Thermococcus profundus 2-Ketoisovalerate Ferredoxin Oxidoreductase, a Key Enzyme in the Archaeal Energy-Producing Amino Acid Metabolic Pathway

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2-Ketoisovalerate ferredoxin oxidoreductase (VOR) is a key enzyme in hyperthermophiles catalyzing the coenzyme A–dependent oxidative decarboxylation of mainly aliphatic amino acid–derived 2-keto acids. The very oxygen-labile enzyme purified under anaerobic conditions from a hyperthermophilic archaea, Thermococcus profundus, is a hetero-octamer (αβγδ)2 consisting of four different subunits, α = 45,000, β = 31,000, γ = 22,000 and δ = 13,000, respectively. Electron paramagnetic resonance and resonance Raman spectra of the purified enzyme indicate the presence of approximately three [4Fe-4S] clusters per αβγδ-protomer, although one of the clusters has a tendency to be converted to a [3Fe-4S] form during purification. The optimal temperature for the enzyme activity is 93 ± 2°C and the cognate [4Fe-4S] ferredoxin serves as an electron acceptor of the enzyme. The purified enzyme is highly oxygen-labile (t1/2, approximately 5 min at 25°C), and is partly protected in the presence of magnesium ions, thiamine pyrophosphate and sodium chloride (t1/2, approximately 25 min at 25°C).

Key words: amino acid, ferredoxin, hyperthermophile, 2-ketoisovalerate, oxygen-sensitive.

Abbreviations: VOR, 2-ketoisovalerate ferredoxin oxidoreductase; PFOR, pyruvate ferredoxin oxidoreductase; IOR, indolepyruvate ferredoxin oxidoreductase; KGOR, 2-ketoglutarate ferredoxin oxidoreductase; TPP, thiamine pyrophosphate; RR, resonance Raman.

Elemental sulfur (S0)–dependent hyperthermophilic archaea were first discovered in the 1980s. A number of modified or new metabolic pathways have been discovered, and in many cases the enzymes involved exhibit novel properties that have been the subject of biochemical and biophysical characterization. A pathway of energy-producing amino acid degradation has been proposed in some peptide-utilizing hyperthermophilic archaea such as Thermococcales (Fig. 1) (1, 2). In this scheme, amino acids produced from peptides by peptidases are converted to the corresponding 2-keto acids by transaminases (reaction I). The resulting 2-keto acids are then oxidized through coenzyme A–dependent decarboxylation by four types of 2-keto acid:ferredoxin oxidoreductases [i.e., pyruvate ferredoxin oxidoreductase (PFOR), 2-ketoisovalerate ferredoxin oxidoreductase (VOR), indolepyruvate ferredoxin oxidoreductase (IOR), and 2-ketoglutarate ferredoxin oxidoreductase (KGOR)], the corresponding acylCoA or arylCoA being produced (reaction II). The above enzymes are thought to catalyze the formation of the corresponding aldehydes in the presence of coenzyme A (reaction III). It is thought that acylCoA (arylCoA) is then converted to the corresponding carboxylic acids, with the concomitant formation of ATP (reaction IV). Thus, this primordial amino acid metabolic pathway is energy producing and involves a number of new hyperthermophilic enzymes that have not been found in any other type of organism.

Among the four types of key ferredoxin-dependent enzymes, PFOR is the best characterized (e.g., 3–5), and has been shown to catalyze the production of acetaldehyde in the presence of coenzyme A (6). The other three types of enzymes (IOR, VOR and KGOR) are relatively poorly characterized, and their physiological functions have not been well established experimentally (7–11). Nevertheless, biochemical and molecular biological studies have unambiguously established that the four types of enzyme form a structurally related superfamily, containing thiamine pyrophosphate, magnesium ions, and 1–3 × [4Fe-4S] cluster(s) as prosthetic groups, albeit with different subunit compositions and substrate specificities (3). For example, PFOR from hyperthermophilic organisms (such as Pyrococcus furiosus, Archaeoglobus fulgidus, and Thermotoga maritima) consist of αβγδ-type protomers, while their mesophilic homologs (such as those from Desulfovibrio africanus and Moorella thermoacetica (f. Clostridium thermoacetaticum) are dimers of a large single subunit (A2): genetic fusion in the αγδβ arrangement has occurred during their molecular evolution (12, 13). The spatial arrangement of the cofactors in D. africanus PFOR (A2-type) was recently clarified by X-ray crystallographic analysis (4). The three-dimensional structures of the other three types of enzyme are not known.

