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Studies on Electron Transport System of Hyperthermophilic Archaeon, *Pyrobaculum islandicum*

A DISSERTATION
submitted in partial satisfaction of
the requirements of the degree
DOCTOR OF PHILOSOPHY (Ph. D)

by

Yasuhiro Nakajima

DEPARTMENT OF BIOSCIENCE
TOKYO INSTITUTE OF TECHNOLOGY

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Abbreviations

2OAFOR	2-oxoacid:ferredoxin oxidoreductase
2OGDH	2-oxoglutarate dehydrogenase
ATP	adenosine 5'-triphosphate
BV	benzyl viologen
CAPS	*N*-cyclohexyl-3-aminopropanesulfonic acid
CM-	carboxymethyl-
CoA	coenzyme A
DAD	2,3,5,6,-tetramethyl-*p*-phenylenediamine
DEAE-	diethylaminoethyl-
EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance
Fd	ferredoxin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAPFOR	glyceraldehyde-3-phosphate:ferredoxin oxidoreductase
H_2ase	hydrogenase
HPLC	high performance liquid chromatography
HOQNO	2-heptyl-4-hydroxyquinoline-*N*-oxide
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NADH	nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PDH	pyruvate dehydrogenase
PMS	phenazine methosulfate
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
SM	sucrose monocaplate
SR	sulfur reductase
SuDH	sulfide reductase
TFA	trifluoro acetic acid
Tris	tris (hydroxymethyl) aminomethane

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CHAPTER 1

General Introduction

Until 1970s, the Archaea were not recognized as a discrete and identifiable grouping. In 1977, Woese *et. al.* proposed the existence of a new Super-Kingdom of Archaea (1) (Fig. 1-1). These organisms can be conveniently divided according to their extreme environmental niche, into three broad phenotypes, the thermophiles, methanogens and extreme halophiles.

When "Life" emerged between 4.0 and 3.5 billion years ago, Earth's environments resembled the anaerobic, high-temperature conditions that today are found at submarine thermal vents. In the microbial world, many intriguing bacteria exist under such extreme environments. Some of the extremophiles grow in geothermally heated, high-temperature (as high as 120°C!) environments. These hyperthermophiles belong to the domain both Archaea and Bacteria.

The hyperthermophiles have ferredoxin-dependent pyruvate dehydrogenase (2,3) which is highly homologous to NAD^+-dependent pyruvate dehydrogenase multienzyme complexes of various aerobic bacteria and eukarya (Fig. 1-2). Furthermore, 2-oxoacid:ferredoxin oxidoreductase which has a broad substrate specificity toward 2-oxoacids such as 2-oxoglutarate, 2-oxobutyrate and pyruvate has been purified from thermophilic archaeon, *Sulfolobus* sp. strain 7 (4). Therefore, ferredoxin-dependent 2-oxoacid dehydrogenase might be earlier type than those of mitochondria and most of aerobic bacteria.

Reduced NADH is then reoxidized by NADH dehydrogenase in the case of most of aerobic bacteria and eukarya. Reduced ferredoxin in fermentative hyperthermophiles like as *Pyrococcus furiosus* is used for electron donor for fermentative electron transfer pathway using the sulfur compounds as terminal electron acceptor (5-9) and all enzymes participated in ferredoxin-dependent fermentative electron transfer pathway are located at cytoplasm or periplasmic fraction and have no proton pumping activity.

On the other hand, a facultative chemoautotrophic archaeon, *Pyrobaculum islandicum*, is considered to produce ATP by anaerobic respiratory chain from H_2 to elemental sulfur (10). Under chemolithotrophical growth condition, the archaeon utilizes H_2 and sulfur

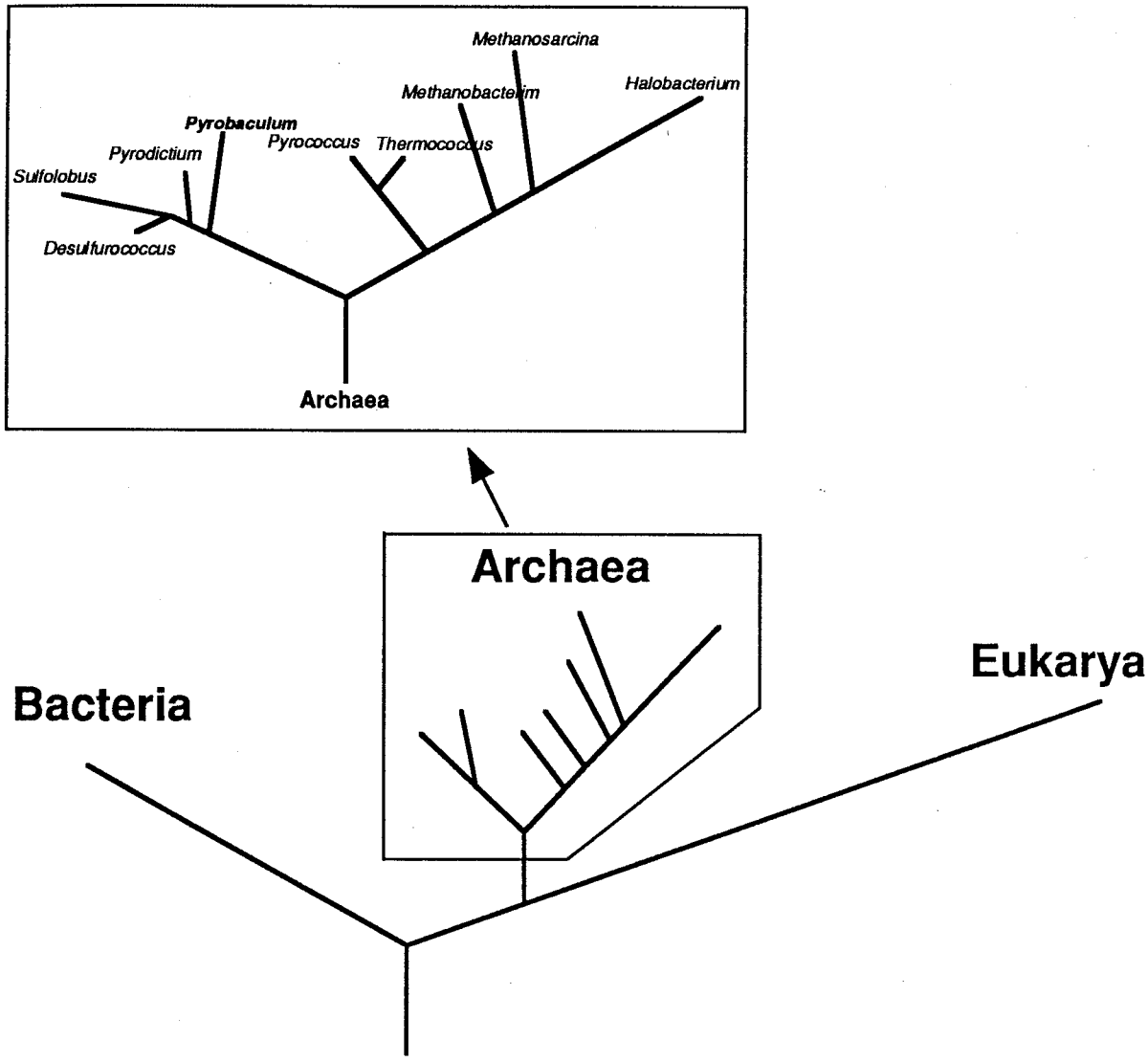

Fig. 1-1. Three different domains of life in a rooted phylogenetic tree proposed by Woese *et al.* (11). *P. islandicum* belongs to the *Archaea* domain.

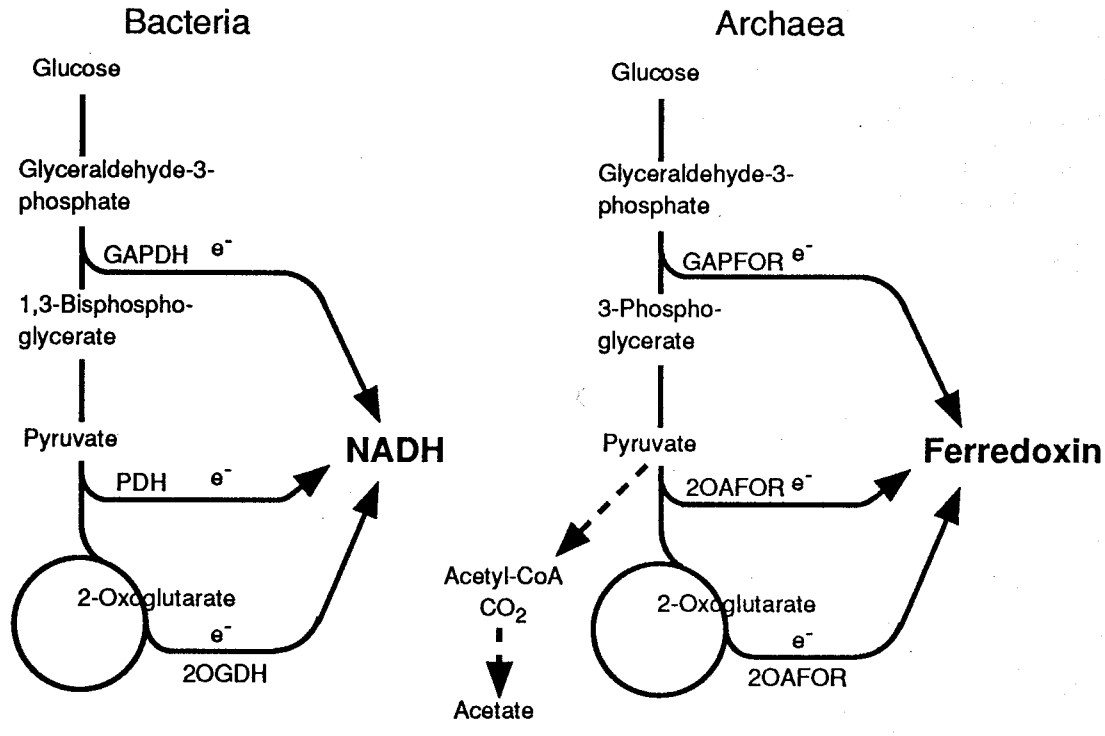

Fig. 1-2. Comparison of Bacterial glycolytic (Embden-Meyerhof) pathway and Archaeal glycolytic pathway.

