted form of the δ-subunit of pyruvate ferredoxin oxidoreductase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TPP, thiamin pyrophosphate; VOR, isovalerate ferredoxin oxidoreductase.

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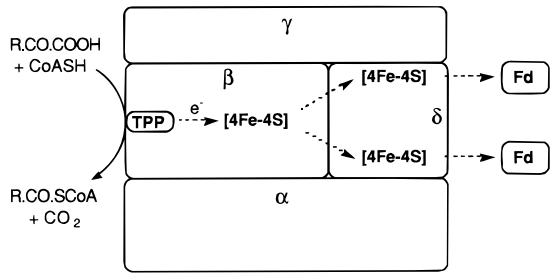

FIGURE 1: Model for the structure of 2-keto acid ferredoxin oxidoreductases. The proposed pathway of electron flow during catalysis is shown by the dotted lines, where ferredoxin (Fd) is the physiological electron acceptor. The hyperthermophilic enzymes consist of four subunits (as indicated) while the mesophilic enzymes typically contain one large subunit (see *2*).

organized as *por*/*vor*$\gamma-vor\delta\alpha\beta-por\delta\alpha\beta$. An interesting feature of the operon organization is that the gene encoding the γ-subunit is common to both enzymes. The amino acid sequences of the other three subunits are 53% identical. Sequence alignments show that the four subunits of POR and VOR show high similarity to discrete domains of the single, large subunit of the mesophilic PORs (*9*). Thus, the small α-subunit aligns with the N-terminal domain of the large mesophilic subunit and is followed by the γ-domain, the δ-domain, and, with one additional 60 amino acid insertion not present in the hyperthermophilic enzymes, the β-domain. When the four subunits of the Pf enzymes are aligned in a single sequence ($\alpha\gamma\delta\beta$) and compared to the single subunit of the mesophilic PORs, the pairwise identities are about 28% (*9*). Thus, the evolution of the mesophilic PORs can be explained by the rearrangement of four ancestral genes of the type now present in POR and VOR from Pf.

POR and VOR purified from Pf contain TPP and, as indicated by metal and spectroscopic analyses, at least two, and more likely three, [4Fe-4S] clusters per catalytic unit (*3*, *6*, *10*). However, the locations of the prosthetic groups within the subunits are not known. Amino acid sequence analyses indicate that the β-subunit contains a putative TPP-binding domain together with four Cys residues which should be capable of coordinating one [4Fe-4S] cluster. In addition, the δ-subunit contains eight Cys residues which could coordinate a further two [4Fe-4S] clusters (*9*). All twelve of these Cys residues are conserved in the mesophilic PORs, as is the TPP-binding motif. It was therefore proposed that all 2-keto acid oxidoreductases contain three [4Fe-4S] clusters per 'catalytic' unit, regardless of subunit composition (*9*). However, the precise mechanism of catalysis by, and electron transfer within, POR and VOR and the role of the individual subunits are unclear, and spectral analyses of the native enzymes are complicated by the presence of multiple [4Fe-4S] clusters (*6*, *10*). The model for the structure of hyperthermophilic and mesophilic 2-keto acid oxidoreductases and the proposed pathway of electron flow during catalysis is shown in Figure 1 (*2*). While TPP is likely to be bound within the β-subunit/domain, it has yet to be established if the twelve conserved Cys do indeed bind three 4Fe clusters and, if so, whether they are coordinated within or between subunits.

To investigate the role of the individual subunits of Pf POR and the nature of the prosthetic groups that each contains, we have embarked upon a study to individually express the genes for the four subunits and to characterize the proteins separately. In view of the high thermal stability of proteins from Pf in general, it was hoped that each subunit would incorporate its cofactors and fold into the native structure, independent of the other subunits. Herein we present data on the heterologous expression of the gene for the POR δ-subunit in *Escherichia coli*. The polypeptide was reconstituted in vitro and does indeed fold into a thermostable form that coordinates two 4Fe clusters. Moreover, its redox, spectral, and biological properties are very similar to Fds that contain two [4Fe-4S] clusters, suggesting a direct evolutionary relationship between them and the corresponding domain of the mesophilic 2-keto acid oxidoreductases.

MATERIALS AND METHODS

Strains, Cultivation, and Cloning. *Epicurian coli* XL1-Blue MRF′ cells (Stratagene, La Jolla, CA) were used for routine maintenance, cloning, and sequencing of plasmid DNA. Strains were grown aerobically in Luria−Bertani (LB) medium (*11*) supplemented with ampicillin (150 μg/mL), in a rotary shaker at 250 rpm and 37 °C. *Epicurian coli* BL21(DE3) cells (Stratagene) were used for the protein expression studies. Expression strains were grown aerobically in the same medium supplemented with ferrous sulfate (50 μM) and were induced at 0.6−0.8 OD unit (590 nm) by the addition of isopropyl β-D-galactopyranoside (IPTG) at a final concentration of 1 mM. Induction was carried out for 6−14 h at varying temperatures. The gene encoding Pf POR-δ (*por*δ) was PCR-amplified by standard techniques using TaqDNA polymerase (Boehringer Mannheim) and Pf genomic DNA as the template. The N-terminus primer (50-mer, Stratagene) included (5′ to 3′) a unique *Not*I restriction site followed by a consensus ribosomal binding site, spacer, unique *Nde*I restriction site [incorporated into the start codon (ATG) of the gene], and the first 20 nucleotides of the coding region of *por*δ. The C-terminus primer (25-mer, Stratagene) included 16 nucleotides of coding region and a unique *Bgl*II site immediately downstream of the stop codon of *por*δ. The PCR product was gel-purified and direct-cloned into pCR-script SK(+) (Stratagene). One clone, pHH1, containing *por*δ oriented behind the T7 promotor, was selected for further analysis. The cloned PCR product in pHH1 was sequenced by the dideoxy chain termination method (*12*) using [α-^{35}S]dATP (600 Ci/mmol; Amersham International), 7-deaza-dGTP, and the Sequenase Version 2.0 Kit (USB Corp., Cleveland, OH). pHH1 DNA was digested with *Nde*I and *Bgl*II restriction enzymes, and the purified insert was subcloned into the expression vector pET21b (Novagen), generating pHH2.

