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2-Oxoacid:Ferredoxin Oxidoreductase from the Thermoacidophilic Archaeon, *Sulfolobus* sp. Strain 7[1]

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The purified 2-oxoacid:ferredoxin oxidoreductase of a thermoacidophilic and aerobic crenarchaeote, *Sulfolobus* sp. strain 7, consists of 70-kDa α and 37-kDa β subunits, and contains one thiamine pyrophosphate (TPP), one [4Fe-4S]$^{2+,1+}$ cluster, and two magnesium atoms per $\alpha\beta$ structure. It exhibits a broad substrate specificity toward 2-oxoacids such as 2-oxoglutarate, 2-oxobutyrate, and pyruvate. The gene encoding the archaeal oxidoreductase was cloned, and the two open reading frames encoding the α (632 amino acids) and β subunits (305 amino acids), respectively, were sequenced. Careful sequence alignment revealed several consensus motifs of this enzyme family, as well as possible cofactor binding residues of the *Sulfolobus* enzyme. This new structural information also indicates that (i) several genetic fusions and reorganization of the early, possibly $\alpha\beta\gamma\delta$-type enzyme similar to those from hyperthermophiles have taken place during evolution of the 2-oxoacid:ferredoxin (flavodoxin) oxidoreductase superfamily, which might have occurred in different ways in early aerobic archaea and early anaerobic bacteria, and that (ii) enzymes with different subunit compositions should have an essentially similar catalytic mechanism with one TPP and at least one [4Fe-4S] cluster as the minimal set of redox centers.

Key words: archaea (archaebacteria), evolution, 2-oxoacid:ferredoxin oxidoreductase, primary structure, *Sulfolobus*.

Ferredoxin serves as an intermediate electron acceptor in a variety of key steps in the central metabolic pathways involved in saccharolytic and peptide fermentation by several strictly anaerobic archaea (archaebacteria), NAD(P)$^+$ usually substituting by bacteria (eubacteria) (*1-5*). Although the ferredoxin-dependent redox system of aerobic respiratory archaea is rather less understood, ferredoxin has been shown to function as a physiological electron acceptor of the archaeal coenzyme A (CoA)-acylating 2-oxoacid:ferredoxin oxidoreductase (*6-10*). The enzyme plays a central role in the decarboxylation of pyruvate and 2-oxoglutarate in archaea, the 2-oxoacid dehydrogenase multienzyme complex (*11*) being involved in aerobic bacteria and eukarya (eukaryotes) with mitochondria.

In the case of conventional 2-oxoacid dehydrogenase multienzyme complexes (2,000-7,000 kDa), thiamine pyrophosphate (TPP), lipoic acid, and flavin nucleotide are involved as prosthetic groups, and each of three different kinds of enzymes catalyzes a partial reaction. In contrast, the archaeal 2-oxoacid:ferredoxin oxidoreductases are much smaller enzymes (100-300 kDa), and contain one TPP and at least one [4Fe-4S]$^{2+,1+}$ cluster (per protomer) as prosthetic groups (*7, 12-16*). Thus, the cofactor composition of the archaeal enzymes is remarkably similar to that of those from fermentative and phototrophic obligatory anaerobic bacteria (*e.g., Clostridia* and *Chlorobia*) (*17-22*), several photosynthetic bacteria (*18, 23*), and anaerobic and amitochondrial protozoa [such as *Trichomonas vaginalis* (*24-26*)] (Table I). Cloning and sequencing of the gene encoding *Halobacterium salinarium* (f. *halobium*) pyruvate:ferredoxin oxidoreductase, which is composed of two non-identical subunits and probably contains two TPP and two [4Fe-4S]$^{2+,1+}$ cluster per $\alpha_2\beta_2$ structures (*12*), has revealed that neither the α nor the β subunit shows considerable homology to any subunit of the mitochondrial 2-oxoacid dehydrogenase multienzyme complexes (*27*). This is also supported by recent molecular biological studies on 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases from several anaerobic bacterial and eukaryal sources, including the *Klebsiella pneumoniae* (*28*), *Enterobacter agglomerans* 333 (*29*), *Rhodospirillum rubrum* (unpublished, EMBL accession number X77515), *Anabaena variabilis* ATCC29413 (*30*), *Anabaena* sp. PCC 7119 (*30*), and *T. vaginalis* enzymes (*26*), which have an α_2 composition.

The strategy used for the breakdown of 2-oxoacids by the *H. salinarium* enzyme is distinctly different from that in the case of mitochondrial multienzyme complexes, and is proposed to proceed through a sequential two-step reaction (*20, 31*). More recently, a modified catalytic mechanism was also proposed for the hyperthermophile enzymes which

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TABLE I. **Properties of 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases.**

Source	Molecular mass (kDa)	Subunit (kDa)	Cofactor composition (per protomer)	Substrate specificity (K_m[mM])		References
				Pyruvate	2-Oxoglutarate	
Archaea:						
***Sulfolobus* sp. strain 7**	103[a]	70, 37	TPP, [4Fe-4S], 2×Mg	0.25	0.87	(*52, 53*; this work)
Halobacterium salinarium	256[b,e]	86×2, 42×2	TPP, [4Fe-4S]	0.10	1.1	(*12, 27, 31*)
	248[b,f]	88×2, 36×2	TPP, [4Fe-4S]?	—[g]	n.d.[h]	(*27*)
Pyrococcus furiosus	115[c,e]	47, 31, 24, 13	TPP, 3×[4Fe-4S], Cu	0.46	—	(*13, 15, 22, 32*)
Archaeoglobus fulgidus	120[c]	45, 33, 25, 13	TPP, 3×[4Fe-4S]?	0.3	—	(*14*)
Bacteria:						
Clostridium acidiurici	240[d]	120×2	TPP, [4Fe-4S]?	2.2	—	(*17*)
Clostridium thermoaceticum	240[d]	120×2	TPP, 2×[4Fe-4S]?	2.0	—	(*20*)
Clostridium acetobutylicum	246[d]	123×2	TPP, [4Fe-4S]?	0.32	—	(*21*)
Klebsiella pneumoniae	240[d]	120×2	TPP, 3×[4Fe-4S]?	2.0	—	(*19, 20, 28*)
Rhodospirillum rubrum	252[d]	114×2	n.d. (9.7 mol of Fe/dimer)	0.18	—	(*23*)
Thermotoga maritima	113[c]	43, 34, 23, 13	TPP, 3×[4Fe-4S]	14.5	—	(*15, 22*)
Anaerobic amitochondrial Eukarya:						
Trichomonas vaginalis	240[d]	123×2	TPP, 3×[4Fe-4S]?	0.14	—	(*24, 26*)

[a] $\alpha\beta$-type enzyme. [b] $\alpha_2\beta_2$-type enzyme. [c] $\alpha\beta\gamma\delta$-type enzyme. [d] α_2-type enzyme. [e] pyruvate:ferredoxin oxidoreductase. [f] 2-oxoglutarate-specific enzyme. [g] not reactive. [h] not determined.

have an $\alpha\beta\gamma\delta$ structure, and contain one TPP cofactor and multiple FeS clusters (*32*).

Sulfolobus sp. strain 7 (originally named *Sulfolobus acidocaldarius* strain 7) is a typical aerobic and thermoacidophilic archaeon isolated from Beppu hot springs, Kyushu, and grows optimally at pH 2-3 and at 75-80°C (*33*). The chemoheterotrophically-grown crenarchaeote acquires biological energy through aerobic respiration rather than simple fermentation, and contains a complex aerobic respiratory system in its membrane (*33-39*). As opposed to most aerobic bacteria and mitochondria, however, *Sulfolobus* sp. strain 7 also contains a ferredoxin-dependent redox system in its cytoplasm (*9, 10*). In earlier studies, we showed that the *Sulfolobus* sp. ferredoxin is of the 7Fe-containing dicluster type, and that only a [3Fe-4S] cluster is reduced during the steady-state turnover of cognate 2-oxoacid:ferredoxin oxidoreductase (*9*) and NADPH:ferredoxin oxidoreductase (Iwasaki, T. and Oshima, T., unpublished result).

In this paper, we report the initial characterization of a 2-oxoacid:ferredoxin oxidoreductase purified from *Sulfolobus* sp. strain 7, and cloning and sequencing of the gene encoding it. We show that the purified enzyme has a heterodimeric $\alpha\beta$ structure, and shows a broad substrate specificity toward 2-oxoacids such as pyruvate and 2-oxoglutarate, implying its bifunctional role in archaeal central metabolism, *viz.*, the CoA-dependent oxidation of pyruvate and 2-oxoglutarate in the tricarboxylic acid cycle. Moreover, in conjunction with careful multiple alignment of the amino acid sequences of the enzymes from other sources including those reported earlier by others (*14, 15, 26*), the primary structure of the archaeal enzyme led to the identification of several conserved amino acid sequences and possible cofactor-binding sites in enzymes with different subunit structures. These findings further indicate that 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases probably have a common catalytic mechanism despite their different domain arrangements. Preliminary accounts of the present work have appeared in abstract form (*52, 53*).

EXPERIMENTAL PROCEDURES

Materials—Sodium pyruvate, 2-oxoglutarate, 2-oxobutyrate, and thiamine pyrophosphate (TPP) were from Nacalai Tesque (Tokyo). Coenzyme A (CoA) was obtained from Kohjin (Tokyo). DEAE-Sephacel, phenyl-Toyopearl 650M, and hydroxylapatite HTP were purchased from Pharmacia LKB Biotechnology, Tosoh, and Bio-Rad, respectively. Water was purified with a Milli-Q purification system (Millipore). Other chemicals mentioned in this study were of analytical grade.

Bacterial Strain and Plasmid—*Sulfolobus* sp. strain 7 (originally named *S. acidocaldarius* strain 7) was cultivated aerobically and chemoheterotrophically at pH 2.5-3 and 75-80°C, and was harvested in the late exponential phase of growth as described previously (*10, 33*). The *Escherichia coli* strain used for cloning was JM 103 grown in LB or 2×YT medium, with 50 μg/ml ampicillin when required. Plasmid pUC118 was used for cloning, and phagemids M13mp18 and M13mp19 were used for sequencing. DNA was manipulated by standard procedures (*40, 41*).