In order to investigate the structure-function basis of why the four enzymes (PFOR, VOR, KGOR, IOR) have
A proposed amino acid metabolic pathway in hyperthermophilic archaea. Taken from Ref. 1. Explanations of reactions I–IV are given in the text. Reaction V is catalyzed by aldehyde ferredoxin oxidoreductase (AOR). Abbreviations: TA, transaminase; KAOR, 2-keto acid ferredoxin oxidoreductase; AOR, aldehyde ferredoxin oxidoreductase; ACS, acetylCoA synthase; Fdox, oxidized ferredoxin; Fdred, reduced ferredoxin.

**Fig. 1. A proposed amino acid metabolic pathway in hyperthermophilic archaea.**

Here we report the purification and initial characterization of VOR from *Thermococcus profundus*, a hyperthermophilic anaerobic archaeon that requires peptides for growth in the presence of inorganic sulfur (S\(^0\)). The purification of the archaean VOR was performed under strictly anaerobic conditions, and TPP, Mg\(^{2+}\) and NaCl were found to stabilize the purified enzyme in a highly active form. This enzyme can be isolated in a relatively large quantity from the archaean cells, suggesting that it can be used as a new tractable model of this enzyme family having a distinctive substrate specificity from those of the well-characterized PFOR enzymes.

**MATERIALS AND METHODS**

**Materials**—Yeast extract and Bactotryptone were obtained from Difco Laboratories. Inorganic sulfur powder was purchased from Wako. Q Sepharose FF was obtained from Pharmacia Biotech Inc. Bathocuproinedisulfonate and zincon were from Dojindo Laboratories. Other chemicals were of analytical grade.

**Strain and Growth**—*Thermococcus profundus* DSM DT5432 was grown on yeast extract and Bactotryptone with inorganic sulfur and sodium sulfide for approximately ten hours at 80°C under anaerobic conditions as described previously (14).

**Purification of 2-Ketoisovalerate Ferredoxin Oxidoreductase**—The purification of 2-ketoisovalerate ferredoxin oxidoreductase was carried out anaerobically basically according to the method described previously (15). All operations were performed anaerobically at ambient temperature (approximately 25°C) and mainly in an anaerobic chamber (Coy Laboratory) filled with nitrogen. Unless indicated otherwise, the anaerobic standard buffer used was 50 mM Tris-HCl, pH 8.0/0.2 mM DTT/2 mM sodium dithionite/0.2 M NaCl/1 mM TPP/1 mM MgSO\(_4\).

Cell-free extracts, which were prepared as described previously (15), were loaded onto a Q Sepharose Fast Flow column (5 × 45 cm) equilibrated with the buffer. After a wash with the buffer (1 liter), the VOR and analogous enzymes (PFOR, IOR and KGOR) were eluted with a NaCl linear gradient, from 0.05 to 0.8 M, in a total volume of 4 liters. The fractions that contained the VOR activity (eluted at ~0.45 M NaCl) were combined and applied to a hydroxypatite column (5 × 30 cm) equilibrated with the buffer. The column was washed with the buffer and then eluted with a linear gradient of 0.05 M to 0.5 M potassium phosphate (pH 6.5)/2 mM DTT/2 mM sodium dithionite (2 liters). The enzymatically active fractions were combined and the buffer for the enzyme solution was changed to a modified standard buffer with 5 mM DTT but without 2 mM sodium dithionite using an Amicon YM50 membrane. Then the enzyme solution was applied to a Red TOYOPEARL column (2.5 × 30 cm) equilibrated with the modified buffer without sodium dithionite. The column was washed with the buffer (500 ml) and the enzyme was eluted with a NaCl linear gradient up to 0.8 M in a total volume of 1,500 ml. The fractions that contained VOR alone (the purity of each fraction was checked by native PAGE) were combined (120 ml) and concentrated with an Amicon YM50 membrane. Then the concentrated enzyme solution was stored at −30°C until use.