Purification of Recombinant POR-δ. Induced pHH2-containing cells were harvested after induction for 14 h at 37 °C [for isolation of inclusion bodies (IBs)] or at 28 °C (for isolation of soluble POR-δ) and used immediately or stored at −80 °C. Unless stated otherwise, all steps were carried out anaerobically wherein all buffers were degassed with argon and maintained under a positive pressure of the inert gas. The cell paste was resuspended in 50 mM TrisHCl (pH 8.0) containing 2 mM dithiothreitol (buffer A) (1 g wet weight/mL of buffer), treated with 100 μg/mL lysozyme and 50 μg/mL DNase I, and broken by two passages through a French press under a stream of argon. Cell extracts were centrifuged at 12000*g* for 30 min at 4 °C. The pellet, which

contained the insoluble POR-δ fraction and consisted primarily of inclusion bodies (IBs), was washed once in buffer A containing 1 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100, followed by two washes with buffer A containing 100 mM NaCl. The washed IBs were solubilized overnight (4 °C) in buffer A containing freshly prepared, deionized 2 M urea and centrifuged at 27000*g* for 30 min at 4 °C. The solubilized supernatant was resolved at 1 mL/min on a column of Superdex 75 (2.6 × 60 cm; Pharmacia LKB, Piscataway, NJ) equilibrated in buffer A containing 2 M urea, and fractions containing partially purified POR-δ were concentrated, washed with buffer A, and used for EPR analyses. IBs solubilized in 2 M urea were also subjected to ion exchange chromatography on a 1.6 × 11 cm DEAE-Sepharose FF column (Pharmacia) and eluted with a nonlinear gradient of 0−0.3 M NaCl in 80 mL followed by 0.3−0.6 M NaCl over 130 mL in buffer A. The 'aggregation state' of POR-δ in IBs solubilized with different concentrations of urea (2, 4, or 6 M) was determined by resolving the protein at 1 mL/min on a column of Superdex 75 (1 × 30 cm) equilibrated in buffer A containing 2, 4, or 6 M urea, respectively. The soluble form of POR-δ was isolated from the 40000*g* supernatant of cell extracts. Ammonium sulfate was added to 30% saturation, and the solution was centrifuged at 12000*g* for 30 min at 12 °C. The precipitate was redissolved in buffer A and dialyzed overnight at 4 °C against the same buffer. The dialysate was centrifuged at 40000*g* for 45 min, and the supernatant was resolved at 2 mL/min on a 3.5 × 60 cm column of Superdex 200 (Pharmacia) in buffer A containing 200 mM KCl. Fractions containing partially purified POR-δ were combined into five pools (I−V), based on contaminating protein patterns, concentrated by ultrafiltration using a PM10 membrane (Amicon), and stored at −20 °C until needed.

In Vitro Reconstitution of [4Fe-4S] Clusters in POR-δ. The protocol for the insertion of Fe-S clusters into apo-POR-δ was adapted from a previously published procedure (*13*). All steps were carried out anaerobically at 24 °C unless specified otherwise. POR-δ, partially purified from the soluble fraction (Superdex 200, pool IV), was precipitated in 10% (w/v) TCA for 15 min at 4 °C and centrifuged at 12000*g* for 10 min. The pellet was washed with 100 mM TrisHCl, pH 8.0 (buffer B), containing 2% (w/v) TCA and redissolved in buffer B containing 10 mM dithiothreitol (buffer C) and 6 M urea, at a final concentration of less than or equal to 2 mg/mL. The sample was diluted 2-fold with buffer C, and a fresh solution of 400 mM ferric chloride in buffer B was slowly added to 2 mM, followed by the addition of fresh 400 mM sodium sulfide in buffer B to 2 mM. After a 15 min incubation with gentle stirring, the urea concentration was diluted to 0.6 M with buffer C. The diluted reconstitution mixture was transferred into an anaerobic hood and filtered through an 0.22 μm filter attached to a syringe, washed 3 times with buffer C, and concentrated approximately 40-fold in an Amicon ultrafiltration unit fitted with a PM10 membrane and loaded onto a HiTrap-Q column (5 mL; Pharmacia) equilibrated in buffer A. The column was washed with 25 mL of buffer A, and the reconstituted protein was eluted with a nonlinear gradient of 0−0.2 M NaCl in 20 mL and 0.2−0.5 M NaCl in 80 mL of buffer A. The holoprotein eluted at approximately 0.23 M NaCl, while the apoprotein remained bound under these conditions. Fractions containing pure, reconstituted POR-δ (recPOR-δ), as determined by SDS−PAGE analyses, were pooled, concentrated, and stored at −20 °C.