compound like elemental sulfur as respiratory substrate and terminal electron acceptor, respectively (10). So the archaeon must has the membrane-bound respiratory enzymes with proton pumping activity in the respiratory chain. Furthremore, the archaeon can produce H_2S under heterotrophic growth conditions (10). Therefore, the archaeon seems to have both ferredoxin-dependent electron transfer pathway and H_2-dependent electron transfer pathway which utilize sulfur compound as terminal electron acceptor.

In the present study, I first purified ferredoxin from the archaeon to an electrophoretically homogeneous state and investigated its molecular properties. I then analyzed the amino acid sequence and compared it with those of several other bacterial-type ferredoxins. Further, I have identified *b*-type cytochromes in the membrane and partially purified the membrane-bound cytochrome *b*-559. Finally I proposed the anaerobic respiratory chain for *P. islandicum.*

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CHAPTER 2

Purification and Characterization of [3Fe-4S][4Fe-4S] Type Ferredoxin from Hyperthermophilic Archaeon *Pyrobaculum islandicum*

Introduction

The hyperthermophilic archaeon *Pyrococcus furiosus* has many kinds of ferredoxin-related enzymes such as aldehyde ferredoxin oxidoreductase (1), formaldehyde ferredoxin oxidoreductase (2,3), glyceraldehyde-3-phosphate ferredoxin oxidoreductase (4), sulfide dehydrogenase and sulfhydrogenase (5-8), indicating that ferredoxin is essentially participated in various metabolic pathways. On the other hand, the ferredoxin from the thermoacidophilic archaeon *Sulfolobus* sp. strain 7 serves as an intermediate electron transfer protein between the coenzyme A-acylating 2-oxoacid:ferredoxin oxidoreductase and a novel iron-sulfur flavoprotein (9,10). Recently, Fujii *et al.* have determined the 2.0 Å resolution crystal structure of the ferredoxin from *Sulfolobus* sp. strain 7 (11,12), and showed that zinc atom is tightly coordinated by three histidine residues in the N-terminal extension region and one aspartate residue in core fold. Furthermore, Iwasaki *et al.* have suggested that the consensus sequence as possible ligands to the novel zinc center in the N-terminal extension region is characteristic of thermoacidophilic archaeon (13).

As described above, the ferredoxin is thought to be a key electron transfer protein in Archaeon. On the other hand, the hyperthermophilic archaeon, *Pyrobaculum islandicum*, is a facultative chemoautotrophic archaeon and produces ATP by anaerobic respiration (14). Recently, Seilig *et al.* have reported that all of the enzymatic activities involved in the citric acid cycle are present in *P. islandicum* (15). Furthermore, the archaeon can utilize thiosulfate and elemental sulfur as terminal electron acceptors and thereby produce CO_2 and H_2S under heterotrophic growth conditions (16). Therefore, the archaeon seems to have metabolic pathways for oxidizing the organic compounds to CO_2 in the presence of elemental sulfur or thiosulfate, suggesting that the ferredoxin plays an essential role as an electron mediator between acetyl-CoA oxidation and elemental sulfur reduction or thiosulfate reduction much like the role of the ferredoxin of *P. furiosus* in glucose

fermentation.

In the present study, I purified ferredoxin from the archaeon to an electrophoretically homogeneous state and investigated its molecular properties. I then analyzed the amino acid sequence and compared it with those of several other bacterial-type ferredoxins.

Materials and Methods

Bacterium and growth condition

Pyrobaculum islandicum (DSM 4184) was cultivated anaerobically at 95 °C for 2 days in a chemically defined medium (16) with slight modifications. The ingredients in the basal medium are summarized in Table 1-1. Sodium thiosulfate was added to the medium as an electron acceptor. The cells were harvested by centrifugation at 10,000 x *g* for 15 min and stored at -80 °C until use.

Physical measurements

Spectrophotometric measurements were performed with a Shimadzu MPS-2000 spectrophotometer and an Hitachi 220A with 1-cm light path cuvettes. The metal content was determined with an inductively coupled Plasma Spectrometer SPS 1500 VR (Seiko Instruments, Tokyo, Japan) after the sample was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA for 18 h. Electron paramagnetic resonance measurements were carried out using a Bruker ESP-300E spectrometer or a JEOL JEX-RE1X spectrometer equipped with an Air Products model LTR-3 Heli-Tran cryostat system. MALDI-TOF mass spectrometry was performed by a PerSeptive Biosystems Voyager RP instrument at an accelerating potential of 25 kV, using 3,5-dimethoxy-4-hydroxycinammic acid.

SDS-PAGE was performed by the method of Schägger *et al.* (17). The sample for SDS-PAGE was denatured by heating at 100 °C with 1% (W/V) SDS and 1% (v/v) ß-mercaptoethanol for 30 min. The molecular weight of the native protein was determined by gel filtration on a Sephadex G-75 column (2.4 x 115 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl. The column was calibrated with the following standard proteins: trypsin inhibitor (M_r = 21,500), horse heart cytochrome *c* (M_r = 12,375), and lysozyme (M_r = 14,300) (lysozyme behaves as a protein species with a molecular weight of 5,000 (18)).

Protein content was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as a standard.

Enzyme Assay

P. islandicum 2-oxoacid:ferredoxin oxidoreductase activity was measured at 70 °C by the method of Zhang *et al.* (10), using *P. islandicum* ferredoxin as

Table. 1. Composition of the growth medium for cultivation of *Pyrobuculum islandicum.*

Na_2S	0.5	g
Yeast extract(Difco)	0.2	g
Peptone(Difco)	0.5	g
Elemental sulfur(powder)	5.0	g
or		
Sodium thiosulfate	1.0	g
or		
Sodium sulfite	1.0	g
Basal mineral mixture		
(Macro elements)*	20.0	ml
(Micro elements)**	1.0	ml
distilled water	979	ml

*Basal mineral mixture macro elements

(per 1000 ml of distilled water)

$(NH_4)_2SO_4$	66.0	g
KH_2PO_4	13.6	g
$MgSO_4$	12.3	g
$CaCl_2$	3.68	g
H_2SO_4	4.9	g
distilled water	1.0	l

**Basal mineral mixture micro elements

(per 1000 ml of distilled water)

$Fe_2(SO_4)_3$	28.6	g
$Na_2WO_4 \cdot 2H_2O$	1.79	g
$MnSO_4$	1.37	g
H_3BO_3	2.82	g
$ZnSO_4$	0.22	g
$CuSO_4$	87	mg
Na_2MoO_4	25.2	mg
$VOSO_4$	32	mg
distilled water	1.0	l

an intermediate electron acceptor. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 2 mM 2-oxoglutarate or pyruvate, 50 μM CoA , 18.8 μM horse cytochrome *c* and 0.3 μM *P. islandicum* ferredoxin. The reaction was started by addition of enzyme to the reaction mixture. Although the reduction of horse cytochrome *c* was followed with time by measuring the increase of the absorbance at 550 nm, the activity could not be measured at over 70 °C , because of the denaturation of horse cytochrome *c* at high temperature.

Determination of amino acid sequence

The chemical digestion of ferredoxin, which was carboxymethylated according to Crestfield *et al.* (19), was performed with formic acid as follows: about 130 μg CM- Fd was incubated in 2% (v/v) formic acid for 2 h at 108 °C by the method of Inglis (20). Enzymatic digestions by trypsin or V8-protease were performed by treating about 130 μg each of CM-Fd with 10 μg *Staphylococcus aureus* V8-protease or 10 μg TPCK-trypsin, respectively, in 50 mM NH_4HCO_3 containing 2 mM EDTA for 24 h at 37 °C. Enzymatic digestion by proline specific endopeptidase was performed by treating about 130 μg of CM-Fd with 10 μg proline specific endopeptidase in 50 mM sodium phosphate buffer, pH 7.0, for 4 h at 37 °C. These digested ferredoxins were lyophilized and fractionated by reverse-phase HPLC on a Cosmosil 5C18 column (4.6 X 250 mm) in 0.1% (v/v) trifluoroacetic acid (TFA) with a linear gradient from 0 to 50% (v/v) acetonitrile. The peptides in the eluate were monitored by the absorbance at 230 nm.