**Analytical Procedures**—The purity of the enzyme preparation was examined by 6% native-PAGE (16) and 12.5% SDS-PAGE (17). The gel obtained on SDS-PAGE was stained with Coomassie Brilliant Blue G250. Subunit molecular weights were calculated using an SDS gel with eight molecular weight standards (MW: 6,500–66,000, Sigma low molecular weight marker). Gel filtration was performed on a TSK gel G3000 SW\(_{XL}\) column in a JASCO model 880-PU HPLC system with eight molecular weight standards (MW: 12,400–443,000). The buffer used for the column chromatography was anaerobic 50 mM Tris-HCl (pH 8.0)/0.2 M NaCl, which had been prepared by repeating the degassing-Ar gas flushing five times.

The protein concentration was estimated by means of the dye-binding assay described in the literature (19) with bovine serum albumin as the standard. Iron was quantified by the bathophenanthroline method (20) and acid-labile sulfide was measured by the methylene blue formation method (21). Metal analyses were carried out with an ICP-Mass spectrometer, PQΩ (VE Elemental), and also by atomic absorption spectroscopy with a Hitachi Z-8100 polarized Zeeman Atomic Absorption Spectrophotometer.

**Analytical Centrifugation**—Sedimentation equilibrium was performed in an Optima XL-I analytical ultracentrifuge (Beckman-Coulter) in a 4-hole An60Ti rotor at 20°C using standard double sector centerpieces and quartz windows. Prior to analysis, a sample was dialyzed against buffer comprising 50 mM Tris-HCl (pH 8.0)/0.2 M NaCl/4 mM DTT. The dialysis buffer was used as a con-

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trol solution. The concentration profiles of the samples were monitored by measuring the absorbance at 400 nm. The sedimentation equilibrium experiments were carried out at an $A_{400}$ of 0.15, 0.3 and 0.45 at rotor speeds of 4,000, 6,000 and 8,000 rpm, respectively. Scans were recorded every 2 h and the equilibrium of the system was judged by superimposing the last three scans. The molecular weight was calculated with a partial specific volume of 0.692 cm$^3$/g, based on the amino acid composition of VOR determined as described previously in ref 18: Asp+Asn (168), Thr (77), Ser (374), Glu+Gln (390), Pro (71), Gly (378), Ala (196), Val (85), Met (24), Ile (58), Leu (72), Tyr (50), Phe (55), Trp (13), Lys (76), His (40), Arg (26), and Cys (13). The partial specific volume and solvent density were calculated with the Sednterp program (22).

Spectral Measurements—Absorption spectra were recorded on a JASCO model 530 UV-visible spectrophotometer using the reducing reagent–free VOR. The reducing reagent–free anaerobic buffer was prepared as described above. The oxidized VOR was prepared by reacting VOR with an aliquot of anaerobic 10 mM thionine for 15 min and then passing the reaction mixture through a small Sephadex G-25 column equilibrated with reducing reagent–free anaerobic 50 mM Tris-HCl (pH 8.0)/0.2 M NaCl. Resonance Raman spectra were recorded at 77 K using a Spex 750M Raman spectrometer fitted with a Spectrum-One 2–8 × 512 CCD camera and a Spectra-Physics 2017 Ar$^+$ laser (output, 500 mW) by collecting 45$^\circ$ backscattering off the surface of a frozen sample in a quartz flat cell with a polyethylene cap (15). The slit width of the spectrometer was 60 µm, and the multiscan signal-averaging technique was employed to improve the signal-to-noise ratio. The spectral data were processed with the use of KaleidaGraph v3.05 (Abelbeck Software). The protein sample for the Raman measurement was prepared by oxidizing the purified VOR as described above for preparing the sample for UV-visible absorption spectra. EPR spectra of the purified VOR were recorded as described previously (23): the buffer for the VOR solution was changed to 0.1 M Tris-HCl (pH 8.0)/0.02 M NaCl/1 mM TPP/1 mM MgSO$_4$/20 mM 2-ketoisovalerate/5 mM CoA SH using an Amicon YM30 membrane under an anaerobic atmosphere, and then the sample was poured into an EPR tube. After being capped with a butyl rubber stopper, the tube was heated up to 80°C for 5 min in the anaerobic chamber. The spin concentration of the VOR solution was determined with the use of the Cu-EDTA complex as the standard. EPR spectra of the oxidized VOR samples were obtained with a sample prepared by oxidizing the purified VOR with thionine for Raman spectral measurements as described above.