Analyses of Recombinant and Reconstituted POR-δ. Metal contents (20 elements including Fe) of unreconstituted and recPOR-δ, isolated from the soluble fraction, were determined using plasma emission spectroscopy at the Chemical Analysis Laboratory, University of Georgia. Iron contents of the samples were also measured colorimetrically (*14*). UV−visible absorption spectra were recorded at 24 °C using a Hewlett-Packard HP8452A diode array spectrophotometer. Molecular masses were estimated by gel filtration on a column (1 × 30 cm) of Superdex 75 (Pharmacia) equilibrated with buffer A containing 200 mM KCl using horse heart cytochrome *c* (12.38 kDa), carbonic anhydrase (29 kDa), and ovalbumin (45 kDa) as standards. The molecular mass of recPOR-δ was more accurately determined by mass spectrometry, using liquid chromatography coupled to electrospray analysis, at the Mass Spectrometry Service at the University of Georgia. The sample was analyzed using a PE-Sciex API I plus mass spectrometer coupled to an Applied Biosystems 140B delivery system and ABI 759A absorbance detector. RecPOR-δ was supplied at 1.6 mg/mL in HPLC-grade water, injected onto a Kromasil C-4 column (1 × 150 mm), and eluted at 30 μL/min with a gradient of 100−0% solvent B (90% acetonitrile in solvent A: water containing 0.1% trifluoroacetic acid). The effluent was loaded at 11 μL/min into the mass spectrometer, and the electrospray voltage used was 4500 V. Thermal stability was determined anaerobically by following the change in absorbance at 394 nm of a 6 μM solution of recPOR-δ in 50 mM HEPES (pH 8.0) in a Spectronic 601 spectrophotometer (Milton Roy) connected to a constant-temperature water bath. EPR spectra were recorded on an IBM-Bruker ER 300D Spectrometer interfaced to an ESP3220 Data System and equipped with an Oxford Instruments ITC-4 flow cryostat. Oxidized EPR samples were prepared by the addition of thionine (1.3 mM) to purified recPOR-δ (∼100 μM) or native Pf POR (∼50 μM) in 50 mM TrisHCl (pH 8.0) or 50 mM CAPS (pH 10). Reduced samples were prepared by the addition of excess sodium dithionite (10 mM). Spin concentrations were estimated by double integration of spectra recorded at 8 K using 10 μW microwave power and comparing them with spectra of 0.997 mM $CuSO_4$/10 mM EDTA or 10 mM $CuSO_4$/10 mM HCl/2 M $NaClO_4$ recorded under the same conditions. Electrochemistry was performed using a three-electrode setup with a glassy carbon working electrode, a platinum counter electrode, and a Ag/AgCl reference electrode (*15*). Neomycin (2 mM) and $MgCl_2$ (20 mM) were added as promoters. The following buffers (100 mM) were used for the pH study: acetate (pH 4.0), MES (pH 6.0), MOPS (pH 7.0), EPPS (pH 8.0), CHES (pH 9.0), and CAPS (pH 10.0). Promoters were made up in buffers at the appropriate pH. Temperature dependence studies were done over a range of 24−82 °C. Results are expressed as midpoint potential vs SHE (standard hydrogen electrode) and corrected for the temperature dependence of the Ag/AgCl reference electrode.

Biological Assays. The efficiency of electron transfer from POR during pyruvate oxidation to recPOR-δ was measured by the POR-δ-dependent reduction of metronidazole, based on the method previously described for Pf Fd (*16*). The

reaction mixture (2 mL) contained 50 mM EPPS buffer (pH 8.0), sodium pyruvate (10 mM), CoA (0.2 mM), and POR (20 μM). The reactions were preincubated at 80 °C for 5 min, metronidazole (100 μM) was added, and base line activity was recorded for 2 min. The reaction was initiated by the addition of recPOR-δ (0−5 μM) or Pf Fd (0−30 μM). The reduced electron acceptor (Fd or recPOR-δ) was reoxidized by metranidazole, the irreversible reduction of which was measured at 320 nm (*17*). A molar absorption coefficient of 9300 M^{-1} cm^{-1} was used for oxidized metronidazole, and bleaching of the chromophore was assumed to be a one-electron process (*16*, *18*). The low rate of metronidazole reduction by POR in the absence of an electron carrier was used to correct all activities. One unit of POR activity is defined as 1 μmol of pyruvate oxidized/min with the specified electron acceptor. A two-enzyme coupled assay system was used to measure the ability of recPOR-δ to transfer electrons from POR to Fd:NADP oxidoreductase (FNOR; *19*) from Pf. The assay was designed to measure the interaction of recPOR-δ with FNOR, and an excess of POR was used to ensure that the reduction of recPOR-δ by POR was not the rate-limiting step. The reaction mixture (1 mL) contained 100 mM EPPS buffer (pH 8.0), sodium pyruvate (10 mM), CoA (0.2 mM), $NADP^+$ (0.3 mM), and native Pf POR (44 μM). After incubation at 80 °C for 5 min, recPOR-δ (2.5 μM) or Fd (5 μM) was added, and the reaction was initiated by the addition of Pf FNOR (0.062 μM). NADP reduction was measured at 365 nm, and an absorption coefficient of 3400 M^{-1} cm^{-1} was used in all calculations. One unit of activity is defined as 1 μmol of $NADP^+$ reduced/min using the specified electron carrier. POR, FNOR, and Fd used in these assays were purified in this laboratory using published procedures (*3*, *19*, *20*).

Other Analytical Techniques. Protein concentrations were routinely estimated by the method of Bradford (*21*) using bovine serum albumin as the standard. Quantitative amino acid analysis of pure recPOR-δ was performed at the Microchemistry Facility at Emory University. The protein content estimated by quantitative amino acid analysis was in good agreement with that determined by the colorimetric assay (101 ± 8.5%). Polypeptides were analyzed by SDS−PAGE in Tris−Tricine-buffered 10% acrylamide gels using the method of Schägger and von Jagow (*22*), and proteins were visualized with Coomassie Blue R-250. For N-terminus amino acid sequence analysis, POR-δ was separated from contaminants in IBs, as described above, and transferred to a ProBlott PVDF [poly(vinylidene difluoride)] membrane (Applied Biosystems) as per the manufacturer's instructions. Sequence analysis was performed on the PVDF membrane-bound protein at the Molecular Genetics Instrumentation Facility, University of Georgia. Individual and multiple sequence alignments were performed using the BESTFIT and PILEUP programs, respectively, from the University of Wisconsins Genetics Computer Group (GCG) package (*23*).