The deblocking of the modified N-terminal amino acid residue as *N*-acetylserine or *N*-acetylthreonine was performed by the method described in Wellner *et al.* (21) with some modifications, the ferredoxin in the SDS-PAGE gel was transferred onto the PVDF membrane filter at room temperature by the method of Towbin *et al.* (22), using 0.5% (w/v) SDS. After the filter was stained with Coomassie Brilliant Blue R-250, the ferredoxin band was cut down and incubated in gas phase anhydrous trifluoroacetic acid (TFA) for 1 h at 60 °C. To deblock pyroglutamate, *P. furiosus* pyroglutamate aminopeptidase was used.

The amino acid sequence of ferredoxin was determined by a gas-phase protein sequencer (Applied Biosystems, model 470A, USA) equipped with an on-line PTH-analyzer (Applied Biosystems, model 120A, USA) and a gas-phase protein sequencer (SHIMADZU, PPSQ-21, Japan) equipped with a

UV-VIS DETECTOR (SHIMADZU, SPD-10A, Japan) and LIQUID CHROMATOGRAPHY (LC-10AS).

Phylogenetic Tree Analysis

The phylogenetic trees were constructed by the parsimony and neighbor-joining methods using the sequence interpretation tool, CLUSTALW (http://www.genome.ad.jp/SIT/SIT.html). All amino acid sequence data of ferredoxins used for phylogenetic calculations in this study were obtained from the GenEMBL, PIR, and SWISS-PROT data banks, except for the ferredoxin from *P. islandicum.*

Reagents

CM-cellulose and Sephadex G-75 were purchased from Pharmacia Fine Chemicals (Sweden). DEAE-Toyopearl was purchased from the Tosoh Corporation (Japan). The molecular weight markers for SDS-PAGE were purchased from Fluka AG (Switzerland). *P. furiosus* pyroglutamate aminopeptidase was purchased from Takara Biomedicals (Japan). Proline specific endopeptidase was purchased from Seikagaku Corporation (Japan). TPCK-trypsin was purchased from Worthington Biochemical (USA). *Staphylococcus aureus* V8-protease was purchased from the Wako Pure Chemical Co. (Japan).

Results and Discussion

Purification of Ferredoxin from P. islandicum

Frozen cells (about 20 g wet weight) harvested from about 150 l culture with sodium thiosulfate were suspended in 270 ml of 10 mM Tris-HCl buffer, pH 8.0, and disrupted with a French pressure cell (1,100 kg/cm^2). All subsequent steps were performed under aerobic conditions at room temperature. Unbroken cells were removed by centrifugation at 10,000 x *g* for 15 min. The resulting supernatant was used as cell-free extract. The cell-free extract was further centrifuged at 104,000 x *g* for 1 h, and the supernatant obtained was charged on a DEAE-Toyopearl column (2.6 x 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0. After the column was washed with 200 ml of the same buffer, ferredoxin was eluted with a linear gradient produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0, and the buffer containing 0.4 M NaCl. The eluates which contained ferredoxin were combined and concentrated by pressure filtration on an Amicon YM-3 membrane under N_2 gas at 4 °C. The concentrated fraction was subjected to gel filtration on a Sephadex G-75 column (2.4 x 115 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl. To the eluates which contained ferredoxin, ammonium sulfate was added to give a final concentration of 2.06 M, and the suspension was stirred for 30 min. After the resulting solution was centrifuged at 10,000 x *g* for 15 min, the supernatant was charged on a CM-cellulose column (1.0 x 3 cm) equilibrated with the 10 mM Tris-HCl buffer, pH 8.0, containing 2.06 M ammonium sulfate. Ferredoxin was eluted with a linear gradient produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0, containing 2.06 M ammonium sulfate and the buffer containing 1.35 M ammonium sulfate. The eluates were used as the purified ferredoxin preparation. These purification steps are summarized in Figure 2-1. About 0.83 mg of purified ferredoxin was obtained from about 20 g (wet weight) cells.

Characterization of P. islandicum Ferredoxin

Figure 2-2 shows Tricine-SDS PAGE of the purified ferredoxin. When the sample was incubated at 100 °C for 30 min in the presence of 1% SDS plus 1% ß-mercaptoethanol before being subjected to the electrophoresis, one major band was observed in the gel. The molecular weight of ferredoxin was estimated to be 11,500 by SDS-PAGE. Furthermore, the molecular weight of

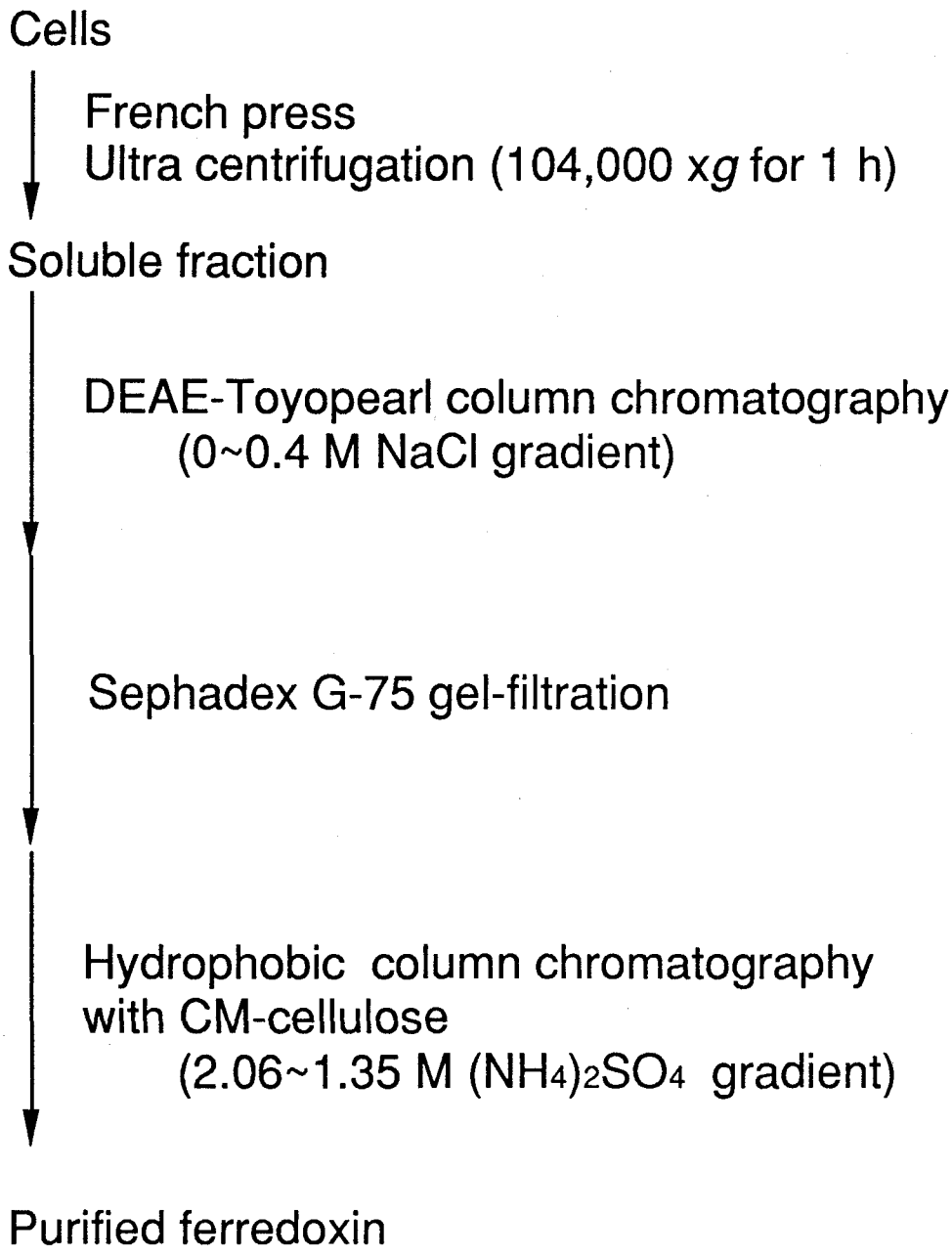

Fig. 2-1. Purification scheme of ferredoxin from *P. islandicum*.