Enzyme Assay—VOR activity is routinely assayed by means of CoA-dependent reduction of methyl viologen with 2-ketoisovalerate at 80°C (8). The standard assay mixture (1 ml) comprised 50 mM TAPS-Na (pH 8.4)/5 mM sodium 2-ketoisovalerate/0.2 mM CoA/1 mM TPP/1 mM MgSO$_4$/1 mM methyl viologen. Then the rate of methyl viologen reduction at 80°C or at the temperatures indicated was determined at 600 nm (ε = 13,000 M$^{-1}$·cm$^{-1}$). For 30–90°C, the enzyme assay was performed as described above. From 90°C to 112°C, the enzyme activity was measured by the instant assay method (24).

VOR activity was also measured by quantitating the product, isobutyrylCoA. The assay for isobutyrylCoA that formed in the standard assay mixture at 30–115°C was based on a method described previously (25). The purified enzyme was incubated in the standard enzyme assay mixture (1 ml) in a serum vial (6 ml) under an Ar atmosphere for 5 min at each temperature. The amount of isobutyrylCoA produced was determined by measuring the absorbance at 260 nm after separating it from non-reacted CoASH on a reversed-phase column (JASCO C18S column, 7.5 × 150 mm).

The pH optimum of the enzyme activity was obtained with the use of Good buffers (CHES, EPPS, TAPS and MOPS) by the standard assay method at 80°C. Thirteen 2-keto acids (pyruvate, glyoxylate, 2-ketoisovalerate, 2-ketoisocaproate, 2-ketobutyrate, 2-ketogluutarate, 2-keto-methyl-n-valerate, 3-hydroxypyruvate, 3-phenylpyruvate, p-hydroxyphenylpyruvate, mercaptopyruvate, 4-methyl-thio-2-ketobutyrate and indole-3-pyruvate), mainly derived from the corresponding amino acids, were examined as substrates for the purified VOR at a concentration of 5 mM with the use of methyl viologen as the electron acceptor.

Thermal Stability and Oxygen Sensitivity—The thermal stability of the enzyme was examined by measuring the remaining activity after incubation at 80°C. Fifteen serum vials (6 ml) containing the purified enzyme in 50 mM Tris-HCl (pH 8.0)/1 mM TPP/1 mM MgSO$_4$/2 mM DTT (1 ml) were prepared and immersed in a water bath or a glycerol bath at 80°C. The stability of the enzyme under aerobic conditions was examined by bubbling moist air quite slowly at 25°C through the enzyme solution, which had been prepared by diluting the concentrated enzyme solution (50 mg/ml) tenfold with either aerobic 50 mM Tris-HCl (pH 8.0) or aerobic 50 mM Tris-HCl (pH 8.0)/1 mM TPP/1 mM MgSO$_4$/0.2 M NaCl. An aliquot of the incubated solution was diluted at an appropriate time point during the incubation with anaerobic 50 mM Tris-HCl (pH 8.0)/0.2 M NaCl/1 mM TPP/1 mM MgSO$_4$/2 mM DTT/2 mM dithionite under anaerobic conditions. The diluted enzyme solution was used for the standard assay.