RESULTS

Purification and Reconstitution of Recombinant POR-δ. The nucleotide sequence of the PCR-cloned gene encoding POR-δ established that the PCR technique had not introduced any mutations into the coding region. The *E. coli* BL21-(DE3)/pHH2 strain used for expression appeared to produce

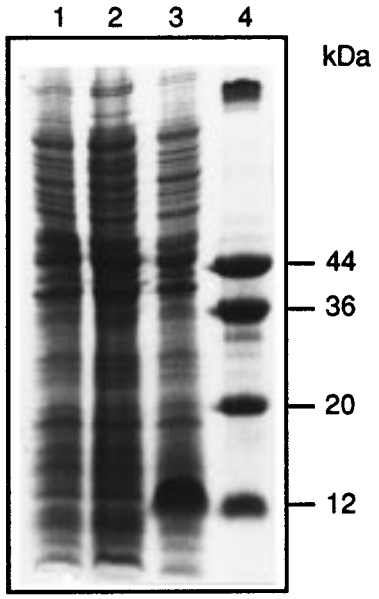

FIGURE 2: Expression of POR-δ in *E. coli* BL21(DE3) as determined by SDS gel analysis. The samples are cell-free extracts (~10 μg) from (1) induced pET21b-containing control cells, (2) uninduced pHH2-containing cells, and (3) induced pHH2-containing cells. (4) Native Pf POR (where the subunits and their masses, in kDa, are: α, 44; β, 36; γ, 20; and δ, 12), 10 μg.

significant amounts of recombinant POR-δ. As shown by SDS−PAGE analysis (Figure 2), cell-free extracts of induced pHH2 cells contained a protein, not present in the pET21b control or uninduced pHH2-containing cells, that corresponded in electrophoretic mobility to the authentic δ-subunit of purified, native POR from Pf. Initial induction experiments were carried out at 37 °C for 6−12 h. Under such conditions, most of the recombinant POR-δ was produced in the form of insoluble inclusion bodies (IBs). However, the in vivo solubility of recombinant POR-δ was determined by the time, temperature, and scale of induction. For example, induction for 4 h at 28 °C led to the production of soluble POR-δ whereas induction for 14 h at 37 °C resulted in the formation of mostly insoluble IBs. Similarly, small-scale cultures induced for 14 h at 28 °C produced roughly equal amounts of soluble and insoluble POR-δ, whereas a culture grown in a 100 L fermentor, under the same conditions, contained higher concentrations of the soluble form of POR-δ. In the latter case, the two forms of POR-δ represented as much as 20% of the total cellular protein (approximately 15 mg/g wet wt or 60 mg/L culture).

The purification of recombinant POR-δ was attempted using both the soluble and insoluble fractions of cell extracts of *E. coli*. POR-δ solubilized from IBs using concentrations of urea up to 6.0 M existed as high molecular mass complexes, as they eluted in the void volume of a Superdex 75 gel filtration column (fractionation range: 3−70 kDa). This was surprising, as all buffers were anaerobic and contained DTT to prevent aggregation likely to arise from the formation of intermolecular disulfides. In addition, although POR-δ solubilized from IBs in 2 M urea bound to DEAE-Sepharose, it eluted over a wide salt concentration (0.17 and 0.4 M NaCl), and very little purification was achieved. These results indicated that POR-δ isolated from IBs was present in the form of heterogeneous, misfolded aggregates, that nonspecifically interacted with contaminating

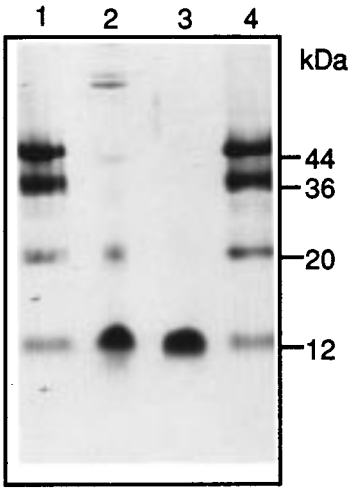

FIGURE 3: In vitro reconstitution of POR-δ monitored by SDS gel electrophoresis. The samples were: 1 and 4, native Pf POR (see legend to Figure 2), 6 μg; 2, unreconstituted, soluble POR-δ from pooled and concentrated Superdex 200 fractions (1.2 μg); 3, purified, reconstituted POR-δ (2.1 μg).

proteins. Similarly, while the soluble form of POR-δ from cell extracts was partially purified, at least 4-fold, by ammonium sulfate fractionation, the protein was not resolved by gel filtration, even using a Superdex 200 column (fractionation range: 10−600 kDa). POR-δ was present in the void volume and evenly distributed across almost the entire molecular size range, presumably as multimers of varying subunit numbers.

N-terminal sequence analysis of recombinant POR-δ, separated from the minor contaminating proteins present in IBs by SDS−PAGE, revealed a single sequence, AESPFKA-, which was identical to that determined for the δ-subunit of native POR purified from Pf, including the absence of the N-terminal methionine (*24*). Thus, while the gene encoding POR-δ includes a Met start codon, this residue appears to be efficiently removed in vivo by the *E. coli* methionine aminopeptidase. Partially purified POR-δ obtained from both the soluble and insoluble fractions was brown in color, suggesting that iron was incorporated into both forms of the protein in *E. coli*. However, given that both forms of the protein were isolated as heterogeneous aggregates, which was a good indication that the protein was misfolded in vivo, it was unlikely that they contained stoichiometric amounts of Fe-S clusters. Indeed, a sample of POR-δ partially purified (~80% pure based on SDS−PAGE analysis) from IBs solubilized in 2 M urea and containing 10 mM sodium dithionite gave a rhombic-type EPR spectrum indicating the presence of reduced $[4Fe\text{-}4S]^{1+}$ clusters, but the signal was so weak that it could not be accurately quantitated. In addition, colorimetric analysis of the iron content of POR-δ partially purified from the soluble fraction indicated the presence of only 0.3−0.4 mol of Fe/mol of POR-δ (assuming 80% purity, based on SDS−PAGE gels). This corresponded to at most a 10% occupancy (assuming one [4Fe-4S] cluster/mol of POR-δ). Hence, although a significant amount of the recombinant form of POR-δ was produced in *E. coli*, only a small fraction contained FeS clusters.