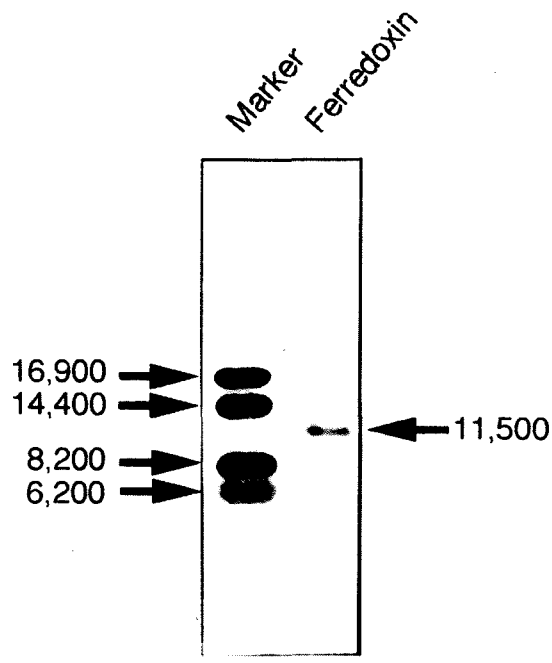

Fig. 2-2. SDS-polyacrylamide gel electrophoresis of *P. islandicum* ferredoxin. The gel was stained with Coomassie Brilliant Blue R-250. The sample was treated at 100 °C for 30 min with 1% SDS plus 1% ß-mercaptoethanol. Marker proteins: Myoglobin (*M*r=16,900), Myoglobin I/II (*M*r=14,400), Myoglobin I (*M*r=8,200), Myoglobin II (*M*r=6,200).

P. islandicum ferredoxin was estimated to be 11,500 by gel filtration analysis and the MALDI-TOF mass spectrometry of the denatured apoprotein by heating at 100 °C with 1% (W/V) SDS and 1% (v/v) ß-mercaptoethanol gave an average mass of 11,910±50 (Fig. 2-3). These results shows that *P. islandicum* ferredoxin exists as a monomeric form in an aqueous solution.

The metal contents of *P. islandicum* ferredoxin were determined with an inductively coupled Plasma Spectrometer. The ferredoxin contained 32.4 μg non-heme irons per mg protein, indicating that it has 7 iron atoms per mol of the protein. Zn, Mo, Ni, Mn and Cu were scarcely detected in the purified preparation.

Figure 2-4 shows the absorption spectra of *P. islandicum* ferredoxin. This ferredoxin showed an absorption peak at 282 nm and broad peak at around 400 nm in the air-oxidized form (Fig. 2-4(A)) and the spectra were not changed by incubation for 2 h at 90 °C. Upon addition of excess solid $Na_2S_2O_4$ to the ferredoxin solution, the broad peak at around 400 nm was 31% decreased in 20 mM sodium phosphate buffer (pH 7.0)(Fig. 2-4(B)). Because further addition of $Na_2S_2O_4$ did not induce spectral changes at around 400 nm, the ferredoxin seems to have been held in the partially-reduced state under the experimental conditions. However, when the ferredoxin was suspended in 600 mM CAPS buffer, pH 9.3, it was more highly reduced with $Na_2S_2O_4$ (Fig. 2-4(C)). Therefore, it seems likely that *P. islandicum* ferredoxin has an [Fe-S] cluster with a pH-dependent and unusual low redox potential.

Figure 2-5 shows the EPR spectra of *P. islandicum* ferredoxin. When the ferredoxin was suspended in 600 mM CAPS buffer, pH 9.3, the oxidized form showed a sharp g=2.03 signal at 12.5 K(Fig. 2-5(A)). The lineshape of the signal is, in general, similar to those observed from proteins containing an oxidized $[3Fe\text{-}4S]^{1+}$ center (23-26). The signal with g=2.03 was also observed in the oxidized form at pH 7.0 (Fig. 2-5 (C)). Reduction of the ferredoxin by excess solid $Na_2S_2O_4$ at pH 9.3 decreased the g=2.03 signal and produced a broad rhombic EPR spectrum with g=2.08, 1.94 and 1.89 signals (Fig. 2-5 (B)). These signals are similar to those of the reduced $[4Fe\text{-}4S]^{1+}$ cluster (26,27). No signals derived from oxidized $[3Fe\text{-}4S]^{1+}$ or reduced $[4Fe\text{-}4S]^{1+}$ cluster were detected at pH 7.0 (data not shown). Therefore, it seems likely that *P. islandicum* ferredoxin has each one of [3Fe-4S] cluster and [4Fe-4S] cluster in the monomer and the redox potential of [4Fe-4S] cluster is lower than that of [3Fe-4S] cluster.

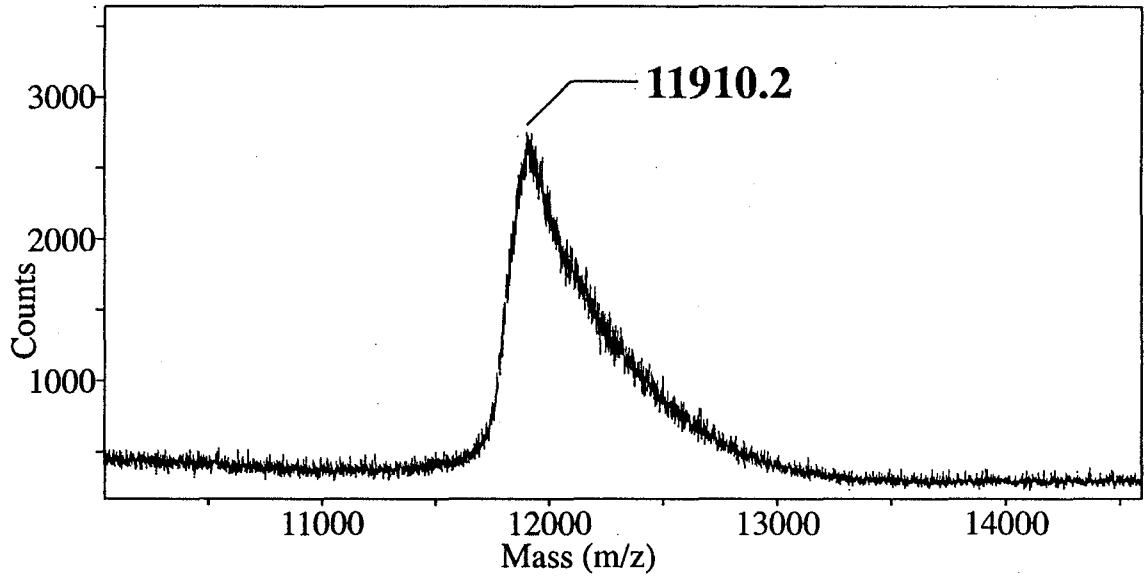

Fig. 2- 3. MALDI-TOF mass spectrum of *P. islandicum* ferredoxin. 3,5-Dimethoxy-4-hydroxycinammic acid was used as matrix. The sample was bombarded by laser beam at an acceleration voltage of 25 kV.

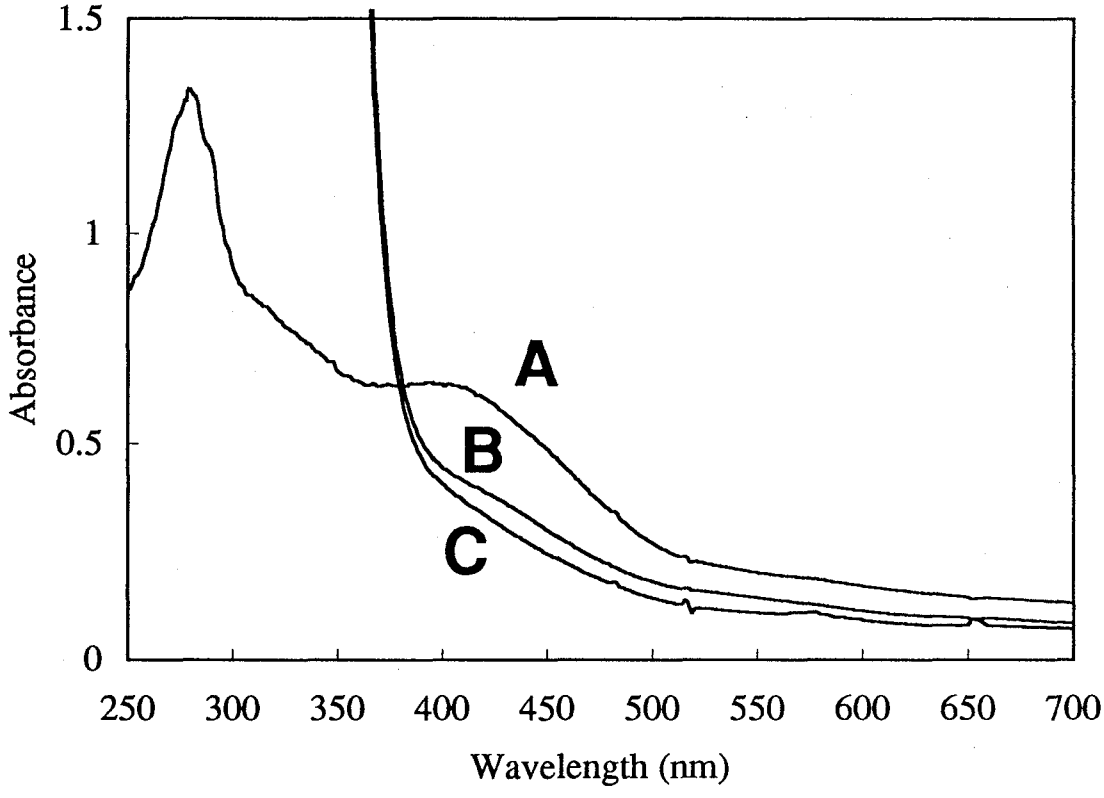

Fig. 2-4. Absorption spectra of *P. islandicum* ferredoxin. The ferredoxin (0.256 mg/ml) was dissolved in 20 mM sodium phosphate buffer, pH 7.0 (A,B) or 600 mM CAPS buffer, pH 9.3 (C). A, air-oxidized form; B and C, the reduced form prepared by the addition of excess amounts of $Na_2S_2O_4$.