RESULTS AND DISCUSSION

Purification and Subunit Composition—An obstacle to the purification of*† /*‡ T. profundus* VOR in the active form is the marked decrease in the enzymatic activity, in addition to the significant sensitivity toward molecular oxygen. For instance, the time required for half of the VOR activity ($t_{1/2}$) of the anaerobically purified enzyme to be lost was 5 h even under anaerobic conditions. After intensive trials under anaerobic conditions, we found that little enzymatic activity was lost during the gel filtration in the presence of TPP, Mg$^{2+}$ and NaCl, whereas a complete loss of the activity was found upon gel filtration in the absence of TPP/Mg$^{2+}$/NaCl. These results indicate that the enzyme-bound TPP/Mg$^{2+}$ tends to be liberated from the archaeal VOR, and that the activity could be recovered upon incubation with the standard assay buffer at ambient temperature. The highest recovery of the enzyme activity was achieved on overnight incubation with TPP/Mg$^{2+}$/NaCl. Therefore, we used TPP, Mg$^{2+}$ and
NaCl as stabilizing reagents for the archaeal VOR, throughout the purification procedure and the characterization, as described below.

VOR was purified from *T. profundus* cells (70 g, wet weight) in the anaerobic chamber. Typical purification results are summarized in Table 1. The final preparation exhibited specific activity of 128 units/mg protein and the yield was 33%. For comparison, the highest specific activity of the enzyme purified in the absence of TPP/Mg\(^{2+}/NaCl\) was only 16 units/mg. A VOR that exhibited specific activity of 46 units/mg protein was purified from *T. litoralis* (8) and one exhibiting that of 6.9 units/mg protein has been reported for *Methanobacterium thermoautotrophicum* (10).

As mentioned above, the purified VOR is extremely oxygen-sensitive under aerobic conditions. The oxygen sensitivity curve (Fig. 2) showed that the apparent 1/2 of the loss of activity was only 5 min. The plot of the incubation time against log (enzyme activity) (Fig. 2, line b) indicates that two straight lines crossed, suggesting that the enzyme denaturation occurred in at least two phases, a fast phase in the initial approximately 10 min and the slower phase. The presence of 1 mM TPP, 1 mM Mg\(^{2+}\) and 0.2 M NaCl in the enzyme solution slightly changed the apparent 1/2 of approximately 25 min (Fig. 2, line a), but failed to completely block the oxygen-dependent inactivation. This suggests that the oxygen sensitivity of the archaeal VOR is not directly associated with loss of the labile TPP/Mg\(^{2+}\) cofactor during the purification.

![Fig. 2. The enzyme activity profile of 2-ketoisovalerate ferredoxin oxidoreductase from *T. profundus* exposed to air at 25°C.](https://academic.oup.com/jb/article-abstract/137/1/101/873838)

Fig. 2. The enzyme activity profile of 2-ketoisovalerate ferredoxin oxidoreductase from *T. profundus* exposed to air at 25°C. The protein concentration in (b) aerobic 50 mM Tris-HCl pH 8.0, or in (a) aerobic 50 mM Tris-HCl (pH 8.0)/1 mM TPP/1 mM MgSO\(_4\)/0.2 M NaCl was 0.06 mg/ml. The incubation time versus log (percent enzyme activity) is plotted.

### Table 1. Typical purification of *Thermococcus profundus* 2-ketoisovalerate ferredoxin oxidoreductase. The results when the cells (70 g) were used are shown.