In vitro reconstitution of the Fe-S clusters was achieved using recombinant POR-δ that was partially purified from the cytoplasmic fraction of *E. coli* (Figure 3). Acid

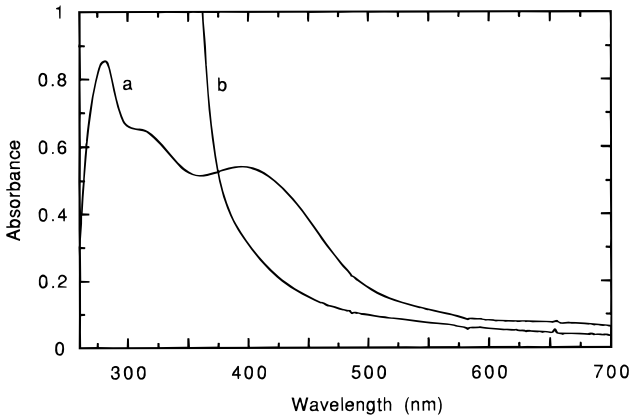

FIGURE 4: UV−visible absorption of recPOR-δ. (a) Protein (0.15 mg/mL) in 50 mM TrisHCl (pH 8.0), as purified anaerobically in the absence of a reducing agent; (b) after reduction with sodium dithionite (6 mM).

precipitation of the protein was used to remove existing metal ions, and the protein was then dissolved in 6 M urea to ensure complete unfolding. The presence of a chaotropic agent to maintain proteins in their unfolded forms has been shown to be critical for FeS cluster reconstitution in some instances (*25*). DTT (10 mM) was also added to ensure that all sulfhydryl groups were in their reduced states. The reconstitution conditions utilized a 24-fold molar excess of Fe and sulfide compared to the apoprotein and represented a 3-fold molar excess of the Fe/S required to convert all the apoprotein into a two [4Fe-4S] form. The putative holoprotein was separated from the reconstitution mixture by filtration and ion-exchange chromatography. Colorimetric protein analyses indicated that 15−20% of the apoprotein had been converted to holoprotein.

Properties of Reconstituted POR-δ. In contrast to the recombinant form of POR-δ produced by *E. coli*, which was isolated in the form of aggregates that could only be partially purified from cell-free extracts (to ~80% purity), the reconstituted form was purified to homogeneity in a single step after reconstitution and corresponded in electrophoretic mobility to the δ-subunit of pure, native POR from Pf (Figure 3). The size of the reconstituted form (hereafter termed recPOR-δ) was estimated to be approximately 9.7 ± 2.0 kDa by gel filtration chromatography, indicating that, unlike the unreconstituted form, recPOR-δ is a monomeric species. Mass spectrometry electrospray analysis yielded a value for the apoprotein of 11 879 ± 1.2 Da. This value is in perfect agreement with the predicted size of the polypeptide from the gene sequence with the N-terminal methionine removed (11 880 Da) and corroborates the N-terminal sequence data obtained with the unreconstituted protein; i.e., the N-terminal Met residue is efficiently removed posttranslationally by *E. coli*.

Pure recPOR-δ exhibited an absorption maximum at 280 nm with a broad shoulder at 394 nm (Figure 4). Analysis of three independent reconstitution preparations gave values of 0.632−0.635 for the absorbance ratio A_{394}/A_{280}. Addition of excess sodium dithionite resulted in a decrease in the A_{394} value of about 40%, demonstrating that the FeS chromophore was redox-active. Surprisingly, the protein was also very thermostable, with no significant change in the absorbance at 394 nm after 2 h at 85 °C. In fact, there was only a 25% loss in the A_{394} value after 24 h at this temperature. The Fe

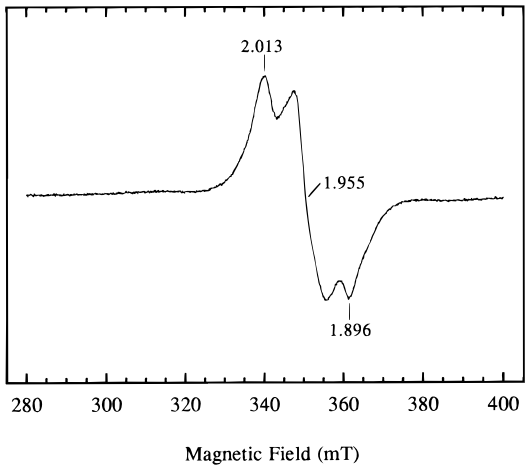

FIGURE 5: EPR spectrum of reduced recPOR-δ. The sample contained the protein (1.3 mg/mL, 109 μM) in 50 mM CAPS (pH 10.0) containing sodium dithionite (10 mM). The spectrum was recorded at 4.7 K. The instrument settings were: microwave power, 100 μW; microwave frequency, 9.596 GHz; receiver gain, 6.3×10^4; modulation amplitude, 0.64 mT; modulation frequency, 100 kHz.

content of recPOR-δ, obtained from three independent reconstitution experiments, was 8.0 $\pm$ 0.7 mol/mol, as determined by colorimetric assays. Plasma emission spectroscopy established that no other metals were present in significant amounts (>0.1 mol/mol).