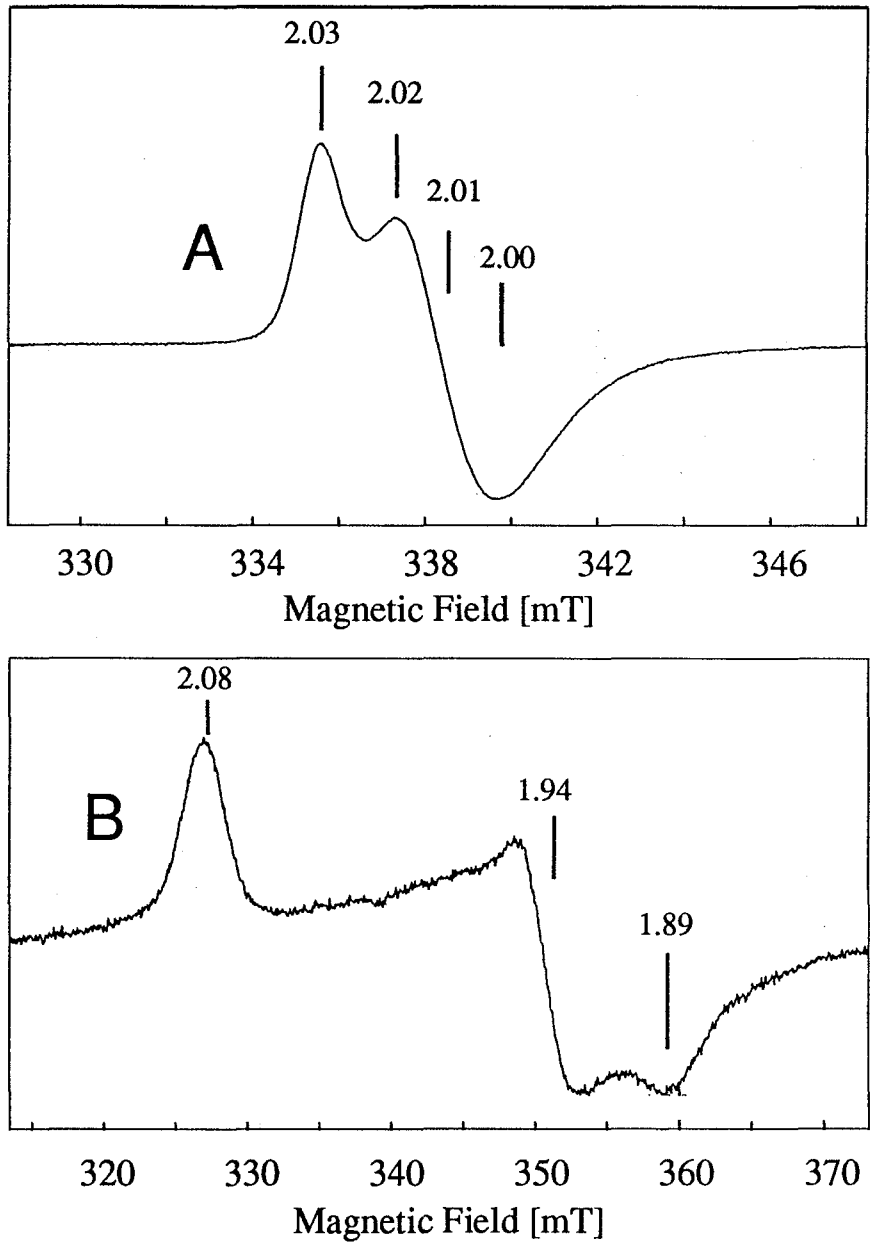

Fig. 2-5. Low temperature EPR spectra of *P. islandicum* ferredoxin in the air-oxidized (A,C) and the dithionite-reduced (B). The sample was dissolved in 600 mM CAPS buffer, pH 9.3 (A,B) or 20 mM sodium phosphate buffer, pH 7.0 (C). Instrument settings for the EPR spectroscopy: temperature, 12.5 K (A), 12.7 K (B); 10.0 K (C); microwave power, 0.51 mW (A), 1.0 mW (B,C); modulation amplitude, 0.31 mT; the g values are indicated.

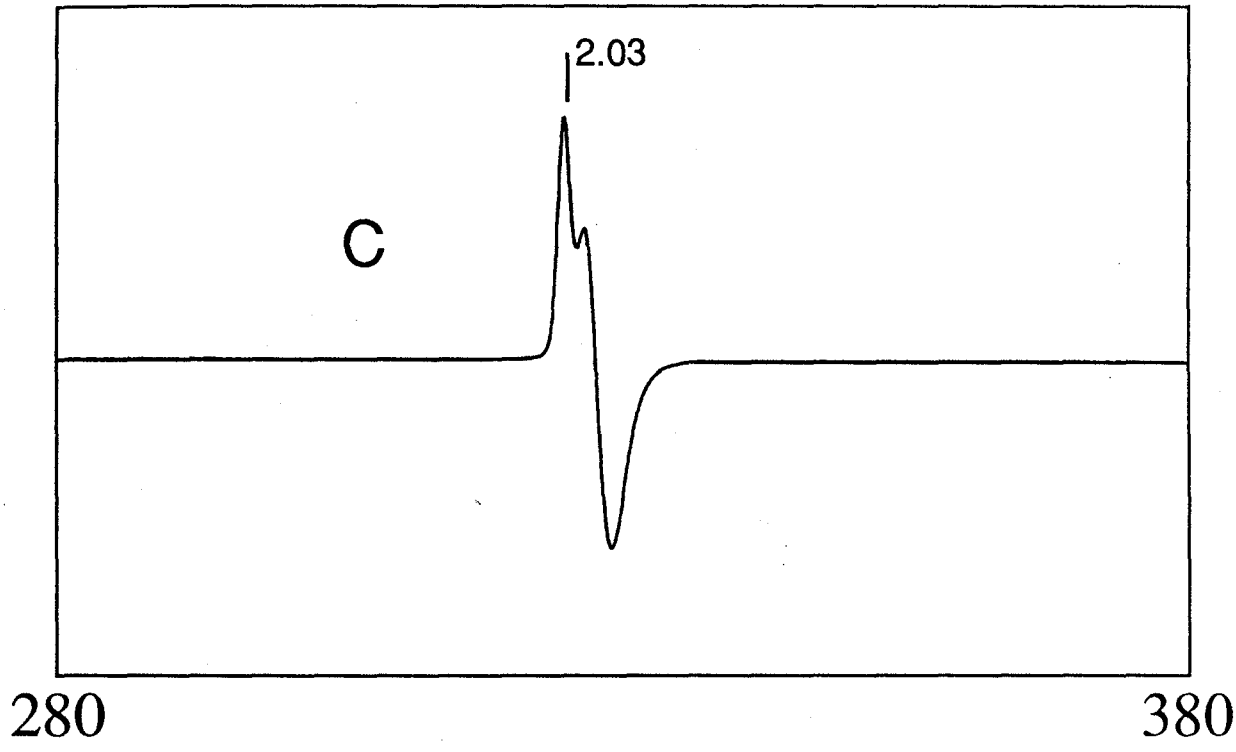

Fig. 2-5.-*Continued.*

Partial Purification of 2-Oxoacid: Ferredoxin Oxidoreductase from P. islandicum

Archaea such *P. furiosus* and *Sulfolobus* sp. strain 7 utilize ferredoxins as electron acceptors for pyruvate: ferredoxin oxidoreductase and 2-oxoacid: ferredoxin oxidoreductase (1,28-30). Recently, Zhang *et al.* have purified 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 and proposed that the enzyme physiologically participates in the ferredoxin-dependent redox system (10). In the present study, I have tried to purify 2-oxoacid: ferredoxin oxidoreductase from *P. islandicum* and reconstitute the electron transfer between *P. islandicum* ferredoxin and 2-oxoglutarate.

Soluble fraction prepared from *P. islandicum* showed 2-oxoglutarate and pyruvate dependent cytochrome *c* reductase activities at 70 °C with 25.0 and 38.6 mU/mg protein, respectively. The activity was not detected in membrane fraction. Figure 2-6 shows the effects of oxygen on the 2-oxoglutarate: ferredoxin oxidoreductase activity of the soluble fraction. The half time for denaturation of the enzyme ($t_{50\%}$) was about 3.7 h under aerobic condition at room temperature. Therefore, the 2-oxoacid:ferredoxin oxidoreductase has been purified from *P. islandicum* under anaerobic conditions.