Purification of Ferredoxin and Measurement of Enzymatic Activities—A 7Fe-containing dicluster ferredoxin of *Sulfolobus* sp. strain 7 was purified as described previously (*9*), and was used for the routine enzymatic assay. 2-Oxoacid:ferredoxin oxidoreductase activities were monitored by means of the horse heart cytochrome *c* reduction assay at 50°C using the *Sulfolobus* ferredoxin as an intermediate electron acceptor (*6*), except that the reaction was carried out in 10 mM potassium phosphate buffer, pH 6.8, and was initiated by the addition of the purified enzyme (*10*). Non-enzymatic reduction of cytochrome *c* by coenzyme A at this temperature was corrected.

Purification of Sulfolobus 2-Oxoacid:Ferredoxin Oxidoreductase—Purification of the 2-oxoacid:ferredoxin oxidoreductase of *Sulfolobus* sp. strain 7 was carried out by following the 2-oxoacid:ferredoxin oxidoreductase activity and the absorption bands at 280, 408, and 450 nm of each fraction at different steps. The cytosol fraction, prepared by French press treatment as described previously (*10*), was applied to a DEAE Sephacel column (3.0×28 cm), which had been previously equilibrated with 30 mM Tris-Cl buffer, pH 7.3, containing 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. The column was washed with 500 ml of the equilibration buffer, and then the

material bound to the column was eluted with a 1,600-ml linear gradient of NaCl (0-400 mM) in the equilibration buffer. The combined peak fraction was made to a 1 M ammonium sulfate solution by the addition of solid ammonium sulfate at 4°C. The suspension was gently degassed with an aspirator, and then directly applied to a phenyl-Toyopearl 650M column (1.2×25 cm), which had been pre-equilibrated with 20 mM potassium phosphate buffer, pH 6.8, containing 1 M ammonium sulfate. The column was washed with 100 ml of the equilibration buffer, and then eluted with a 200-ml linear gradient between 1 M and 0 M ammonium sulfate in 20 mM potassium phosphate buffer, pH 6.8. The combined peak fraction was dialyzed against 20 mM potassium phosphate buffer, pH 6.8, and then applied to a hydroxylapatite column (1.0×23 cm), which had been pre-equilibrated with 10 mM potassium phosphate buffer, pH 6.8. The enzyme bound to the column was eluted with a 90-ml linear gradient of potassium phosphate buffer, pH 6.8 (10-300 mM), and the peak fraction was collected and concentrated by pressure filtration through an Amicon YM-10 membrane at 4°C. The concentrated material (~2 ml per tube) was loaded onto a 34-ml glycerol density gradient (5-20%, v/v) in 20 mM potassium phosphate buffer, pH 6.8, which had been placed on 0.5 ml of a 25% glycerol (v/v) cushion (containing 20 mM potassium phosphate buffer, pH 6.8), and then ultracentrifuged in a Beckman SW 28 rotor at 28,000 rpm for 20 h at 10°C. The combined fraction collected by tubing was made to 2 M ammonium sulfate in 20 mM potassium phosphate buffer, pH 6.8, and then gently degassed with an aspirator. It was then applied to a phenyl-Toyopearl 650M column (1.0×25 cm) which had been pre-equilibrated with 20 mM potassium phosphate buffer, pH 6.8, containing 2 M ammonium sulfate. The column was first washed with 40 ml of the equilibration buffer, and eluted with a 160-ml linear gradient of 1-0 M ammonium sulfate in 20 mM potassium phosphate buffer, pH 6.8, and then further with 10 mM potassium phosphate buffer, pH 6.8. Although 2-oxoacid: ferredoxin oxidoreductase activity in the cytosol fraction was not very stable on storage at room temperature, at 4°C or −80°C, the purified enzyme was considerably stable against oxygen and could be stored at −80°C after dialysis against 20 mM potassium phosphate buffer, pH 6.8.

Analytical Methods—Absorption spectra were recorded with a Hitachi U-3210 spectrophotometer equipped with a thermoelectric cell holder, or with a Beckman DU7400 spectrophotometer. EPR measurements were carried out with a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system, in which the temperature was monitored with a Scientific Instruments series 5500 temperature indicator/controller.

The apparent molecular masses of the purified proteins were estimated by gel filtration analysis on a calibrated Tosoh G-3000XL column in a Tosoh HPLC 8030, which had been equilibrated with 50 mM 3-[*N*-morpholino]-propanesulfonic acid (MOPS)-NaOH buffer, pH 6.8, containing 200 mM NaCl. Protein was measured by the BCA assay (Pierce Chemical) with bovine serum albumin as a standard. Metal content analyses of the purified proteins were carried out by inductively coupled plasma atomic emission spectrometry with a Seiko SPS 1500 VR instrument. The TPP content of the purified 2-oxoacid:ferredoxin oxidoreductase was determined as described in Ref. *12* using a Shimadzu spectrofluorophotometer RF-540.

Cross-linking between 2-oxoacid:ferredoxin oxidoreductase and ferredoxin was examined with a water soluble carbodiimide, *N*-ethyl-3-(3-dimethylaminopropyl)carbodiimide, as described by Zanetti *et al.* (*42*).

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (*43*) was carried out on 12 to 20% gels after treating the proteins with 2% SDS in the presence or absence of 2% 2-mercaptoethanol at 90°C for 15 min, and the proteins were visualized by Coomassie Brilliant Blue (CBB) staining and scanned with a densitometer (Shimadzu dual-wavelength scanner CS-930). Analytical PAGE (in the absence of SDS) was carried out with the Laemmli-discontinuous system on 5 or 7.5% gels in the absence of SDS, and proteins were visualized by CBB staining.

N-Terminal Amino Acid Sequencing—N-Terminal amino acid sequence analysis of each subunit of the purified 2-oxoacid:ferredoxin oxidoreductase was carried out with a 470A Protein Sequencer (Applied Biosystems), after separation of the subunits on SDS-PAGE gels and recovery by electroelution in the presence of 0.1% SDS, the subunits having been visualized by reversible imidazole/Zn/SDS-staining (*44*).

Cloning Procedures—A 23-mer of 32-fold degeneracy, 5′-CC TTG (TA)GC (TA)CC (TA)CC (TG)AT (TA)AC CCA-3′, the antisense sequence corresponding to the 5th to 12th amino acid residues of the α subunit of the purified *Sulfolobus* sp. enzyme, was synthesized and used to probe Southern blot of the chromosomal DNA digests. Genome DNA from *Sulfolobus* sp. strain 7 was completely digested with *Eco*RI, *Hin*dIII, or *Bam*HI. The fragments were separated on a 1% agarose gel and then transferred to a nylon membrane. Hybridization was carried out with the oligonucleotide probe labeled with dUTP digoxigenin (DIG) (Boehringer) at its 3′-terminal. Hybridization and subsequent washing were carried out with a 5×SSC solution at 45°C. The probe designed above hybridized exclusively with the bands corresponding to 5.4, 3.6, and 2.5 kbp (data not shown), with the fragments digested by *Eco*RI, *Xba*I, and *Hin*dIII, respectively.

A genomic DNA library was constructed by ligating the *Eco*RI digested genome DNA and pUC118. The library was screened with the oligonucleotide probe labeled with [γ-^{32}P]ATP by T_4 polynucleotidyl kinase (Takara). Colony hybridization was carried out under the same conditions as for Southern hybridization. A single clone containing a 5.4 kbp *Eco*RI insert, designated as pZE91, was isolated on the screening of 8,000 colonies from the *Eco*RI library. A subclone, pZES91, was constructed by removing the 2.1 kb fragment between the *Bcl*I and *Eco*RI sites of pZE91 (see Fig. 4A).

Sequence Analysis—The nucleotide sequence of the gene encoding the *Sulfolobus* enzyme in pZES91 (Fig. 4A) was determined on both strands by the dideoxy chain termination method with an Applied Biosystem 373A sequencer. The DNA sequence was processed using Genetyx software (SDC Software, Tokyo). The nucleotide sequence reported in this paper has been submitted to the DNA Data Bank of Japan (DDBJ), and will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D64024.

RESULTS AND DISCUSSION

Purification and Catalytic Properties of Sulfolobus 2-Oxoacid:Ferredoxin Oxidoreductase—A 2-oxoacid:ferredoxin oxidoreductase was purified to electrophoretically homogeneity from the cytosol fraction of *Sulfolobus* sp. strain 7, as described under "EXPERIMENTAL PROCEDURES." The purified enzyme exhibited specific activities of 69 units/mg with 2-oxoglutarate and 39 units/mg with pyruvate, respectively, which typically corresponded to ~52- and ~43-fold purification, respectively, from the cytosol fraction. Approximately 6-8 mg of pure enzyme could be obtained from ~100 g (wet weight) of cells. The ratio of the specific activities toward pyruvate/2-oxoglutarate of the purified enzyme was ~0.6, which was very close to the corresponding values for the cytosol fractions of *Sulfolobus* sp. strain 7 (~0.7) and *S. acidocaldarius* strain DSM 639 [~0.68 (*6*)]. No pyruvate-specific enzyme was detected during the purification of soluble redox proteins (data not shown). For comparison, purified pyruvate:ferredoxin and 2-oxoglutarate:ferredoxin oxidoreductases of *H. salinarium* exhibited ratios of specific activities toward pyruvate/2-oxoglutarate of 1.7 and 0.01, respectively, while its cytosol fraction exhibited a ratio of ~0.78 (*12*).

Table II summarizes the substrate specificities toward 2-oxoacids and the effects of the *Sulfolobus* 7Fe ferredoxin on the enzymatic activities. As opposed to the cases of the enzymes from other sources, most of which are specific to either pyruvate or 2-oxoglutarate (*12-14, 20-24, 45*), the *Sulfolobus* enzyme showed a broad substrate specificity toward 2-oxoacids; under the present experimental conditions, the apparent K_ms for pyruvate and 2-oxoglutarate were 250 and 870 μM, respectively, while the apparent V_{max} for 2-oxoglutarate (86 units/mg in the presence of 25 μM *Sulfolobus* ferredoxin) was about twofold larger than that for pyruvate. The specific activities of the purified enzyme in the presence of the cognate ferredoxin were in the order of 2-oxoglutarate > 2-oxobutyrate > pyruvate, while the V_{max}/K_m for pyruvate was ~1.9-fold higher than that for 2-oxoglutarate.