Pure recPOR-δ reduced by excess sodium dithionite exhibited a broad, rhombic-type EPR signal with g values of 2.013, 1.955, and 1.896 at temperatures below 30 K (Figure 5). The general line shape of the spectrum remained unchanged when the temperature was varied from 5 to 30 K and when the microwave power was varied from 10 μW to 200 mW (at 8 K). The rhombic spectrum of reduced recPOR-δ accounted for 1.7 $\pm$ 0.3 spins/mol. All of these data taken together therefore suggest that reduced recPOR-δ contains two $[4Fe\text{-}4S]^{1+}$ clusters with very similar properties, and the presence of "wings" in the EPR spectrum near $g =$ 2.044 and 1.874 indicates some spin$-$spin interaction between them. The oxidized form of POR-δ, obtained by treating the protein with excess thionine ($E_m = +60$ mV), gave rise to an isotropic-type signal at $g = 2.01$ at 8 K (data not shown), characteristic of an oxidized $[3Fe\text{-}4S]^{+}$ cluster (*26*). However, the spectrum represented less than 0.1 spin/mol, indicating that virtually all of the iron in the oxidized protein was present as intact $[4Fe\text{-}4S]^{2+}$ clusters. Additional resonances at low fields ($g \sim 4.3$) were not apparent (at 8 K, 100 mW), suggesting little if any adventitiously bound iron was present in the recPOR-δ sample.

As shown in Figure 6a, the POR holoenzyme in its reduced state exhibits a complex EPR spectrum, and part of this obviously arises from its δ-subunit (Figure 6c). In fact, comparing the two, it is clear that the predominant features of the spectrum seen from the holoenzyme do not arise from the δ-subunit. These must originate from another paramagnetic species, and this is presumably the putative $[4Fe\text{-}4S]^{1+}$ cluster in the β-subunit of the reduced enzyme. From the difference spectrum (Figure 6b), this reduced cluster exhibits a rhombic spectrum with g values of 2.043, 1.932, and 1.891. The additional complexity seen in the spectrum from the holoprotein, e.g., at $g = 2.065$, must arise from the interactions between the two clusters in the δ-subunit and

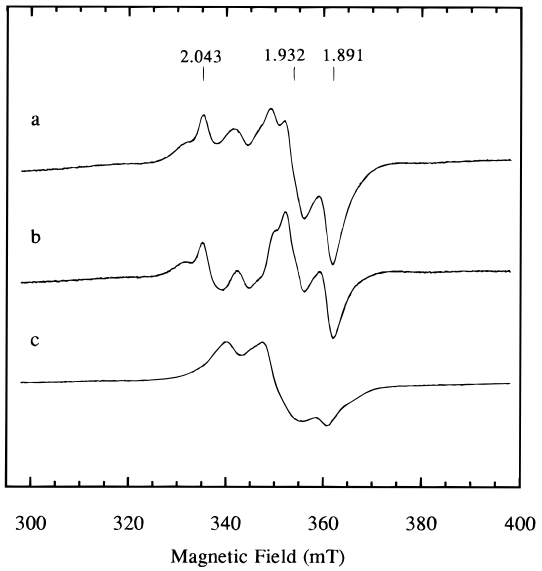

FIGURE 6: EPR properties of native POR and recPOR-δ. The samples were: (a) POR (12.6 mg/mL, 56.5 μM) and (c) recPOR-δ (1.36 mg/mL, 114 μM) in 50 mM TrisHCl (pH 8.0) containing 10 mM sodium dithionite. Spectrum b was obtained by subtracting spectrum c from spectrum a. The spectra were recorded at 5 K. The instrument settings were: microwave power, 10 mW; microwave frequency, 9.592 GHz; receiver gain, 5×10^3 for (a) and 2.5×10^4 for (c); modulation amplitude, 0.511 mT; modulation frequency, 100 kHz.

the single cluster in the β-subunit. It should be noted, however, that the holoenzyme is thought to contain two copies of each subunit, i.e., $\alpha_2\beta_2\gamma_2\delta_2$, so an alternative explanation is that the clusters of the two β-subunits are close enough within the holoenzyme to interact with each other, as well as with those in the δ-subunits. A third explanation is that the $[4Fe\text{-}4S]^{1+}$ cluster of the β-subunit exists in two different conformations and that each gives rise to a similar but distinct EPR signal. In any event, the EPR properties of the δ-subunit provide evidence for a third $[4Fe\text{-}4S]^{1+}$ cluster in the holoenzyme, located in the β-subunit, and show that the complexity of the EPR spectrum of the holoenzyme does not arise directly from the properties of the two $[4Fe\text{-}4S]^{1+}$ clusters within the δ-subunit itself. An examination of the EPR features of the β-subunit in isolation is needed to provide further insight into this issue.

The midpoint potential of recPOR-δ was determined by direct electrochemistry using cyclic voltammetry. The voltammograms were characterized by a rather large peak to peak distance of $\sim$115 mV, instead of the expected 57 mV (at 25 °C) for a reversible one-electron transfer (*27*). This suggests that the two redox centers present in POR-δ have slightly different redox potentials and are reduced successively (*28*). From the peak width of the voltammogram, an estimate can be made for this difference in potential using the working curve reported in Myer and Shain (*29*). In this way, it was calculated that the first cluster to be reduced is 30$-$50 mV more positive than the second cluster. However, it is likely that the broadening of the voltammogram is a result of both the difference in potential between the two clusters as well as slow electron-transfer kinetics between the protein and the electrode. Since it is difficult to distinguish between these two effects, a single value was assigned to both clusters using the average of the cathodic and anodic peak potentials. This yielded a value of -403

± 8 mV at pH 8.0 and 24 °C. The midpoint potential of the protein was unchanged over the pH range 6.0−10.0 (at 24 °C) but exhibited a linear, temperature-dependent decrease with increasing temperature over the range 24−82 °C (pH 8.0; data not shown). The average change was −1.55 mV/°C, and the measured reduction potential at the physiologically relevant temperature of 82 °C was −500 ± 2.0 mV. This is more negative than that (−450 mV) reported by direct electrochemical analysis at 80 °C of the holoenzyme (*30*). Since the latter reduction potential reflects all of the redox centers in the enzyme, it would appear that either (a) the 4Fe center of the β-subunit has a higher potential than those of the δ-subunit or (b) the reduction potential of the isolated δ-subunit increases (by ∼50 mV) upon incorporation into the holoenzyme. At present, we cannot distinguish between these two possibilities.