Frozen cells were suspended in 10 mM Tris-HCl buffer, pH 8.0 which had been degassed and flushed with pure N_2, and disrupted at 4 °C with a French pressure cell at 1,100 kg/cm^2. After the unbroken cells were removed by centrifugation at 10,000 x *g* for 15 min, the supernatant obtained was degassed and flushed with N_2, and further centrifuged at 104,000 x *g* for 1 h. The supernatant which had been degassed and flushed with N_2 was charged on a DEAE-Toyopearl column (2.6 x 12 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0 in the presence of N_2. After the column had been washed with 200 ml of the same buffer, 2-oxoacid:ferredoxin oxidoreductase was eluted with a linear gradient produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0 and the buffer containing 0.4 M NaCl (Fig. 2-7). Both 2-oxoglutarate:ferredoxin oxidoreductase activity and pyruvate:ferredoxin oxidoreductase activity were eluted at the same NaCl concentration from a DEAE-Toyopearl column. The eluates with 2-oxoacid: ferredoxin oxidoreductase activity were concentrated by ultrafiltration in a Amicon unit (centriflo CF25) and stored under N_2 gas at -80 °C.

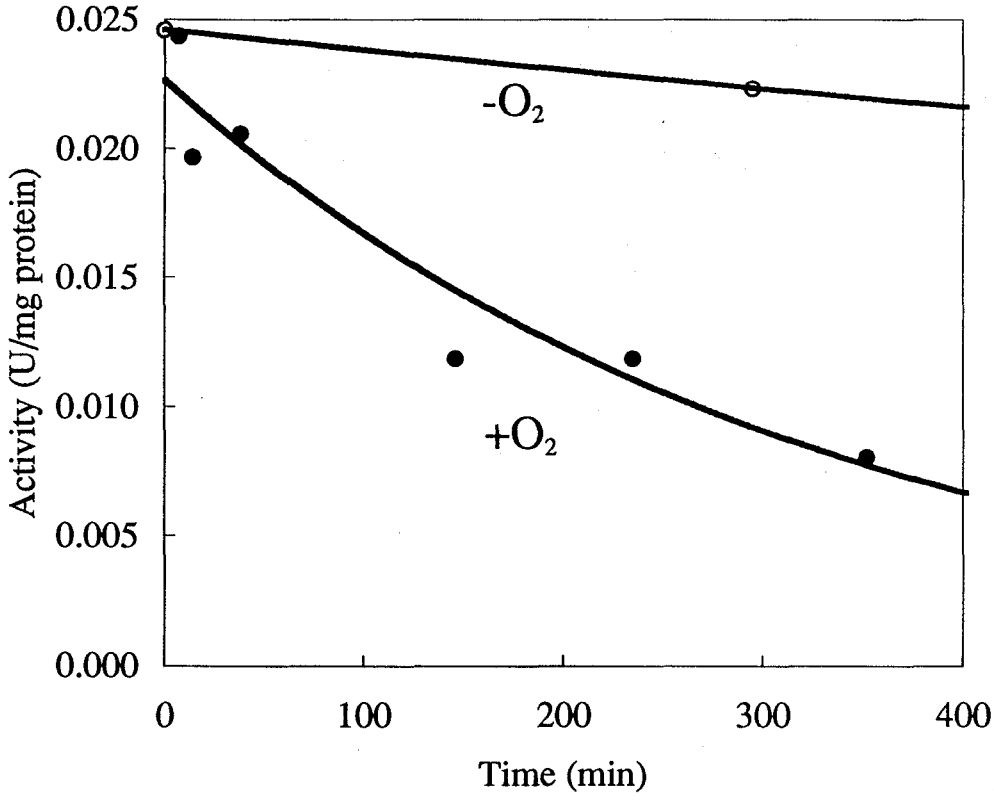

Fig. 2-6. Stability of 2-oxoglutarate:ferredoxin oxidoreductase of *P. islandicum* under aerobic condition. Soluble fraction was incubated under aerobic (*close circle*) or under 100% Ar atmosphere as a gas phase (*open circle*) at room temperature.

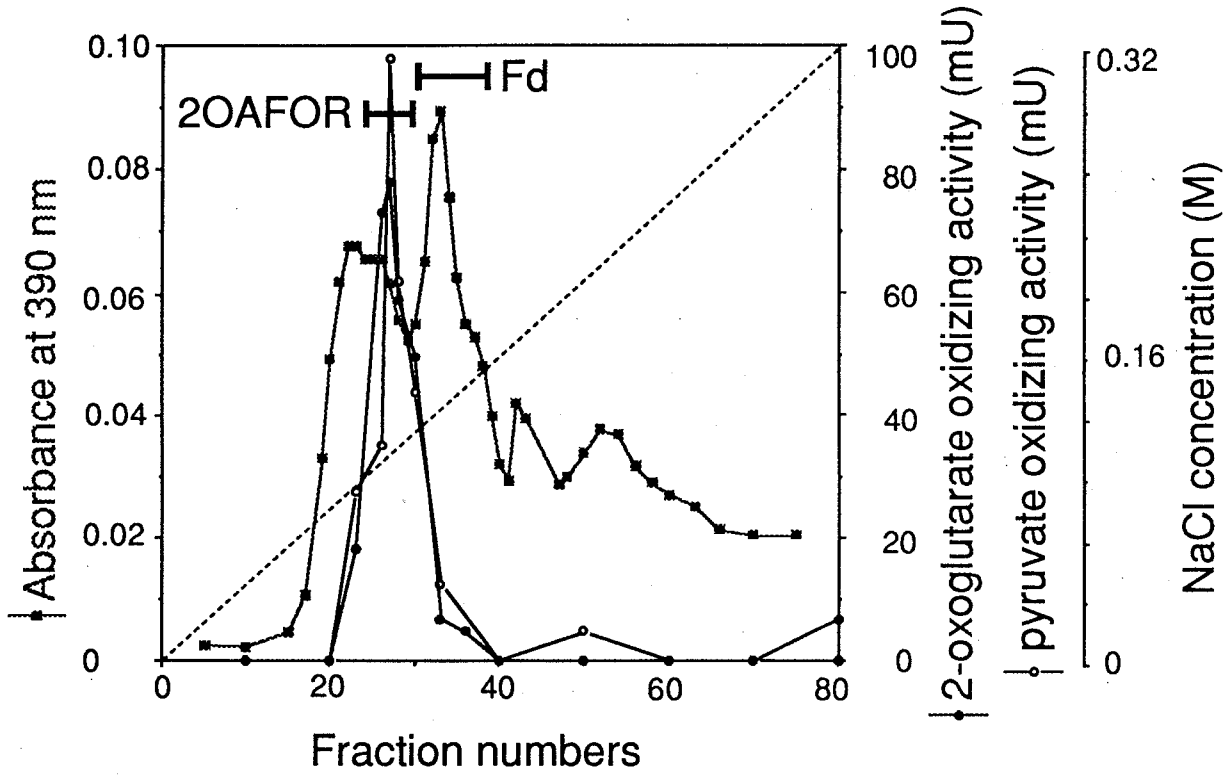

Fig. 2-7. DEAE-Toyopearl elution profiles of ferredoxin and 2-oxoacid: ferredoxin oxidoreductase. The elution of ferredoxin was monitored with absorbance at 390 nm. 2-Oxoglutarate: ferredoxin oxidoreductase activity, pyruvate: ferredoxin oxidoreductase activity and ferredoxin were indicated by *filled circle*, *open circle* and *filled box*, respectively.

Reconstitution of the Ferredoxin-dependent Redox System of P. islandicum

Figure 2-8 shows the increase of absorbance at 550 nm of horse ferrocytochrome *c* in the presence of *P. islandicum* ferredoxin and 2-oxoacid:ferredoxin oxidoreductase. The cytochrome *c* was about 3-fold rapidly reduced with the enzyme in the presence of ferredoxin than in its absence. The occurrence of the enzymatic activity in the absence of ferredoxin may be caused by the impurity of the enzyme preparation.

As described above, the absorption spectra and EPR spectra indicated that *P. islandicum* ferredoxin has [3Fe-4S] cluster and [4Fe-4S] cluster in the monomer. In the case of [3Fe-4S][4Fe-4S] type ferredoxin of *Sulfolobus* sp. strain 7, only the [3Fe-4S] cluster is reduced by 2-oxoacid:ferredoxin oxidoreductase, while extremely low potential [4Fe-4S] cluster remains in the oxidized state (25). Indeed, the redox potential of [4Fe-4S] cluster of *Sulfolobus* sp. strain 7 ferredoxin (-530 mV) is lower than that of (CO_2 and succinylCoA) / (2-oxoglutarate and CoA) (-468 mV). Similar observations have been reported for *Clostridium pasteurianum* and *Clostridium acidiurici* 2[4Fe-4S] type dicluster ferredoxins, both of which have been partially reduced with hydrogen and hydrogenase (31,32). Thus, in some of the dicluster-type ferredoxins, only one Fe-S cluster is used as a single electron carrier *in vivo*. Therefore, it seems likely that one of iron-sulfur clusters of *P. islandicum* ferredoxin participates in the reaction with *P. islandicum* 2-oxoacid:ferredoxin oxidoreductase.