Comparison of the specific activities in the presence of 4 mM 2-oxoacids indicated a correlation between the sizes of 2-oxoacids and the ferredoxin dependence of the reaction (Table II). Thus, when the acyl group of a 2-oxoacid was bulky, as in the cases of 2-oxoglutarate and 2-oxobutyrate, the 2-oxoacid-dependent cytochrome *c* reductase activity was clearly ferredoxin-dependent. On the other hand, when a small 2-oxoacid such as pyruvate was used as a substrate, a non-physiological electron acceptor (such as horse heart cytochrome *c*) was reduced even in the absence of ferredoxin, although ferredoxin dependence was still observed. These data imply that the microenvironment of the putative ferredoxin binding site of the enzyme may be affected by the binding of a 2-oxoacid, possibly indicating a conformational and/or structural change of the enzyme. When glyoxylate, which is even smaller than pyruvate, was used, no activity was detected in either the presence or absence of ferredoxin.

TABLE II. **Substrate specificity.**

R: $^{-}$OOC-$\overset{O}{\overset{\|}{C}}$-	Substrate (4 mM)	Cyt. *c* reductase activity [U/mg][a]	
		− ferredoxin	+ 25 μM ferredoxin
R-**H**	Glyoxylate	~1	~1
R-**CH_3**	Pyruvate	19	39
R-CH_2-**CH_2**-CH_3	2-Oxobutyrate	8.8	46
R-CH_2-CH_2-**COO**$^{-}$	2-Oxoglutarate	2.9	69[b]

[a] The specific activity was measured at 50°C in 20 mM potassium phosphate buffer, pH 6.8, containing 50 μM coenzyme A and 50 μM horse heart cytochrome *c*, in either the absence or presence of the *Sulfolobus* 7Fe ferredoxin. [b] K_m for 2-oxoglutarate = 870 μM; V_{max} = 86 U/mg; apparent K_m for the cognate ferredoxin = ~0.1 μM.

Molecular Composition and Spectroscopic Properties—The purified *Sulfolobus* enzyme gave a single band on 10% analytical PAGE in the absence of SDS, and had an apparent molecular mass of ~103 kDa, as estimated on gel filtration analysis (data not shown). This apparent size is similar to those of the hyperthermophile pyruvate:ferredoxin oxidoreductases (*13-15, 22*), corresponding to roughly half the sizes of other prokaryotic enzymes (Table I). Two different subunits with apparent molecular masses of 70 and 37 kDa, respectively (designated as the α and β subunits, respectively), were observed on SDS-PAGE, of which the 37-kDa β subunit was less stained by CBB (Fig. 1). These data suggest that the *Sulfolobus* enzyme is a dimeric protein, $\alpha\beta$. In order to identify possible ferredoxin-binding subunit(s), a cross-linking experiment was performed with a water soluble carbodiimide, *N*-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (*42*), but with no success. Even under low ionic strength conditions and under various conditions in either the presence or absence of substrates, no cross-linked complex (except for the intramolecular ones) could be obtained (Iwasaki, T. and Oshima, T., unpublished results). These data imply that the *Sulfolobus* enzyme may not form any electrostatically-stabilized ferredoxin-enzyme intermediate complex *in vitro*.

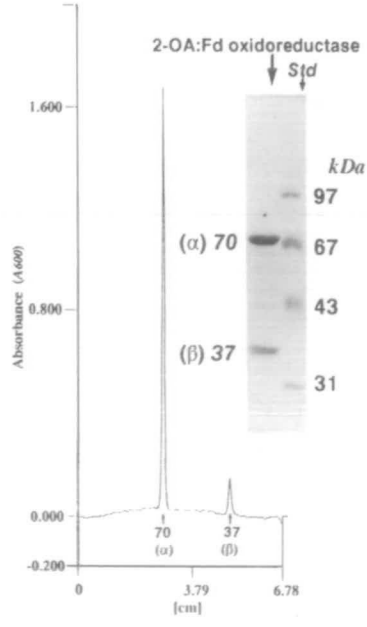

Fig. 1. **Polyacrylamide gel electrophoresis in the presence of SDS of the purified *Sulfolobus* 2-oxoacid:ferredoxin oxidoreductase, as analyzed by densitometric scanning.** The purified enzyme was analyzed by 13% SDS-PAGE and stained with Coomassie Brilliant Blue. Std, standard molecular markers (Bio-Rad).

The purified *Sulfolobus* enzyme contained 2.8 mol of non-heme Fe (27.2 nmol of Fe/mg of protein), 1.4 mol of Mg (13.7 nmol of Mg/mg of protein), and 0.4–0.5 mol of TPP per mol of protein. These values are probably underestimated due to the overestimation of protein, as often experienced for other FeS proteins (*e.g.*, Refs. *4, 9, 16, 36*). On the basis of the spectroscopic and primary structural evidence presented below, we suggest that the native *Sulfolobus* enzyme contains 4 mol of non-heme Fe, 2 mol of Mg, and 1 mol of TPP per mol of protein. No copper (<0.2 mol of Cu/mol of protein), flavin, or heme could be detected in the purified enzyme (data not shown).

The optical spectrum of the air-oxidized *Sulfolobus* enzyme had a peak at 280 nm and a broad shoulder around 410 nm, characteristic of FeS proteins with [4Fe-4S] clusters (Fig. 2, trace 1). Upon the addition of excess of a 2-oxoacid such as 2-oxoglutarate and pyruvate, the formation of a 320-nm chromophore was detected (Fig. 2, traces 2 and 3, and inset). Since this species was formed only after the binding of a 2-oxoacid, but not upon the addition of excess glyoxylate, it is due probably to an "activated" hydroxyalkyl-TPP radical intermediate of the enzyme. Preliminary experiments suggested that the formation of a 320-nm chromophore upon the addition of pyruvate occurred faster than in the case of 2-oxoglutarate, being consistent with the respective apparent K_m values (Iwasaki, T. and Oshima, T., unpublished results).

The properties of the bound cofactors were further investigated by X-band EPR spectroscopy (Fig. 3). The air-oxidized (as isolated) enzyme elicited a radical feature at $g=2.01$ (Fig. 3A). This signal showed a temperature dependence distinctly different from those of $[3Fe\text{-}4S]^{1+}$ clusters, and was readily detectable at 70 K (data not shown). The EPR lineshapes of the $g=2.01$ signal were altered and slightly broadened upon the addition of excess 2-oxoacids (Fig. 3B), indicating the formation of an activated hydroxyalkyl-TPP radical intermediate (*6, 12, 46*).

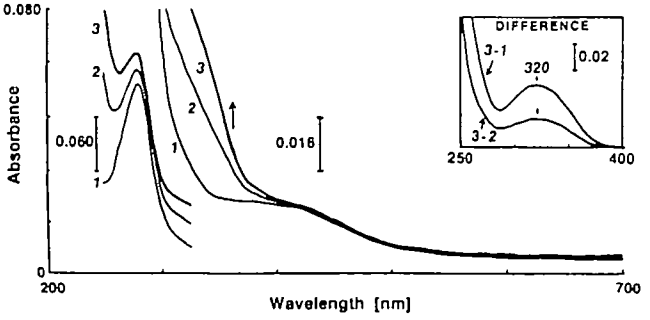

Fig. 2. **Optical properties of the purified 2-oxoacid:ferredoxin oxidoreductase of *Sulfolobus* sp. strain 7 in the air-oxidized (trace 1) and 2-oxoacid-bound (traces 2 and 3) states.** Formation of the 2-oxoacid-bound enzyme was monitored spectroscopically at room temperature after the addition of 2 mM 2-oxoglutarate in the absence of CoA (traces 2 and 3, 5 and 105 s after the addition of 2-oxoglutarate, respectively; scan speed, 2 s per scan). The difference spectra show the formation of the 320-nm chromophore in the 2-oxoacid-bound state (inset). Identical spectra were obtained for the pyruvate-bound enzyme (the formation of the pyruvate-bound species was faster than in the case of the 2-oxoglutarate-bound species, and was completed within ~10 s; data not shown). The protein concentration was ~0.86 mg/ml in 20 mM potassium phosphate buffer.

Upon reduction with excess dithionite, the *Sulfolobus* enzyme gave a complex EPR spectrum which was attributed to a partially-reduced $S=1/2[4Fe\text{-}4S]^{1+}$ cluster ($g_{z,y,x}=2.07$, 1.94, and 1.90) and an overlapping radical signal ($g=2.01$) (Fig. 3C). The relative intensity of the radical species was dependent on the extent of dithionite treatment, although it was not possible to achieve complete abolition of the radical feature at pH 6.8 (data not shown). No low-field resonance was detected under the conditions used. Double integration of the EPR signals in the dithionite-reduced state resulted in only ~6-9% of that of the $g=2.0$ radical in the air-oxidized state. Since the visible absorption around 400 nm of the purified enzyme was only slightly bleached on the addition of excess dithionite under the conditions used, it is suggested that the bulk of the [4Fe-4S] cluster still remained in the oxidized state even under the conditions used due to its low midpoint redox potential (presumably below −450 mV). In the partially-reduced state, the [4Fe-4S] and radical centers exhibited a spin–spin interaction (Fig. 3C), indicating the close proximity of these centers as a functional unit. The EPR lineshape

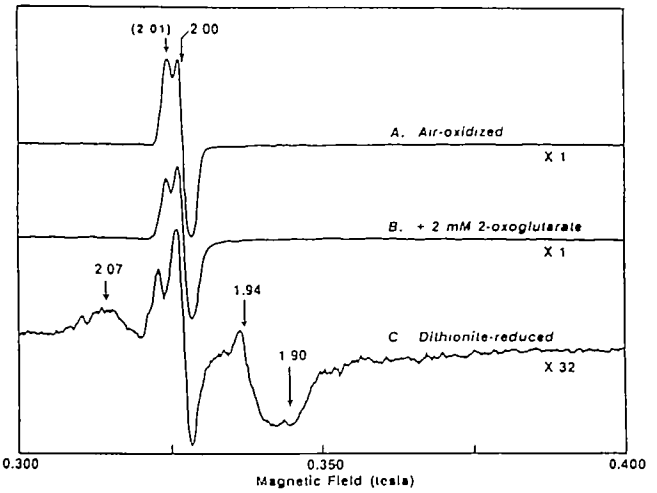

Fig. 3. **EPR spectra of the purified 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7.** The protein concentration was ~1.7 mg/ml in 20 mM potassium phosphate buffer. Instrument settings: temperature, 10 K; microwave power, 1.0 mW; modulation amplitude, 0.79 mT; accumulation, 4 times; the g values and the relative intensity scales are indicated in the figure.