The function of the POR holoenzyme within Pf is to oxidize pyruvate to acetyl-CoA and reduce Fd, which in turn is oxidized by FNOR and the electrons are used to reduce $NADP^+$. Fd therefore has the ability to both accept electrons from POR and donate them to FNOR. The ability of recPOR-δ (E_m = −403 mV, 24 °C) to substitute for Pf Fd (E_m = −370 mV, 24 °C; *15*) in these two electron-transfer reactions was investigated. In the POR assay, the reduction of recPOR-δ was measured by coupling its reoxidation to the irreversible reduction of metronidazole (*16*, *17*). In this assay, recPOR-δ very efficiently replaced Fd as an electron acceptor for POR. Kinetic constants were estimated at 80 °C from linear double reciprocal plots using concentrations of recPOR-δ and Fd in the range 0−5 μM and 0−30 μM, respectively. In fact, the POR holoenzyme displayed a higher apparent affinity for its own subunit, recPOR-δ (apparent K_m = 1 μM at 80 °C), than it did for its physiological electron carrier, Fd (K_m = 4.45 μM at 80 °C), although the rate of pyruvate oxidation with recPOR-δ (V_m = 2.9 units/mg) was slightly lower than with Fd (4.0 units/mg).

RecPOR-δ also functioned as an electron donor to FNOR in place of Fd. In this assay system, Fd is reduced with excess POR, and the ability of reduced Fd to transfer electrons to FNOR is estimated by the rate of reduction of NADP by FNOR (*16*, *19*). In the absence of an electron carrier (Fd or recPOR-δ), there was a low rate of direct electron transfer from native POR to FNOR (0.5 units/mg) at 80 °C. The rate of NADP reduction increased to 3.6 units/mg when Fd (5 μM) was added, but an even higher rate, 4.9 units/mg, was observed if recPOR-δ (2.5 μM) was added instead. There was no NADP reduction in the presence of either recPOR-δ or Fd if native POR was not added to the reaction mixture.

DISCUSSION

The recombinant form of the δ-subunit of POR was produced in relatively large amounts in *E. coli*, but only a fraction of the molecules contained [4Fe-4S] clusters and the majority appeared to be apoprotein in the form of misfolded aggregates. *E. coli* has been used as a heterologous host to express the genes encoding many iron−sulfur-containing proteins. While this has been successful for several small proteins such as HiPiPs and some 2Fe- and 4Fe-Fds (*31*−*37*), there are many analogous redox proteins that are expressed in *E. coli* as apoproteins (*25*, *38*−*43*). Heterologous expression of genes encoding proteins containing Fe-S clusters is apparently influenced by a number of factors, such as the presence of chaperones (*44*, *45*), the cysteine content of the expressed protein (*46*), the type of Fe-S cluster (*47*), the availability and redox state of iron and sulfide (*48*, *49*), the protein environment around the cluster (*50*), and in vivo turnover rates (*51*). However, there appears to be no general conclusion on how to maximize the likelihood of successful heterologous expression of an FeS-containing holoprotein. Hence, POR-δ of Pf was produced in *E. coli* mainly as apoprotein, although the relative amounts of soluble and insoluble forms appeared to vary with induction time and temperature. Nevertheless, the recombinant protein could be readily reconstituted with Fe and sulfide to give a form which is assumed to represent the native state within the POR holoprotein.

The reconstituted, recombinant form of Pf POR-δ contained two [4Fe-4S] clusters per molecule, as demonstrated by colorimetric Fe analysis and EPR spectroscopy. Moreover, the resultant recPOR-δ was a thermostable monomer that obviously did not require interactions with the other three types of subunits in the octomeric POR holoprotein ($\alpha_2\beta_2\gamma_2\delta_2$) to maintain a stable structure, which, again, is assumed to represent the native form. The presence of two [4Fe-4S] clusters within the δ-subunit agrees with that predicted based on sequence analysis (Figure 1), and a comparison of its EPR properties with those of the POR holoprotein provides evidence that the latter contains three distinct [4Fe-4S] centers. In light of the Cys content of the four subunits, the third 4Fe cluster must be coordinated by the β-subunit (Figure 1). The EPR analysis also suggests a strong interaction between the [4Fe-4S] centers in the two subunit types (δ and β), in contrast to the interaction between the two clusters present in the δ-subunit itself. RecPOR-δ also served as an in vitro electron carrier for two Pf enzymes, POR and FNOR. This interaction with native POR was surprising, since recPOR-δ is in all likelihood accepting electrons from the δ-subunit of the holoenzyme, as shown in Figure 1, where Fd is replaced by recPOR-δ. Moreover, native POR displayed a higher affinity for recPOR-δ (apparent K_m = 1.0 μM) than it did for Fd (apparent K_m = 4.4 μM), its physiological electron acceptor. Similarly, recPOR-δ was able to substitute for Fd (at the same cluster concentration) and donate electrons to FNOR but with a 50% increase in rate (as measured by NADP reduction). These results clearly demonstrate the stability and electron-transfer ability of recPOR-δ at 80 °C, although why it should be as, or even more, efficient than the physiological redox protein is not clear.