Amino acid Sequence of P. islandicum Ferredoxin

After the chemical digestion of the ferredoxin with formic acid as described in Materials and Methods, two peptides, F-3 and F-10, were obtained in pure states by fractionation on a Cosmosil 5C18 column (Fig. 2-9(A)). Peptide F-3 was sequenced up to residue 11. *Staphylococcus aureus* V8-protease-cleaved peptides (V-5, V-18, V-25, and V-27), TPCK-trypsin-cleaved peptides (T-6, T-7, T-12, and T-32), and proline specific endopeptidase cleaved peptide (P-11) were obtained in pure state by fractionations on a Cosmosil 5C18 column (Fig. 2-9(B)-(D)) and completely sequenced by the method as described in Materials and Methods.

Recently, Hulmes *et al.* have reported that polypeptides are selectively cleavaged with TFA (33). In the present study, the ferredoxin was treated with TFA to deblock *N*-acetylserine or *N*-acetylthreonine as described in

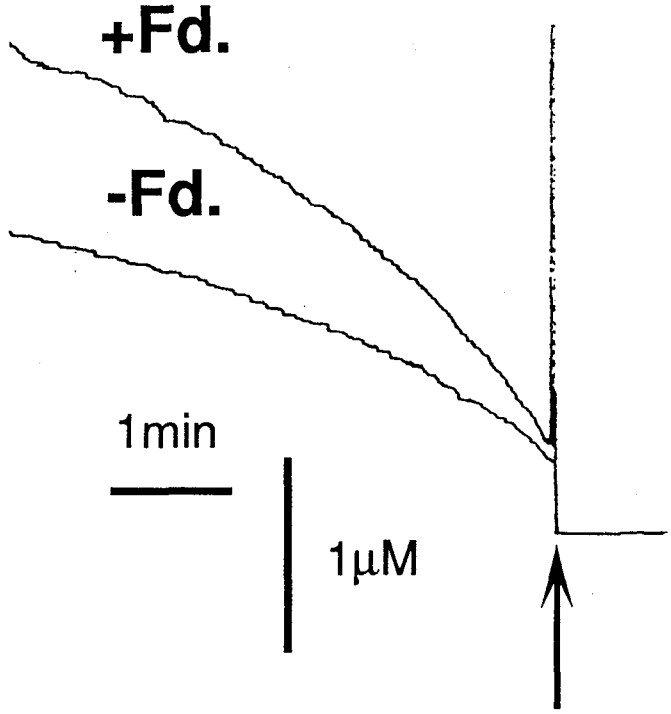

Fig. 2-8. Activation of electron transfer from 2-oxoglutarate to cytochrome *c* with 2-oxoglutarate:ferredoxin oxidoreductase by *P. islandicum* ferredoxin. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.5, 2 mM 2-oxoglutarate, 50 μM CoA and 18.8 μM horse cytochrome *c* and 0.3 μM *P. islandicum* ferredoxin. The reduction of horse heart cytochrome *c* was spectrophotometrically followed with time at 70 °C as described in Materials and Methods. The reaction was started by addition of 2-oxoglutarate:ferredoxin oxidoreductase indicated by the arrow.

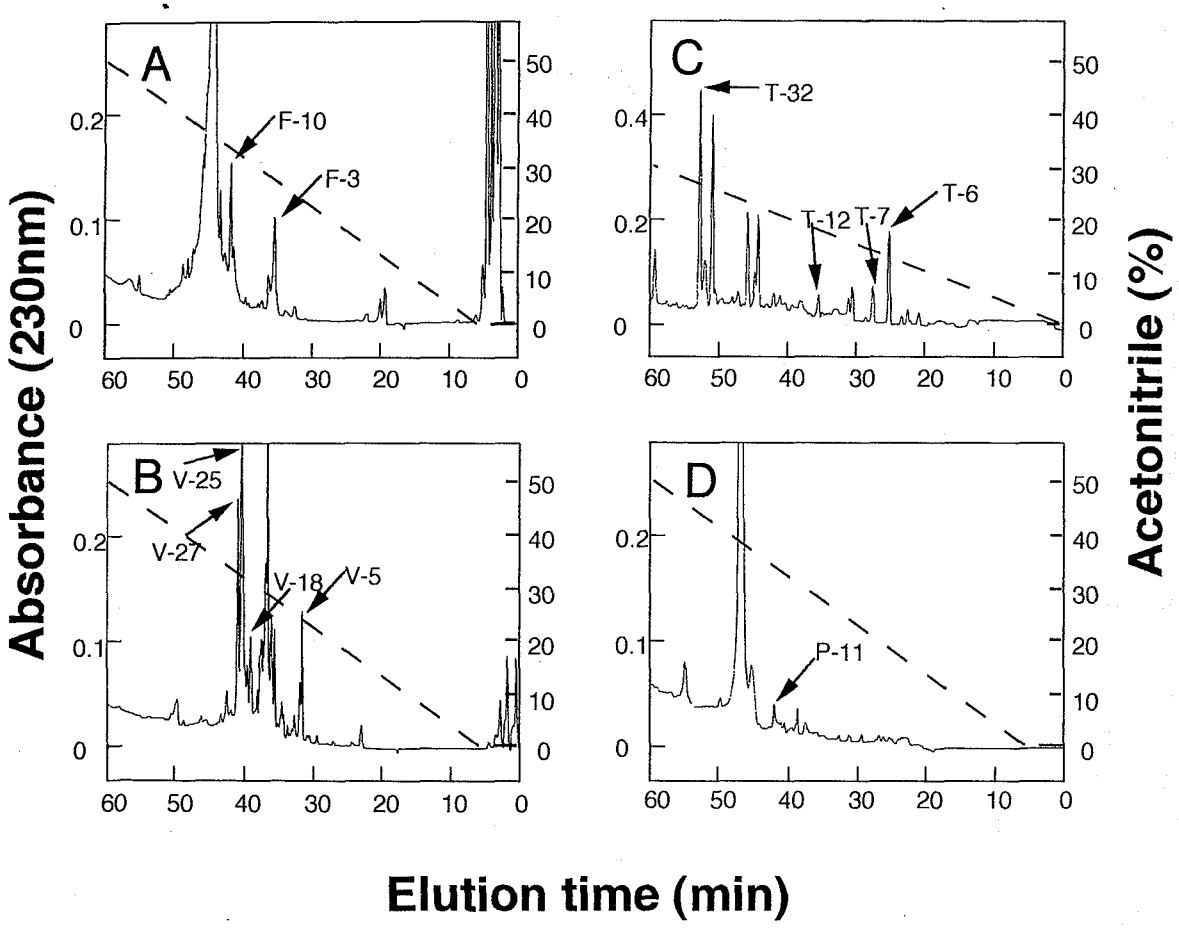

Fig. 2-9. Separation of peptides from digested of *P. islandicum* ferredoxin. The digests were chromatographed by HPLC under the conditions as described in Materials and Methods. Dashed line is concentration of acetonitrile. (A) formic acid digests; (B) *Staphylococcus* V8-protease digests; (C) trypsin digests; (D) proline specific endopeptidase digests

Materials and Methods. After TFA treatment, I could have one TFA-polypeptide and determine the amino acid sequence.

Although the N-terminal sequence of the *P. islandicum* ferredoxin was analyzed with a gas phase protein sequencer, no amino acids were detected, indicating that the N-terminus of the *P. islandicum* ferredoxin was blocked. Accordingly, I tried to deblock *N*-formylmethionine, pyroglutamate, *N*-acetylserine, and *N*-acetylthreonine by the conventional methods as described in Materials and Methods. However, no amino acids were detected with a gas phase protein sequencer.

The sequence studies of ferredoxin are summarized in Fig. 2-10. The total number of amino acid residues of *P. islandicum* ferredoxin was 101, giving a molecular weight of 11,214. On the other hand, the molecular weight of the ferredoxin was estimated to be 11,910 from the mass spectrometry. These results strongly indicate that the ferredoxin has extra 4-11 amino acids at the N-terminus.

Sequence Analysis and Function of C-terminal Extention of P. islandicum Ferredoxin

Figure 2-11 shows the amino acid sequence of *P. islandicum* ferredoxin aligned with those of ferredoxins from other organisms. In consideration of the C-terminal extension and the composition of [Fe-S] clusters, *P. islandicum* ferredoxin would seem to be similar to *Azotobacter vinelandii* ferredoxin I, although the homology between the two ferredoxins is low.

Figure 2-12 shows the schematic [Fe-S] cluster binding model of bacterial-type ferredoxins. *P. furiosus* ferredoxin has one clusters co-ordinated by first Fe-S binding motif. *A. vinelandii* Fd I has two clusters, one comprising a [3Fe-4S] core co-ordinated by first Fe-S binding motif, which consists of ^{8}Cys, ^{16}Cys and ^{49}Cys, and the other having a [4Fe-4S] core co-ordinated by second Fe-S binding motif, which consists of ^{20}Cys, ^{39}Cys, ^{42}Cys and ^{45}Cys (34). However, it should be noted that ^{13}Tyr and ^{42}Cys in *A. vinelandii* Fd I is replaced with cysteine and aspartate in *P. islandicum* ferredoxin, respectively. Therefore, it seems likely that the [4Fe-4S] core is bound at the first motif and the [3Fe-4S] core is bound at the second motif. This is a matter for future investigation.