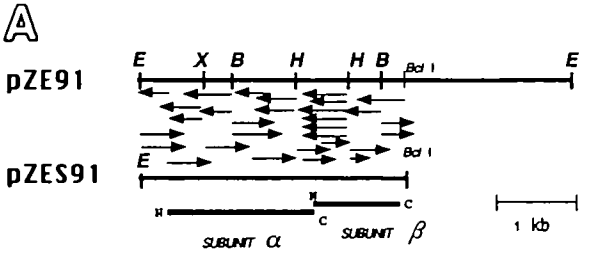

Fig. 4. **Gene cloning and sequencing strategy (A), and nucleotide sequence and deduced amino acid sequence (B) of the 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7.** The putative promoter (box A) and terminator elements are indicated. RBS, possible ribosomal RNA binding site. The amino acid sequence confirmed by protein analysis is underlined.

B

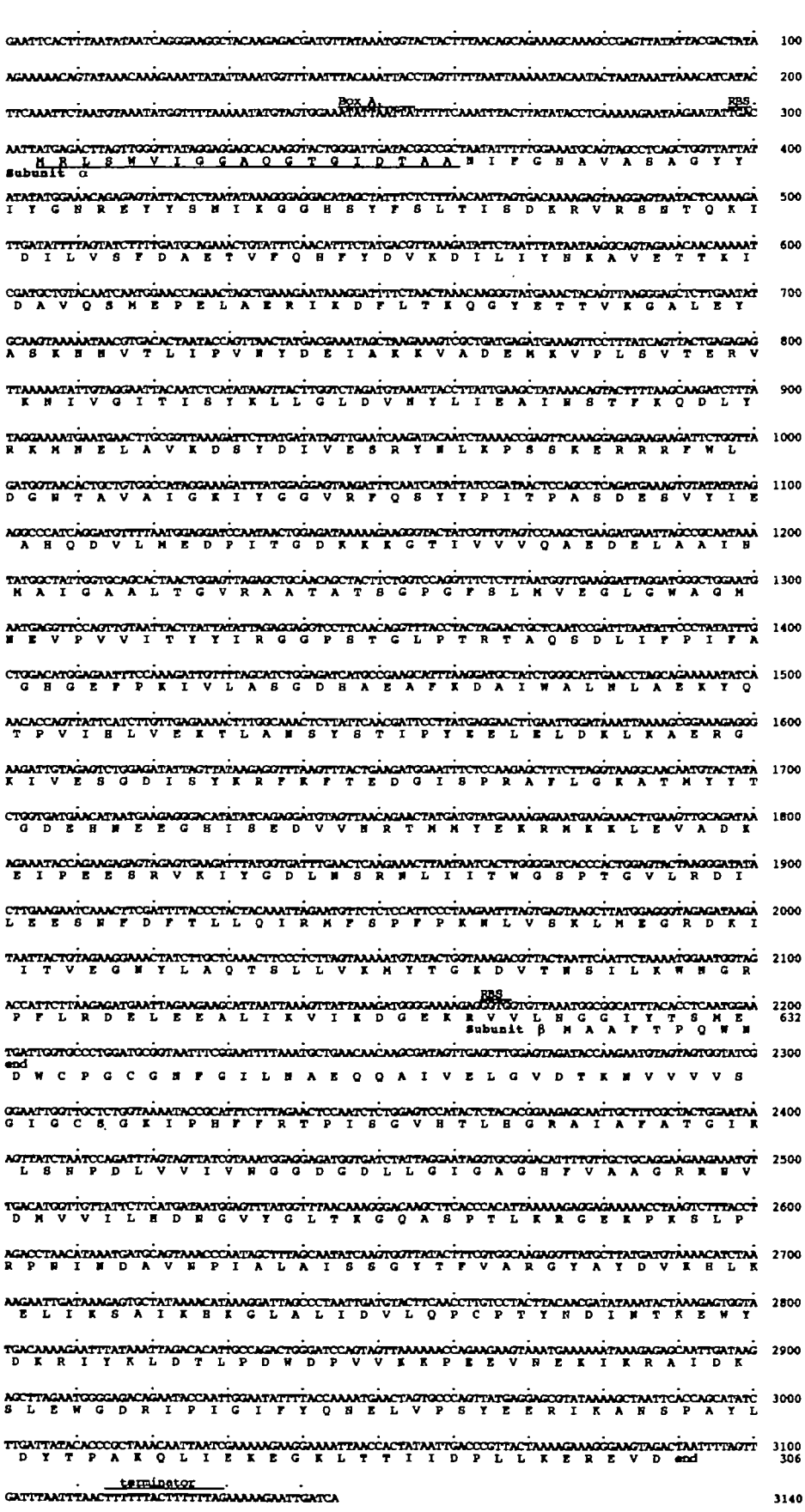

Fig. 4B

of the $S=1/2[4Fe\text{-}4S]^{1+}$ cluster was not affected by the addition of 5 mM cyanide (data not shown). On the other hand, although the catalytic cycle model of a 2-oxoacid: ferredoxin oxidoreductase of Kerscher and Oesterhelt (*7, 31*) predicts the reduction of a [4Fe-4S] center on the addition of a 2-oxoacid alone, this [4Fe-4S] cluster was not reduced on the addition of a 2-oxoacid alone even under anaerobic conditions, as in the cases of several other pyruvate:ferredoxin oxidoreductases (*20, 32*).

These data suggest that the *Sulfolobus* enzyme contains one TPP, one low-potential $[4Fe\text{-}4S]^{2+,1+}$ cluster, and at least two Mg atoms per $\alpha\beta$ structure. One of the bound Mg atoms may be coordinated to the phosphate groups of the TPP cofactor, by analogy to the case of *Lactobacillus plantarum* TPP-containing pyruvate oxidase (*47*).

Cloning and Sequencing of the Sulfolobus Oxidoreductase Gene—The N-terminal amino acid sequence of the α subunit of the *Sulfolobus* 2-oxoacid:ferredoxin oxidoreductase was determined up to 23 residues: MRLSWVIGGAQGTGIDTAANIFG---. It is highly homologous to the corresponding subunit of the *H. salinarium* enzyme (*27*). On the other hand, the N-terminal amino acid sequence of the β subunit of the *Sulfolobus* enzyme was blocked (data not shown).

The peptide sequence of the α subunit thus obtained was used to design mixtures of oligonucleotide probes for library screening, and the genes encoding the α and β subunits of the *Sulfolobus* enzyme were cloned and sequenced as described under "EXPERIMENTAL PROCEDURES" (Fig. 4A). Figure 4B shows the resultant nucleotide sequences of two open reading frames, and promoter- and terminator-like elements. The first open reading frame (positions 305-2200; 1,896 bases) corresponds to the α subunit, as confirmed by protein analysis of the N-terminus (underlined in Fig. 4B), and the deduced molecular mass of 70,758 Da is in good agreement with that obtained on SDS-PAGE (Fig. 1). Its deduced amino acid sequence was 27.2% identical to that of the α subunit gene of the *H.*