<table>
<thead>
<tr>
<th>Step</th>
<th>Enzyme activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free extract</td>
<td>2,800</td>
<td>3,700</td>
<td>0.76</td>
<td>100</td>
</tr>
<tr>
<td>Q Sepharose</td>
<td>1,390</td>
<td>320</td>
<td>4.3</td>
<td>50</td>
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<tr>
<td>Hydroxyapatite</td>
<td>830</td>
<td>220</td>
<td>3.8</td>
<td>30</td>
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<tr>
<td>Red TOYOPEARL</td>
<td>920</td>
<td>7.2</td>
<td>128</td>
<td>33</td>
</tr>
</tbody>
</table>
units/mg protein; 2-ketobutyrate, 33 units/mg protein; pyruvate, 33 units/mg protein; 2-ketoisocaprate, 29 units/mg protein; 2-ketomethylvalerate, 29 units/mg protein; 4-methylthio-2-ketobutyrate, 18 units/mg protein; and indole-3-pyruvate, 16 units/mg protein. Other chemicals such as glyoxylate, 2-ketoglutarate, 3-hydroxy-2-ketobutyrate, phenylpyruvate and mercaptopyruvate did not serve as substrates for the archaeal VOR. The apparent $K_m$ value of the enzyme for 2-ketoisovalerate was 170 µM, and that for coenzyme A was 21 µM.

The optimal temperature for the 2-ketoisovalerate methyl viologen oxidoreductase activity of the purified enzyme was determined to be 93 ± 2°C (see "MATERIALS AND METHODS") (Fig. 5). The same value (93 ± 2°C) was also estimated by quantitation of isobutyrylCoA, a product of the enzyme reaction, using the reversed-phase column chromatography method (25).

The Arrhenius plot was obtained from the data shown in Fig. 5 (inset). At least two lines crossed at approximately 63°C and a small decrease above 80°C was observed. The activation energy was calculated to be 68 kJ/mol in the high temperature region (63–80°C), while the value of 172 kJ/mol was calculated for 30–63°C. A crossing point of the Arrhenius plot has been seen in many cases of thermophilic enzymes, and it is possible that, at temperatures above this crossing point, the protein conformation of VOR changed to a more active state.

When cognate 4Fe ferredoxin was added instead of methyl viologen in the standard assay mixture, the absorbance in the absorption spectrum of the ferredoxin in the entire visible region decreased drastically upon incubation at 80°C for 2 min (or at 25°C for 2 h). The absorbance at 390 nm of the T. profundus ferredoxin decreased by approximately 35%. On the other hand, NAD$^+$ and NADP$^+$ failed to serve as electron acceptors for T. profundus VOR. These results indicate that this 4Fe ferredoxin is a probable physiological electron acceptor of the archaeal VOR.

**Molecular Properties and Characterization of the Iron-Sulfur Clusters**—The sensitivity of the purified T. profundus VOR to oxygen is characteristic of many metalloenzymes from strictly anaerobic organisms. In addition to TPP and Mg$^{2+}$, which could not be estimated quantitatively as they are present as stabilizing reagents for the enzyme, the purified VOR in the active form contained 22.8 mol of Fe$^{2+/(3+)}$ (as judged on colorimetric assay with bathophenanthroline; 19.0 mol/mol on ICP-Mass spectral analysis), and 24.5 mol of acid-labile sulfide per mol of the (αβγδ)$_2$ enzyme. The following metals were not detected in stoichiometric amounts (below 0.1 mol/mol) on ICP-Mass and/or atomic absorption analyses: Zn, Cu, Ni, W and Mo. Chelating reagents such as bathocuproinedisulfonate for Cu$^{2+}$ and zincon (2-(1-(2-hydroxy-5-sulfophenyl)-3-phenyl-5-formazanobenzoate) for Zn$^{2+}$ did not inhibit the VOR activity of the purified enzyme. Taken together, in addition to the TPP/Mg$^{2+}$ center, these results indicate the presence of multiple iron-sulfur centers (up to six) in the purified VOR, which are investigated in greater detail below.

The UV-visible absorption spectrum of the purified VOR showed a wide absorption band in the visible region...
with a shoulder at around 400 nm, supporting the presence of iron-sulfur clusters. With the addition of substrates, 2-ketoisovalerate and coenzyme A, and incubation at 80°C for 2 min or 25°C for 2 h, the absorbance in the visible region decreased to about half, indicating that the iron-sulfur cluster(s) was reduced by the substrates (data not shown).