The properties of recPOR-δ are in many ways similar to those of [4Fe-4S]-type Fds. For example, this subunit is a small, acidic protein (predicted p*I* 5.02) that contains two low-potential [4Fe-4S] centers, each of which is presumably coordinated by four Cys residues. Similarly, the prototypical Fd, that from *Clostridium pasteurianum* (Cpa, *13*), is a small (6 kDa), acidic (p*I* 3.7) protein containing two low-potential [4Fe-4S] clusters coordinated by eight Cys residues (see, for example, *52*). The reduction potentials of the [4Fe-4S] centers in both the Pf and Cpa proteins are almost the same (∼−400 mV), and neither shows any pH dependence. Although the temperature dependence of the midpoint

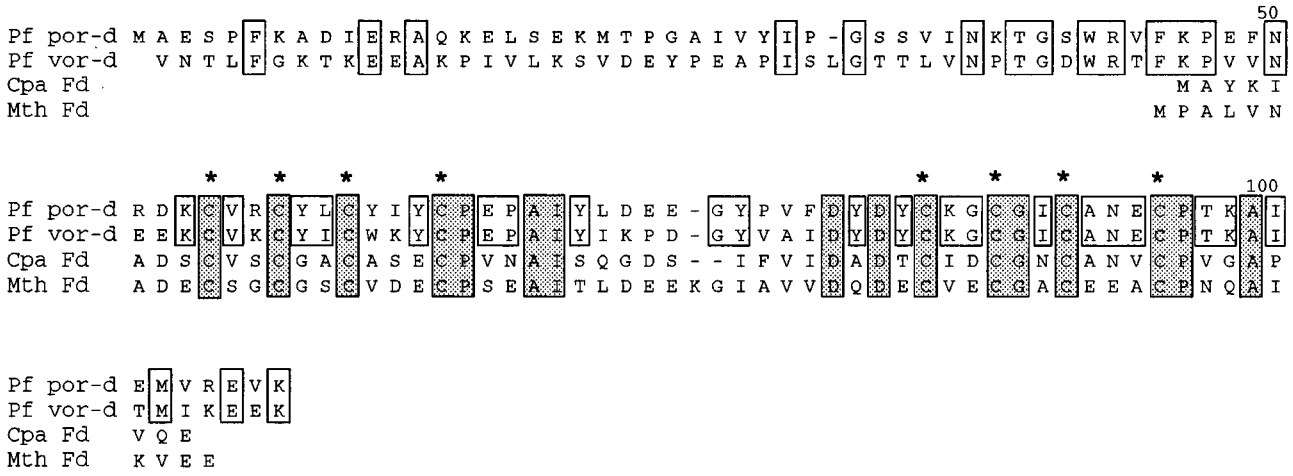

FIGURE 7: Alignment of the amino acid sequences of Pf POR-δ, Pf VOR-δ, Cpa Fd, and Mth Fd. Identities between all four proteins are indicated using shaded boxes, while those between POR-δ and VOR-δ are indicated by clear boxes. The eight cysteine residues that coordinate the two [4Fe-4S] clusters are indicated by asterisks. The accession numbers are: Pf POR-δ and Pf VOR-δ; X85250 (*9*); Cpa Fd, A94028 (*56*); and Mth Fd, A42960 (*57*).

potential of Cpa Fd has not been reported, the change observed for recPOR-δ is in the same range as that seen for other redox proteins (*15*, *53*, *54*). The EPR spectra of the reduced forms of both recPOR-δ and Cpa Fd are also similar and arise from the magnetic interaction between two $S = 1/2$ spin systems, each contributed by one [4Fe-4S] cluster about 1 nm apart (*55*).

Given the remarkable similarity in the properties of recPOR-δ and the Cpa Fd, it was of interest to compare their amino acid sequences (*9*, *56*). These are given in Figure 7, together with that of the δ-subunit of the closely related VOR from Pf (*9*), and that of the Fd from *Methanosarcina thermophila* (Mth; *57*), a moderately thermophilic, methanogenic archaeon. As indicated, the Fds align with the C-terminal part of both δ-subunits, and all four proteins are highly similar, with Cpa/Mth Fds showing 42%/44% and 40%/34% identity with the POR and VOR subunits, respectively. Interestingly, while the 2 δ-subunits are very similar, and share an overall identity of 53% (*9*), the first 40 or so amino acids are conserved (26% identity) to a much lesser extent than the remainder of the proteins (69% identity). Thus, the δ-subunits of both POR and VOR are, in essence, equivalent to an 8Fe-type Fd but with an N-terminal extension of $\sim$45 residues. Furthermore, it would seem likely that it is this extension which determines the nature of the subunit (whether specific to POR or VOR). It has been proposed that the modern Fds evolved from a common ancestor (*58*), although there is controversy as to whether the monocluster 4Fe-type were the precursors to the dicluster 8Fe-type (*59*, *2*), or vice versa (*60*−*62*). In any event, it seems clear that the δ-subunit of both POR and VOR evolved from such an ancestral Fd, be it 4Fe- or 8Fe-type, and that at some point in evolution it acquired an N-terminal extension which allowed it to specifically interact with and become a subunit of an 'ancestral' 2-keto acid oxidoreductase. The concept of a common, ancestral, four-subunit 2-keto acid oxidoreductase has already been proposed (*2*). Thus, the highly conserved 'Fd-like' domain of the δ-subunit conferred electron carrier activity, while the much less conserved N-terminal domain diverged during evolution so as to allow specific interactions within different oxidoreductases (POR, VOR, KGOR, and IOR).

This development of an in vitro reconstitution system for POR-δ is the first step in establishing a flexible system to answer some of the questions regarding the catalytic mechanism and pathway of electron transfer in this enzyme and in related hyperthermophilic 2-keto acid oxidoreductases. The future individual expression of the other subunits of POR and of related enzymes should also help define which are directly involved in enzyme catalysis as well as the "minimum catalytic unit". For example, as suggested by Figure 1, can the β-subunit, alone or in combination with the δ-subunit, catalyze pyruvate decarboxylation?

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