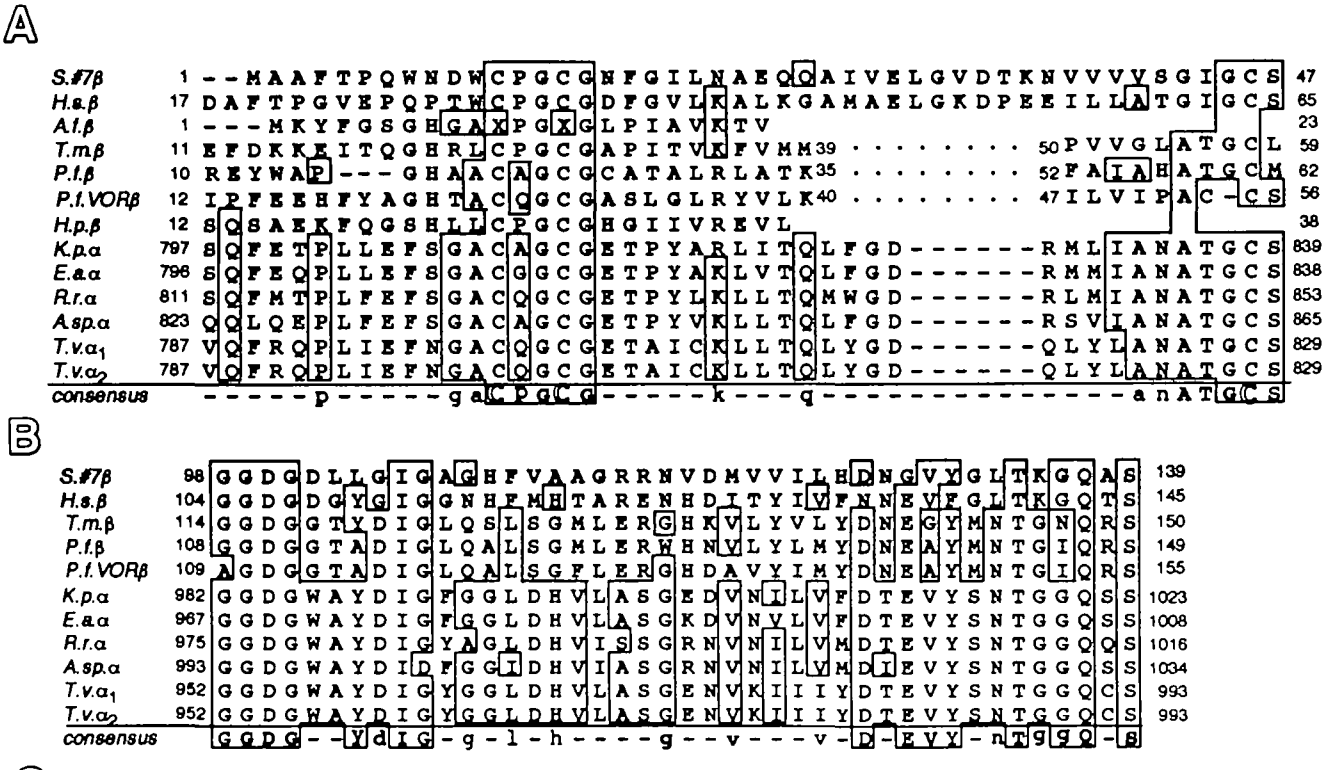

Fig. 5. **Multiple alignment of the amino acid sequence of the β subunit of the $\alpha\beta$-type 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 with the available sequences of homologous regions in $\alpha_2\beta_2$-, $\alpha\beta\gamma\delta$-, and α_2-type enzymes from other sources.** Subunits are denoted by α, β, and γ. The residues exhibiting significant identity are boxed. The consensus sequence is indicated by capital and small letters, in decreasing order of conservation. Highlighted Cys residues indicate possible ligands to the β subunit-bound common [4Fe-4S] cluster located in the vicinity of the TPP center. Only the several conserved regions discussed in the text are indicated. Abbreviations: *S.#7*, $\alpha\beta$-type enzyme of *Sulfolobus* sp. strain 7 (this work); *H.s.*, $\alpha_2\beta_2$-type enzyme of *H. salinarium* (*27*); *A.f.*, $\alpha\beta\gamma\delta$-type enzyme of *A. fulgidus* (*14*); *T.m.*, $\alpha\beta\gamma\delta$-type enzyme of *T. maritima* (*15, 22*); *P.f.*, $\alpha\beta\gamma\delta$-type enzyme of *P. furiosus* (*13, 15, 22*); *P.f.VOR*, branched-chain 2-oxoacid-specific $\alpha_2\beta_2\gamma_2\delta_2$-type enzyme of *P. furiosus* (*15, 16*); *H.p.*, $\alpha\beta\gamma\delta$-type enzyme of *H. pylori* (*45*); *K.p.*, α_2-type enzyme of *K. pneumoniae* (*28*); *E.a.*, α_2-type enzyme of *E. agglomerans* 333 (*29*); *R.r.*, α_2-type enzyme of *R. rubrum* (unpublished, EMBL accession number X77515); *A.sp.*, α_2-type enzyme of *Anabaena* sp. PCC 7119 (*30*); *T.v.* α_1 and *T.v.*α_2, two non-identical α_2-type enzymes of *T. vaginalis* (*26*).

salinarium enzyme (*27*) (data not shown). A promoter-like element (box A) (*48*) was found upstream of the open reading frame at positions 246-254, and a putative ribosome-binding sequence (*49*) at positions 297-300. The second open reading frame encodes a 305 amino acid protein of 33,608 Da (positions 2175-3089, 915 bases),

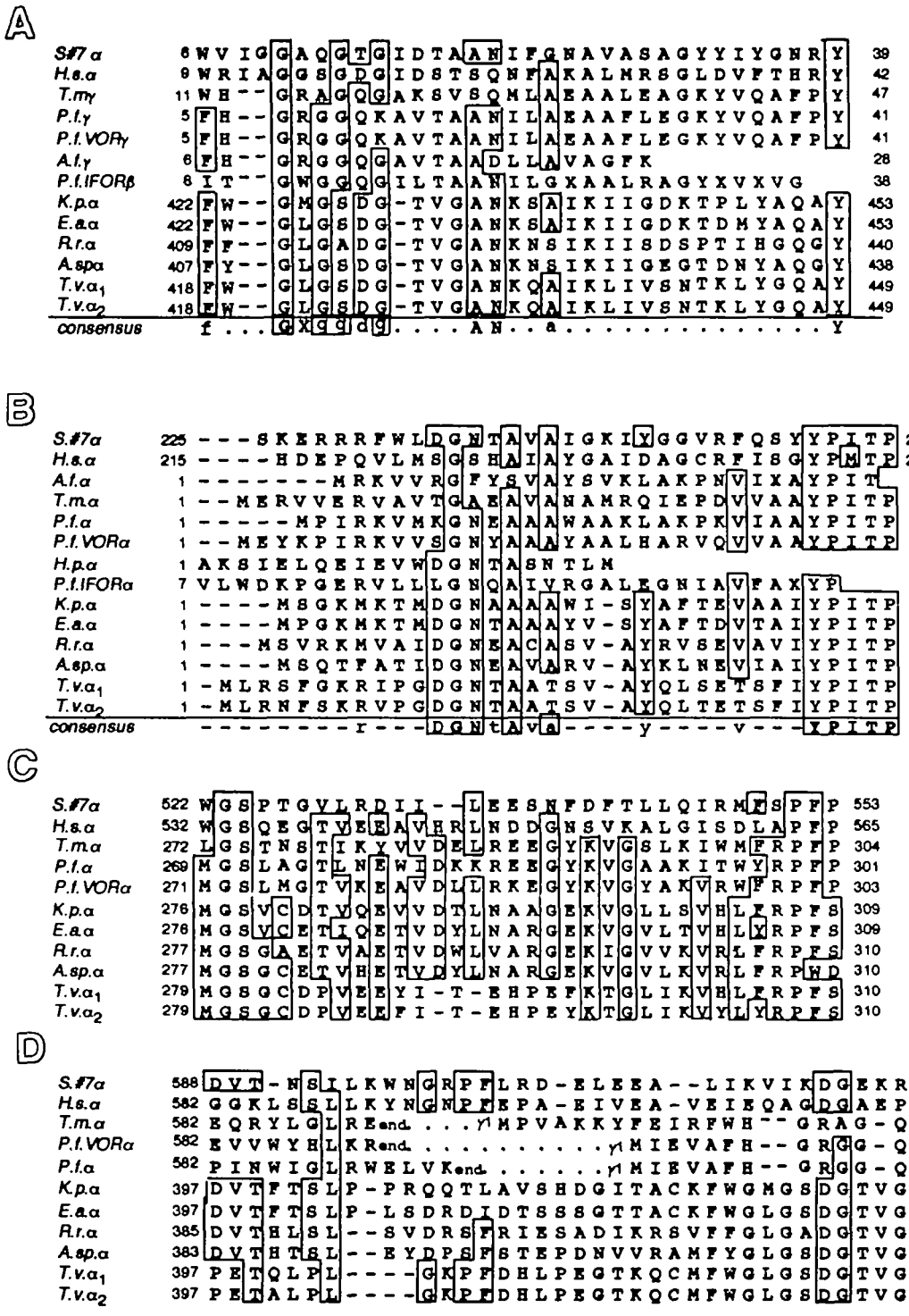

Fig. 6. **Multiple alignment of the amino acid sequence of the α subunit of the $\alpha\beta$-type 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 with the available sequences of homologous regions in $\alpha_2\beta_2$-, $\alpha\beta\gamma\delta$-, and α_2-type enzymes from other sources (only strictly conserved regions are indicated).** Subunits are denoted by α, β, and γ. Note that the arrangement of protein domains differ among different oxidoreductases. The last half of the sequence alignment in panel D overlaps with that in panel A. *P.f.IFORα* and *β* denote the 66-kDa *α* and 23-kDa *β* subunits of indolepyruvate:ferredoxin oxidoreductase from *P. furiosus*, respectively (*4*). The domain arrangement of this enzyme probably differs from those of $\alpha\beta$-/$\alpha_2\beta_2$-type enzymes from aerobic archaea, *Sulfolobus* sp. strain 7 and *H. salinarium* (*cf.* panels A and B; see also Ref. *15*). Other indications and abbreviations are the same as in Fig. 5.

whose deduced amino acid sequence was 38.7% identical to that of the β subunit gene of the *H. salinarium* enzyme (*27*) (data not shown). Because the N-terminal amino acid sequence of the β subunit of the *Sulfolobus* enzyme was blocked, the average mass of the β subunit apoprotein was estimated by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy using 2,5-dihydroxybenzoic acid as a matrix, which gave a $[M+H]^+$ of ~33,566 Da (Iwasaki, T., Oshima, T., and Ikezawa, H., unpublished result). This value is in good agreement, within experimental error, with the deduced molecular mass of the second open reading frame (33,608 Da). Thus, it corresponds to the β subunit gene. At this stage it is not possible to state which kind of modification occurs in the N-terminus of the β subunit. An overlap of upto 26 base pairs was found between the α and β subunit genes. Putative ribosome binding sequence was found at positions 2163-2166, upstream of the open reading frame, and a T-rich terminator like element (*50*) at positions 3114-3127 shortly after the termination codon.

The *H. salinarium* oxidoreductase operon contains a single promoter upstream and a single terminator downstream of the two structural genes, possibly producing a polycistronic RNA for the genes of both subunits (*27*). As shown in Fig. 4, the *Sulfolobus* sp. oxidoreductase genes are organized in a similar manner.

Identification of Possible Ligand Residues to the Redox Centers—The deduced sequence of the N-terminal region of the β subunit of the *Sulfolobus* sp. oxidoreductase showed significant homology to the corresponding regions of the β subunits of the *H. salinarium*, *Pyrococcus furiosus*, *Thermotoga maritima*, and *Archaeoglobus fulgidus* enzymes (*14, 15, 22, 27*) (Fig. 5A). Four strictly conserved Cys residues were found at positions 12, 15, 47, and 197 in the β subunit of the *Sulfolobus* sp. enzyme, of which three were arranged in a conserved motif -CPGCG-//-GCS- (designated as the CPGCG//GCS motif in this paper; Fig. 5A), the fourth one being downstream of this motif (Fig. 5C). Because of the complete absence of any Cys residue in the α subunit of the *Sulfolobus* enzyme (Fig. 4B), these four conserved Cys residues in the β subunit are inevitably responsible for the binding of a [4Fe-4S] cluster without the aid of other subunits (Fig. 5, A and C). Intriguingly, the same Cys arrangement (CPGCG//GCS//C motif) was also found in the central region of the α_2-type enzymes (*e.g.*, around positions 750-1102 in the case of the *K. pneumoniae* enzyme (*28*); Fig. 5, A and C).