The low temperature EPR spectra are depicted in Fig. 6. The enzyme incubated with 2-ketoisovalerate and coenzyme A at 80°C for 2 min exhibited complexed resonances centered at around g = 2 (line A). Further incubation did not change the spectrum. The spectrum consisted of at least two separate types of EPR signals; near-axial signals centered at g = 1.94, and a radical-like feature at g = 2.0. The broadening of the complex signals above 30 K indicates that the signals are due to the [4Fe-4S] clusters, and the spin-spin interactions of these signals indicate the spatial close proximity of these clusters (26). The latter radical signal was detected at 50 K, indicating that it is derived from the hydroxyisobutyl radical produced through the reaction of the enzyme-bound TPP and 2-ketoisovalerate (8). Spin quantitation analysis suggested that substrate-reduced VOR contains approximately 4.6 mol of [4Fe-4S]1+ cluster per mol of (αβγδ)2 enzyme. Regarding the spin concentration of T. litoralis VOR, it has been reported that the substrate-reduced T. litoralis enzyme contains 4.6 spins per αβγδ protomer. This value is twice that of the T. profundus VOR reduced by the substrates.

The purified enzyme oxidized with excess thionine showed an almost isotropic signal at g = 2.01 (line B), which broadened above 30 K and thus was deduced to be derived from a [3Fe-4S]1+ cluster [0.9 spin/mol of the (αβγδ)2 enzyme]. Thus, the purified (αβγδ)2 enzyme apparently contains approximately five [4Fe-4S] clusters and one [3Fe-4S] cluster. The detection of only a substoichiometric amount of spin for the [3Fe-4S] cluster (i.e. ~0.5 spin per αβγδ protomer) indicates that it probably derived from a [4Fe-4S] cluster through oxidative damage during purification.

The resonance Raman (RR) spectra of the archaeal VOR (Fig. 7) exhibited bands at 251, 265, 276, 285, 298, 337, 345, 358, 376 and 399 cm−1. By analogy to other proteins containing one or more [4Fe-4S] clusters, the overall features of the RR spectra are consistent with dominant contributions from (multiple) [4Fe-4S] clusters. The assignment of each Raman band to Fe-S clusters is possible by referring to the literatures (Ref. 27 for [4Fe-4S] clusters and ref 28 for [3Fe-4S] clusters): the RR bands attributable to the [4Fe-4S] cluster are 251, 265, 276, 337, and 358 cm−1, while other bands at 285, 298, 376 and 376 cm−1 can be attributed to a [3Fe-4S] cluster. Thus the [3Fe-4S] cluster indeed exists only in a small amount in the as-isolated enzyme, thus being consistent with the EPR results (Fig. 6).

Considering also the marked oxygen sensitivity of the archaeal VOR, our results collectively indicate that one of three [4Fe-4S]2+/1+ clusters per αβγδ protomer in VOR is highly sensitive to oxidative damage, and that it is readily converted partially to the corresponding cubane [3Fe-4S] form upon loss of one iron atom from an oxygen-labile [4Fe-4S] cluster.

**CONCLUSION**

A novel (αβγδ)2-type 2-ketoisovalerate ferredoxin oxidoreductase, a key enzyme in the energy-producing amino acid degradation pathway of the S0-dependent hyperthermophilic archaeon *T. profundus*, has been purified in an active form, and initially characterized. The intact enzyme is expected to contain six [4Fe-4S] clusters per (αβγδ)2 enzyme, although one of the clusters per αβγδ protomer is extremely oxygen-labile and is readily (partially) converted to the corresponding cubane [3Fe-4S] cluster even under the applied conditions. In addition to this sensitivity to oxygen, the archaeal VOR was found to be inactivated even under anaerobic conditions, which could be effectively prevented by the addition of excess TPP, Mg2+, and NaCl. The purified *T. profundus* enzyme could be obtained in an active form in a relatively large amount, and attempts to crystallize this VOR under anaerobic conditions are being made to understand the variations in subunit structures and substrate specificity in this enzyme family in greater detail.

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