In addition to the conserved ligand residues to the [4Fe-4S] cluster, a possible TPP-binding motif, arranged in the sequence -GGDG-//-TXGQXS- (designated as the GGDG motif in this paper) (*51*), was also found in the same regions of the β subunits of the *Sulfolobus* sp. and *H. salinarium* enzymes (*27*), and the α subunit of the *K. pneumoniae* enzyme (*28*), at positions 97-, 104-, and 962-, respectively (Fig. 5B). Thus, the primary structure of the *Sulfolobus* enzyme clearly indicates the spatial proximity of one [4Fe-4S] cluster and one TPP, both located exclusively in the β subunit, which is consistent with the EPR data (Fig. 3). The CPGCG//GCS//C- and GGDG//TXGQXS- motifs are strictly conserved in all types of 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases with different subunit compositions (*i.e.*, the α_2-, $\alpha\beta/\alpha_2\beta_2$-, and $\alpha\beta\gamma\delta$-types; Fig. 5, A-C), implying essentially the same spatial arrangement of the common TPP and [4Fe-4S] centers in the enzymes.

Identification of a Possible CoA-Binding Site—In order to further elucidate the structure-function relationship among 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases with different subunit compositions, the deduced amino acid sequence of the α subunit of the *Sulfolobus* sp. enzyme was carefully aligned with the available sequences of several hyperthermophile and mesophile enzymes (Fig. 6, A-D). We previously found that the N-terminal region of the α subunit of the *Sulfolobus* sp. enzyme was not only homologous to that of the α subunit of the *H. salinarium* enzyme (*27, 52*), but also to the N-terminal portions of the γ subunits of the *A. fulgidus* and *P. furiosus* enzymes (*13, 14, 22, 53*). The same conclusion was recently made by Kletzin and Adams (*15*), who reported the complete amino acid sequences of three $\alpha\beta\gamma\delta$-type 2-oxoacid:ferredoxin

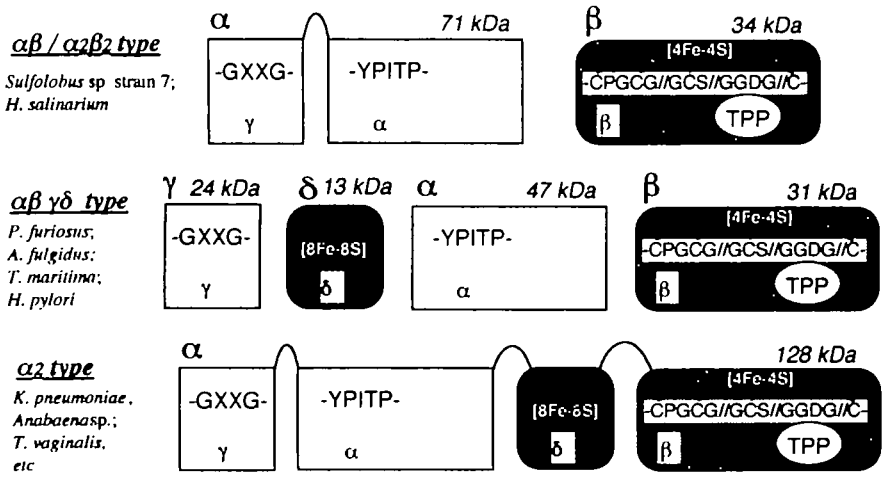

Fig. 7. **The similarities in the protein domain among the 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases, despite the different arrangements.** The structural relations between three representative oxidoreductases [*Sulfolobus* sp. strain 7 enzyme (this work) for $\alpha\beta$-/$\alpha_2\beta_2$-type enzymes from aerobic archaea; *P. furiosus* enzyme (*15*) for $\alpha\beta\gamma\delta$-type enzymes from anaerobic archaea and bacteria; and *K. pneumoniae* enzyme (*28*) for α_2-type enzymes from bacteria and amitochondrial protozoon (*26*)] are schematically illustrated. Each subunit and its apparent size is indicated at the top of each subunit box, in bold greek letters and italicized numbers, respectively. Each domain is designated in small greek letters in each domain box. Four consensus sequence motifs, -GXXG-, -YPITP-, -CPGCG//GCS//C-, and -GGDG- (see the text), are labeled in the figure for convenience. The two common redox centers of the oxidoreductase superfamily (*i.e.*, a TPP cofactor and a [4Fe-4S] cluster) are probably located in the β domain (Fig. 5). The putative CoA-binding site is proposed to be primarily in the γ domain (Fig. 6). Note that the δ domain/subunit potentially carrying two additional [4Fe-4S] clusters in α_2-/$\alpha\beta\gamma\delta$-type enzymes (*15, 26, 28*) (indicated as [8Fe-8S] in the figure) is missing in the *Sulfolobus* sp. enzyme.

oxidoreductases from hyperthermophiles. In Fig. 6A, sequence alignment of the N-terminal regions of domains homologous to the hyperthermophile γ subunits (*15*) is presented, including that of the α subunit of the *Sulfolobus* enzyme. This region, namely the "γ domain," is characterized by several conserved Gly residues, mainly arranged in either a -GXXGXG-, -GXGG-, or -GXGXXG- motif (designated as the GXXG motif; Fig. 6A): This motif is similar to the conserved "adenine recognition loop" identified in the three-dimensional structures of pig heart and *Thermoplasma acidophilum* citrate synthases (*54, 55*), and is most likely the adenine recognition site of 2-oxoacid: ferredoxin (flavodoxin) oxidoreductases for CoA binding. Although several other Gly-rich regions were found in the sequences of the archaeal, bacterial, and eukaryal enzymes at different locations, none of them appeared to be strictly conserved, as opposed to the GXXG motif in Fig. 6A (data not shown). We therefore suggest that the CoA-binding site of the *Sulfolobus* sp. enzyme is located exclusively in the γ domain of the α subunit.

Kletzin and Adams (*15*) suggested the presence of an aldehyde dehydrogenase active-site motif (gElgekNa) in the C-terminal regions of the γ subunits of $\alpha\beta\gamma\delta$-type hyperthermophile enzymes. In the case of the γ domain of the *Sulfolobus* sp. enzyme, the corresponding sequence, -QDLYRMNE- (Fig. 4B), is rather poorly conserved.

Other Conserved Motifs—The central domain of the ~70-kDa α subunit of the *Sulfolobus* sp. enzyme (positions 210-270) showed significant homology with the ~45-kDa α subunits of hyperthermophile and mesophile $\alpha\beta\gamma\delta$-type enzymes (*13-15, 22, 45*), and is called the "α domain." It is also homologous with the N-terminal domains of the ~120-kDa α subunits of mesophile α_2-type enzymes (*26, 28, 30*) (Fig. 6B). One of the strictly conserved stretches found in the α domain is -EQXAVV-//-YP-I(or M)-TP- (designated as the YPITP motif). Several other consensus stretches were also found in this domain, some of which are primarily short clusters composed of hydrophobic amino acid residues (data not shown; *e.g.*, Fig. 3 in Ref. *15*). Although the functions of these consensus stretches remain to be studied, they might have certain regulatory roles in the catalytic cycle of the enzyme.

Evolutionary Implications—Using the several consensus motifs mentioned above as guidelines (Figs. 5 and 6), it can be concluded that 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases with different subunit compositions exhibit remarkable similarities in their protein domains despite different arrangements (schematically illustrated in Fig. 7). Thus, as recently proposed by Kletzin and Adams (*15*), three different types, *viz.*, α_2-, $\alpha\beta/\alpha_2\beta_2$-, and $\alpha\beta\gamma\delta$-type oxidoreductases, appear to be a functionally and structurally related superfamily; each of these protein domains is homologous to a corresponding subunit of $\alpha\beta\gamma\delta$-type enzymes. The primary structure of the *Sulfolobus* enzyme reported in this paper strengthened this proposal (*53*).

Because of the differences in the protein domain arrangements (Fig. 7), we suggest that rearrangement of early genes encoding smaller protein domains (*viz.*, early α, β, γ, and δ genes) and genetic fusion events might have occurred in the course of evolution of the 2-oxoacid:ferredoxin

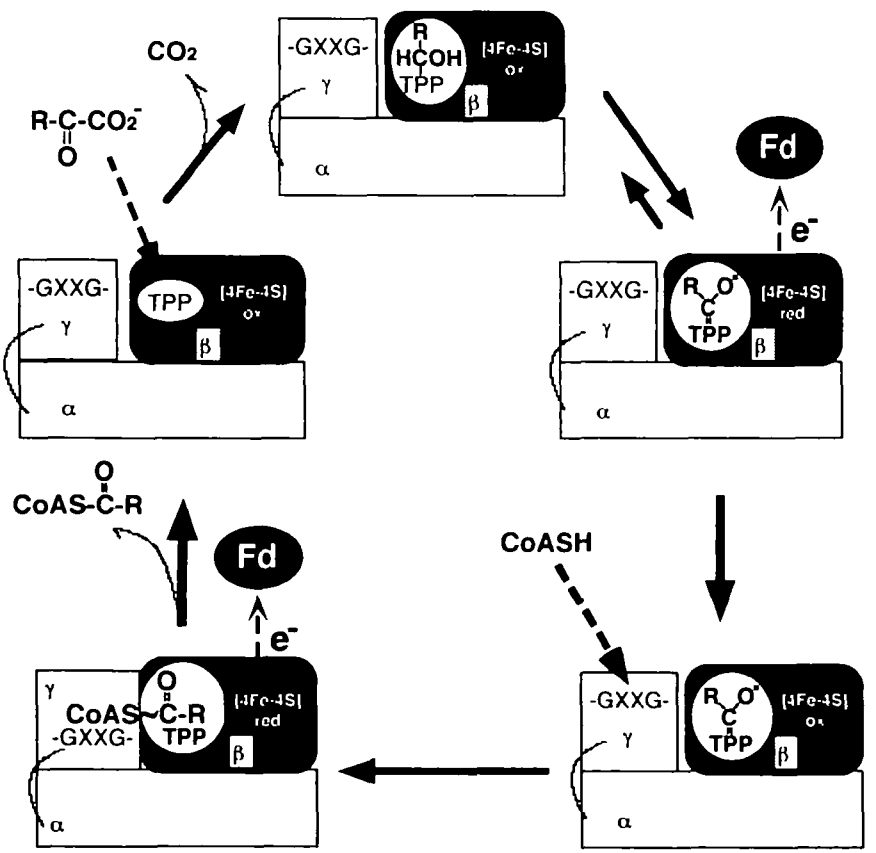

Fig. 8. **Putative catalytic cycle of the $\alpha\beta$-type 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 on the basis of the proposed domain arrangement.** A putative catalytic cycle model of the *Sulfolobus* sp. enzyme is illustrated on the basis of a slightly modified version of the Kerscher-Oesterhelt two-step model (*7, 31*) proposed here (see the text). This model would require one TPP and one [4Fe-4S] cluster as a minimal set of redox centers, but no disulfide groups (*20, 31*). In the β subunit (denoted as β; shaded), both the TPP and [4Fe-4S] centers are bound in close vicinity. In the catalytic cycle, the β subunit-bound common [4Fe-4S] cluster provides reducing equivalent for the cognate 7Fe ferredoxin which probably acts as a single electron carrier (*9*). The α subunit of the enzyme contains the γ and α domains (denoted as γ and α, respectively; bridged white boxes). The γ domain is proposed to have a putative CoA-SH binding site (-GXXG-), which may be in spatial proximity to the β subunit-bound TPP cofactor. The putative CoA-SH binding site is presumably oriented such that no electron transfer from CoA-SH to [4Fe-4S] cluster would occur unless the TPP center is occupied by an acyl group or 2-oxoacid-derived reaction intermediate. The possible function of the α domain is not known, but it is linked to the γ domain in $\alpha\beta$-/$\alpha_2\beta_2$-/α_2-type enzymes (see Fig. 7), implying that it might play a certain regulatory role. In the vicinity of the β subunit-bound common [4Fe-4S] cluster, there is assumed to be a putative ferredoxin docking site in the *Sulfolobus* enzyme, which might be occupied by the δ domain in the cases of α_2-/$\alpha\beta\gamma\delta$-type enzymes (see Fig. 7).

(flavodoxin) oxidoreductase family, in different ways in early aerobic archaea and early anaerobic bacteria: Given the order of the genes in the 2-oxoacid:ferredoxin oxidoreductase operon of the enzymes of "slow-clock" anaerobic hyperthermophiles, *i.e.*, γ-δ-α-β (*15*), it can be postulated that several recombination and fusion events for the early α, γ, δ, and β genes of early $\alpha\beta\gamma\delta$-type enzymes, in that order, might have been required to form a subunit of mesophilic α_2-type enzymes (see Fig. 7). In the cases of the $\alpha\beta/\alpha_2\beta_2$-type enzymes from "fast-clock" aerobic respiratory archaea, loss of the early δ gene, and the subsequent fusion of the early γ and α genes of early $\alpha\beta\gamma\delta$-type enzymes, in that order, might have given rise to an α subunit (see Fig. 7). It could be argued in this connection that the enzymes present in the hydrogenosomes of amitochondrial protozoa (*25*) might be more closely related to and presumably derived from those of early anaerobic and mesophilic bacteria, rather than those of early archaea.

Thus, the molecular evolution of the 2-oxoacid:ferredoxin (flavodoxin) oxidoreductase superfamily clearly demonstrates a unique evolutionary event for the enzymes in fast-clock aerobic archaea, which also have a unique membrane-bound aerobic respiratory chain (*37-39*). The most notable feature of the $\alpha\beta$-/$\alpha_2\beta_2$-type enzymes from fast-clock aerobic archaea is the absence of a δ subunit homologue; this domain carries two sets of typical -$CX_2CX_2CX_3CP$- stretches characteristic of [4Fe-4S] (or [3Fe-4S]) cluster-binding motifs (*15*), and is present not only in the $\alpha\beta\gamma\delta$-type enzymes from slow-clock anaerobic hyperthermophiles (*14, 22*), but also in all the α_2-type enzymes from anaerobic bacteria and an amitochondrial protozoon reported so far (*26, 28, 30*). As opposed to the cases of the latter enzymes potentially carrying as many as three FeS clusters, the $\alpha\beta$-/$\alpha_2\beta_2$-type enzymes from fast-clock aerobic archaea might have evolved to carry only a single [4Fe-4S] cluster per protomer, and presumably thereby acquired resistance toward aerobic conditions. In this connection, it is intriguing that all the enzymes from slow-clock anaerobic archaea obtained so far seem to retain the δ subunit or a homologue of it (*15*); even an $\alpha_2\beta_2$-type indolepyruvate-specific enzyme of *P. furiosus* (*4*) probably has a δ subunit homologue, and its domain arrangement may be different from those of the $\alpha\beta/\alpha_2\beta_2$-type enzymes of fast-clock aerobic archaea (*27* and this work; *cf.* Fig. 6, A and B).

Possible Common Catalytic Mechanism of 2-Oxoacid: Ferredoxin (Flavodoxin) Oxidoreductases—In spite of considerable variations in the numbers of subunits and FeS clusters among 2-oxoacid:ferredoxin (flavodoxin) oxidoreductases from different sources, as emphasized in recent studies (Table I), the results presented here and those recently reported by others (*15*) clearly demonstrate that they are in fact a functionally and structurally related superfamily (Fig. 7). The simplest catalytic scheme for this enzyme superfamily would therefore require only one TPP and one [4Fe-4S] cluster as a minimal set of redox centers, and should be interpreted by the Kerscher-Oesterhelt two-step model (*7, 31*), or the slightly modified version of it proposed here (schematically illustrated in Fig. 8): In the initial step, the binding of 2-oxoacid to the TPP cofactor located in the β subunit causes decarboxylation of the 2-oxoacid and intramolecular electron transfer to the conserved [4Fe-4S] cluster bound to the β subunit in the vicinity of the TPP cofactor (Fig. 3), resulting in the formation of an activated hydroxyalkyl-TPP radical and the reduction of one ferredoxin (or possibly oxygen); in the subsequent step, CoA binds to the γ domain of the α subunit, and upon release of the acyl group from the stable radical intermediate, a second ferredoxin is reduced *via* intramolecular electron transfer through the [4Fe-4S] cluster located in the β subunit. Because the addition of CoA alone did not reduce the [4Fe-4S] cluster in the β subunit, we suggest that the reduction of ferredoxin in the second step should occur prior to release of the final product, acyl-CoA, from the enzyme, to complete the catalytic cycle (Fig. 8).

In order to satisfy this modified model, the putative CoA binding site in the γ domain of the α subunit should be in proximity to the TPP cofactor in the β subunit (*i.e.*, 2-oxoacid binding site), but distantly located from the [4Fe-4S] cluster in the β subunit, which is in the vicinity of the TPP cofactor and possibly near the electron acceptor site (*i.e.*, ferredoxin binding site) (Fig. 8). Considering the significant sequence similarity, we propose this spatial arrangement in the protein molecule to be one of the fundamental structures of this enzyme superfamily. On the other hand, the two additional [4Fe-4S] clusters located in the δ domain/subunit of α_2-/$\alpha\beta\gamma\delta$-type enzymes (*15*) (Fig. 7) are *absent* in the $\alpha\beta$-type enzyme of *Sulfolobus* sp. strain 7 (Fig. 4). We therefore suggest that these additional redox centers are functionally not essential for this enzyme superfamily; rather, they may be intramolecular electron transmitters from the β domain-bound common [4Fe-4S] center to the putative electron acceptor site, presumably functioning as an electron sink in the catalytic cycle of $\alpha\beta\gamma\delta$-/α_2-type enzymes. The results of EPR analyses of several $\alpha\beta\gamma\delta$-type hyperthermophile enzymes (*16, 32*) seem to be in line with this proposal.

The present paper reported the purification, characterization, cloning and sequencing of the $\alpha\beta$-type 2-oxoacid: ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7, providing some new structural and functional insights into this enzyme superfamily. This new information will be useful for further studies involving protein engineering techniques.

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REFERENCES

1. Mukund, S. and Adams, M.W.W. (1991) The novel tungsten-iron-sulfur-protein of the hyperthermophilic archaebacterium, *Pyrococcus furiosus*, is an aldehyde ferredoxin oxidoreductase: Evidence for its participation in a unique glycolytic pathway. *J. Biol. Chem.* **266**, 14208-14216
2. Adams, M.W.W. (1993) Enzymes and proteins from organisms that grow near and above 100°C. *Annu. Rev. Microbiol.* **47**, 627-658
3. Schicho, R.N., Ma, K., Adams, M.W.W., and Kelly, R.M. (1993) Bioenergetics of sulfur reduction in the hyperthermophilic archaeon, *Pyrococcus furiosus*. *J. Bacteriol.* **175**, 1823-1830
4. Mai, X. and Adams, M.W.W. (1994) Indolepyruvate ferredoxin

oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. A new enzyme involved in peptide fermentation. *J. Biol. Chem.* **269**, 16726-16732
5. Adams, M.W.W. (1994) Biochemical diversity among sulfur-dependent hyperthermophilic microorganism. *FEMS Microbiol. Rev.* **15**, 261-277
6. Kerscher, L., Nowitzki, S., and Oesterhelt, D. (1982) Thermoacidophilic archaebacteria contain bacterial-type ferredoxins acting electron acceptors of 2-oxoacid:ferredoxin oxidoreductases. *Eur. J. Biochem.* **128**, 223-230
7. Kerscher, L. and Oesterhelt, D. (1982) Pyruvate:ferredoxin oxidoreductase: New findings on an ancient enzyme. *Trends Biochem. Sci.* **7**, 371-374
8. Danson, M.J. (1989) Central metabolism of the archaebacterium: An overview. *Can. J. Microbiol.* **35**, 58-64
9. Iwasaki, T., Wakagi, T., Isogai, Y., Tanaka, K., Iizuka, T., and Oshima, T. (1994) Functional and evolutionary implications of a [3Fe-4S] cluster of the dicluster-type ferredoxin from the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7. *J. Biol. Chem.* **269**, 29444-29450
10. Iwasaki, T., Wakagi, T., and Oshima, T. (1995) Ferredoxin-dependent redox system of a thermoacidophilic archaeon, *Sulfolobus* sp. strain 7: Purification and characterization of a novel reduced-ferredoxin-reoxidizing iron-sulfur flavoprotein. *J. Biol. Chem.* **270**, 17878-17883
11. Koike, M. and Koike, K. (1976) Structure, assembly and function of mammalian α-keto acid dehydrogenase complexes. *Adv. Biophys.* **9**, 187-227
12. Kerscher, L. and Oesterhelt, D. (1981) Purification and properties of two 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. *Eur. J. Biochem.* **116**, 587-594
13. Blamey, J.M. and Adams, M.W.W. (1993) Purification and characterization of pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Biochim. Biophys. Acta* **1161**, 19-27
14. Kunow, J., Linder, D., and Thauer, R.K. (1995) Pyruvate: ferredoxin oxidoreductase from the sulfate-reducing *Archaeoglobus fulgidus*: Molecular composition, catalytic properties, and sequence alignments. *Arch. Microbiol.* **163**, 21-28
15. Kletzin, A. and Adams, M.W.W. (1996) Molecular and phylogenetic characterization of pyruvate and 2-ketoisovalerate ferredoxin oxidoreductases from *Pyrococcus furiosus* and pyruvate ferredoxin oxidoreductase from *Thermotoga maritima*. *J. Bacteriol.* **178**, 248-257
16. Heider, J., Mai, X., and Adams, M.W.W. (1996) Characterization of 2-ketoisovalerate ferredoxin oxidoreductase, a new and reversible coenzyme A-dependent enzyme involved in peptide fermentation by hyperthermophilic archaea. *J. Bacteriol.* **178**, 780-787
17. Uyeda, K. and Rabinowitz, J.C. (1971) Pyruvate-ferredoxin oxidoreductase. III. Purification and properties of the enzyme. *J. Biol. Chem.* **246**, 3111-3119
18. Gehring, U. and Arnon, D.I. (1972) Purification and properties of α-ketoglutarate synthase from a photosynthetic bacterium. *J. Biol. Chem.* **247**, 6963-6969
19. Shah, V.K., Stacey, G., and Brill, W.J. (1983) Electron transport to nitrogenase: Purification and characterization of pyruvate: flavodoxin oxidoreductase, the *nifJ* gene product. *J. Biol. Chem.* **258**, 12064-12068
20. Wahl, R.C. and Orme-Johnson, W.H. (1987) Closteridial pyruvate oxidoreductase and the pyruvate-oxidizing enzyme specific to nitrogen fixation in *Klebsiella pneumoniae* are similar enzymes. *J. Biol. Chem.* **262**, 10489-10496
21. Meinecke, B., Bertram, J., and Gottschalk, G. (1989) Purification and characterization of the pyruvate-ferredoxin oxidoreductase from *Clostridium acetobutylicum*. *Arch. Microbiol.* **152**, 244-250
22. Blamey, J.M. and Adams, M.W.W. (1994) Characterization of an ancestral type of pyruvate ferredoxin oxidoreductase from the hyperthermophilic bacterium, *Thermotoga maritima*. *Biochemistry* **33**, 1000-1007
23. Brostedt, E. and Nordlund, S. (1991) Purification and partial characterization of a pyruvate oxidoreductase from the photosynthetic bacterium *Rhodospirillum rubrum* grown under nitrogen-fixing conditions. *Biochem. J.* **279**, 155-158
24. Williams, K., Lowe, P.N., and Leadlay, P.F. (1987) Purification and characterization of pyruvate:ferredoxin oxidoreductase from the anaerobic protozoon *Trichomonas vaginalis*. *Biochem. J.* **246**, 529-536
25. Müller, M. (1988) Energy metabolism of protozoa without mitochondria. *Annu. Rev. Microbiol.* **42**, 465-488
26. Hrdy, I. and Müller, M. (1995) Primary structure and eubacterial relationships of the pyruvate:ferredoxin oxidoreductase of the amitochondriate eukaryote *Trichomonas vaginalis*. *J. Mol. Evol.* **41**, 388-396
27. Plaga, W., Lottspeich, F., and Oesterhelt, D. (1992) Improved purification, crystallization and primary structure of pyruvate: ferredoxin oxidoreductase from *Halobacterium halobium*. *Eur. J. Biochem.* **205**, 391-397
28. Arnold, W., Rump, A., Klipp, W., Priefer, U.B., and Pühler, A. (1988) Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of *Klebsiella pneumoniae*. *J. Mol. Biol.* **203**, 715-738
29. Kreutzer, R., Dayananda, S., and Klingmuller, W. (1989) Identification and characterization of the *nifH* and *nifJ* promoter regions located on the *nif*-plasmid pEA3 of *Enterobacter agglomerans* 333. *Gene* **78**, 101-109
30. Schmitz, O., Kentemich, T., Zimmer, W., Hundeshagen, B., and Bothe, H. (1993) Identification of the *nifJ* gene coding for pyruvate:ferredoxin oxidoreductase in dinitrogen-fixing cyanobacteria. *Arch. Microbiol.* **160**, 62-67
31. Kerscher, L. and Oesterhelt, D. (1981) The catalytic mechanism of 2-oxoacid:ferredoxin oxidoreductases from *Halobacterium halobium*. *Eur. J. Biochem.* **116**, 595-600
32. Smith, E., Blamey, J.M., and Adams, M.W.W. (1994) Pyruvate ferredoxin oxidoreductases of the hyperthermophilic archaeon, *Pyrococcus furiosus*, and the hyperthermophilic bacterium, *Thermotoga maritima*, have different catalytic mechanisms. *Biochemistry* **33**, 1008-1016
33. Wakagi, T. and Oshima, T. (1986) Membrane bound ATPase and electron transport system of *Sulfolobus acidocaldarius*. *Syst. Appl. Microbiol.* **7**, 342-345
34. Wakao, H., Wakagi, T., and Oshima, T. (1987) Purification and properties of NADH dehydrogenase from a thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. *J. Biochem.* **102**, 255-262
35. Denda, K., Konishi, J., Hajiro, K., Oshima, T., Date, T., and Yoshida, M. (1990) Structure of an ATPase operon of an acidothermophilic archaebacterium, *Sulfolobus acidocaldarius*. *J. Biol. Chem.* **265**, 21509-21513
36. Iwasaki, T., Isogai, T., Iizuka, T., and Oshima, T. (1995) Sulredoxin: A novel iron-sulfur protein of the thermoacidophilic archaeon *Sulfolobus* sp. strain 7 with a Rieske-type [2Fe-2S] cluster. *J. Bacteriol.* **177**, 2576-2582
37. Iwasaki, T., Matsuura, K., and Oshima, T. (1995) Resolution of the aerobic respiratory system of the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7: I. The archaeal terminal oxidase supercomplex is a functional fusion of respiratory complexes III and IV with no *c*-type cytochromes. *J. Biol. Chem.* **270**, 30881-30892
38. Iwasaki, T., Wakagi, T., Isogai, Y., Iizuka, T., and Oshima, T. (1995) Resolution of the aerobic respiratory system of the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7: II. Characterization of the archaeal terminal oxidase subcomplexes and implication for the intramolecular electron transfer. *J. Biol. Chem.* **270**, 30893-30901
39. Iwasaki, T., Wakagi, T., and Oshima, T. (1995) Resolution of the aerobic respiratory system of the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7: III. The archaeal novel respiratory complex II (succinate:caldariellaquinone oxidoreductase complex) inherently lacks heme group. *J. Biol. Chem.* **270**, 30902-30908
40. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
41. Ausubel, F.M., Brent, R., Lingston, R.E., Moore, D.D., Seidman,

J.G., and Struhl, K. (1987) *Current Protocols in Molecular Biology*, Wiley, New York
42. Zanetti, G., Aliverti, A., and Curti, B. (1984) A cross-linked complex between ferredoxin and ferredoxin-$NADP^+$ reductase. *J. Biol. Chem.* **259**, 6153-6157
43. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
44. Ortiz, M.L., Calero, M., Patron, C.F., Castellanos, L., and Mendez, E. (1992) Imidazole-SDS-Zn reverse staining of proteins in gels containing or not SDS and microsequence of individual unmodified electroblotted proteins. *FEBS Lett.* **296**, 300-304
45. Hughes, N.J., Chalk, P.A., Clayton, C.L., and Kelly, D.J. (1995) Identification of a novel four-subunit pyruvate:flavodoxin oxidoreductase from *Helicobacter pylori*. *J. Bacteriol.* **177**, 3953-3959
46. Cammack, R., Kerscher, L., and Oesterhelt, D. (1980) A stable free radical intermediate in the reaction of 2-oxoacid:ferredoxin oxidoreductases of *Halobacterium halobium*. *FEBS Lett.* **118**, 271-273
47. Muller, Y.A. and Schulz, G.E. (1993) Structure of the thiamine- and flavin-dependent enzyme pyruvate oxidase. *Science* **259**, 965-967
48. Reiter, W.-D., Palm, P., and Zillig, W. (1988) Transcriptional termination in the archaebacterium *Sulfolobus*: Signal structures and linkage to transcription initiation. *Nucleic Acids Res.* **16**, 2445-2459
49. Olsen, G.J., Pace, N.R., Nuell, M., Kaine, B.P., Gupta, R., and Woese, C.R. (1985) Sequence of the 16S rRNA gene from the thermoacidophilic archaebacterium *Sulfolobus solfataricus* and its evolutionary implications. *J. Mol. Evol.* **22**, 301-307
50. Wich, G., Hummel, H., Jarsch, M., Bar, U., and Bock, A. (1986) Transcription signals for stable RNA genes in *Methanococcus*. *Nucleic Acids Res.* **14**, 2459-2479
51. Hawkins, C.F., Borges, A., and Perham, R.N. (1989) A common structural motif in thiamin pyrophosphate-binding enzymes. *FEBS Lett.* **255**, 77-82
52. Iwasaki, T., Wakagi, T., and Oshima, T. (1993) The ferredoxin-dependent redox system of the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7. *International Workshop on Molecular Biology and Biotechnology of Extremophiles and Archaebacteria*, pp. 69-70, Wako, Japan (abstr.)
53. Zhang, Q., Wakagi, T., Iwasaki, T., and Oshima, T. (1995) Molecular structure, function, and evolution of 2-oxoacid:ferredoxin oxidoreductase from the thermoacidophilic archaeon, *Sulfolobus* sp. strain 7. *The 18th Annual Meeting of the Japanese Society for Molecular Biology* (in Japanese), p. 208 (abstr.)
54. Remington, S., Wiegand, G., and Huber, R. (1982) Crystallographic refinement and atomic models of two different forms of citrate synthase at 2.7 and 1.7 Å resolution. *J. Mol. Biol.* **158**, 111-152
55. Russell, R.J., Hough, D.W., Danson, M.J., and Taylor, G.L. (1994) The crystal structure of citrate synthase from the thermophilic archaeon, *Thermoplasma acidophilum*. *Structure* **2**, 1157-1167