Figure 2-13(A) shows the model of common fold of bacterial-type ferredoxins. Thus, the core-fold part has the $(\beta\alpha\beta)_2$ fold, which has two α-helixes and four β-strands, and consists two antiparallel β-sheets and two

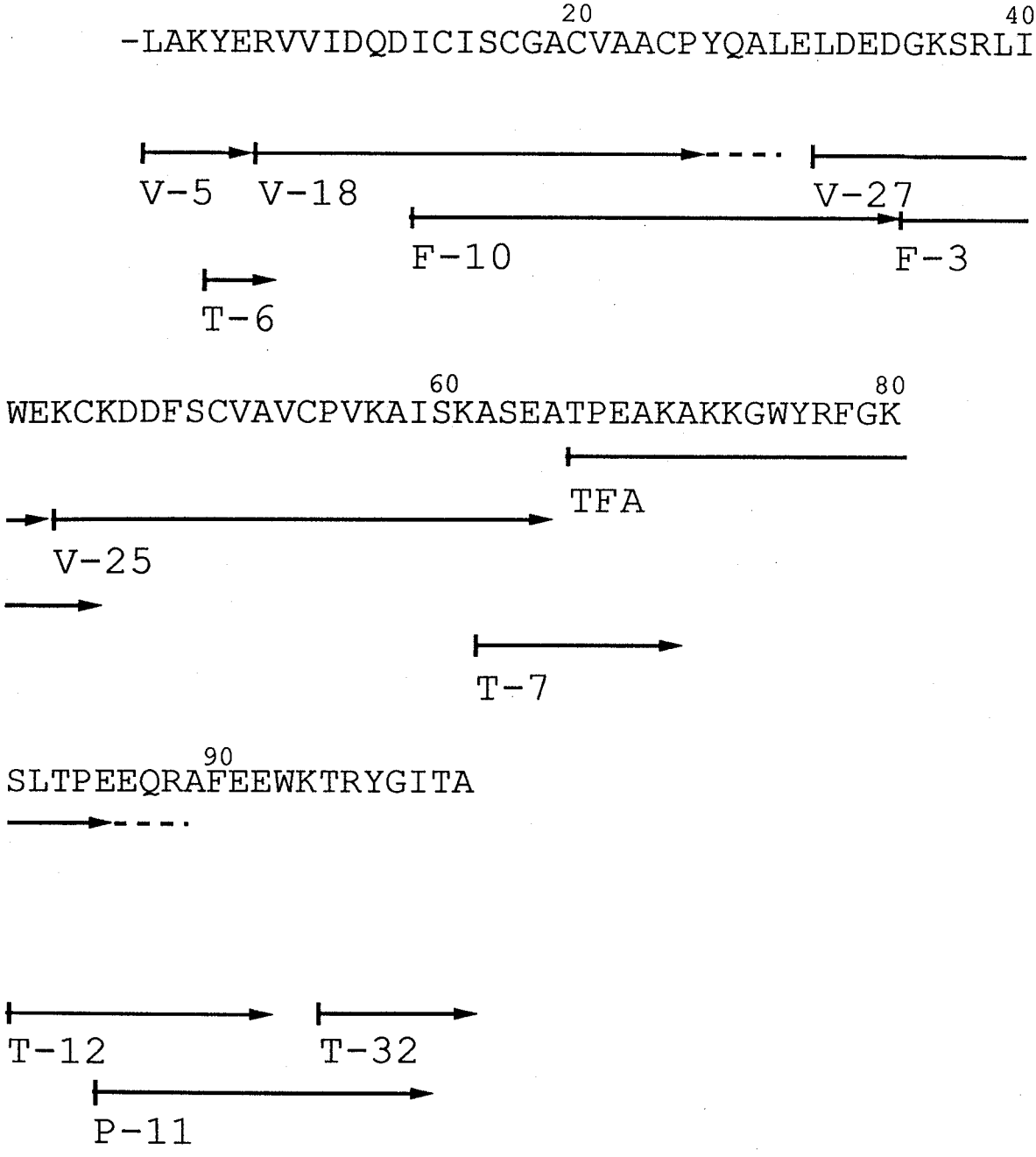

Fig. 2-10. Summary of sequence studies of *P. islandicum* ferredoxin. *V-5, V-18, V-25, V-27 Staphylococcus* V8-protease peptides; *T-6, T-7, T-12, T-32* tryptic peptides; *F-3, F-10* formic acid peptides; *TFA* trifluoro acetic acid peptides; *P-11* proline specific endopeptidase peptides.

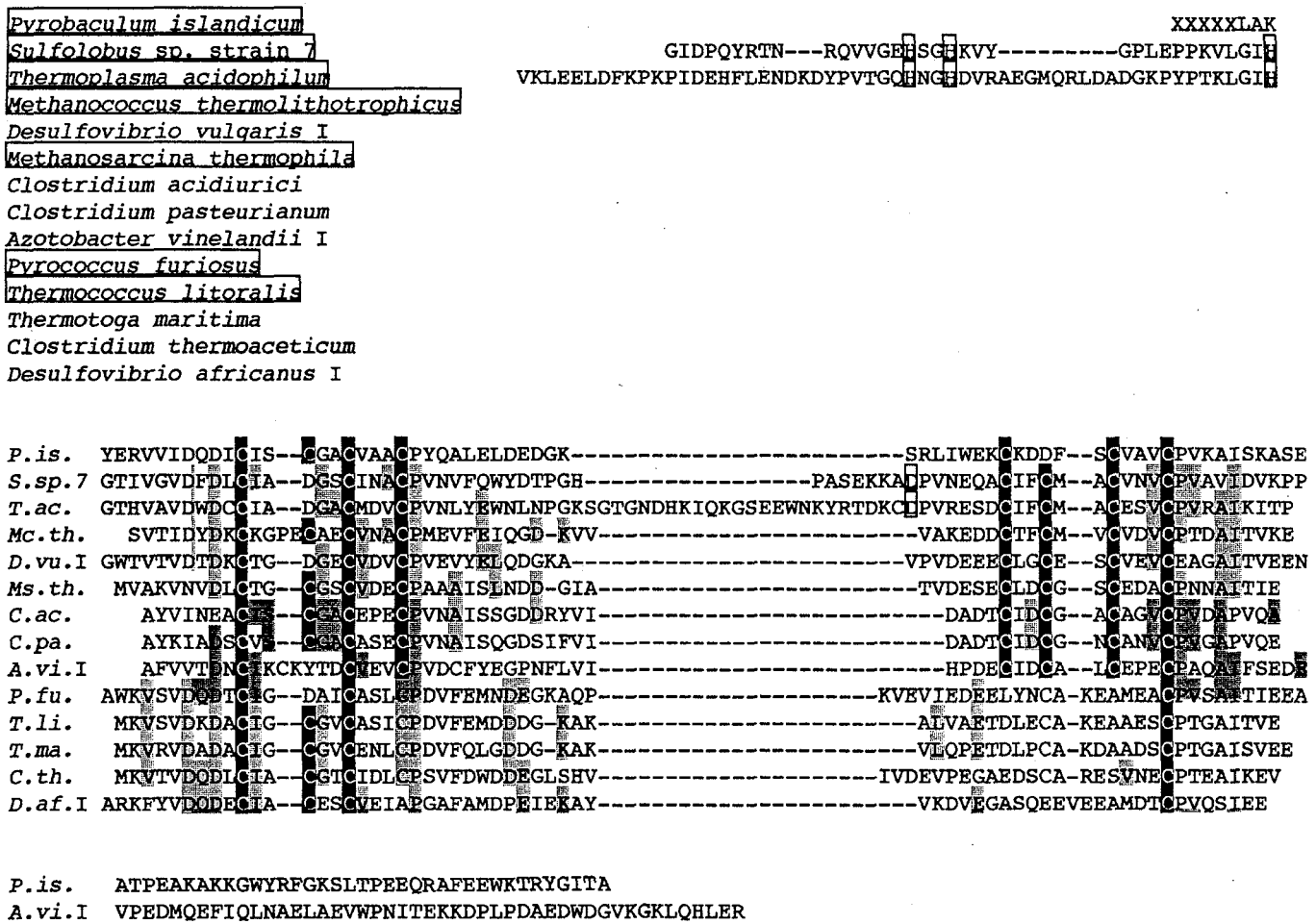

Fig. 2-11. Amino acid sequence alignment of ferredoxins. All amino acid sequence data shown in the figure are from the GenEMBL, PIR, and SWISS-PROT databases, except for ferredoxin from *P. islandicum*. The *boxed* microorganisms are archaea. Residues identical with *P. islandicum* ferredoxin are *shaded* and cysteine residues for Fe-S cluster are emphasized by *white letters on the black background.* The ligand residues to the zinc center in thermoacidophilic archaea are *boxed.*

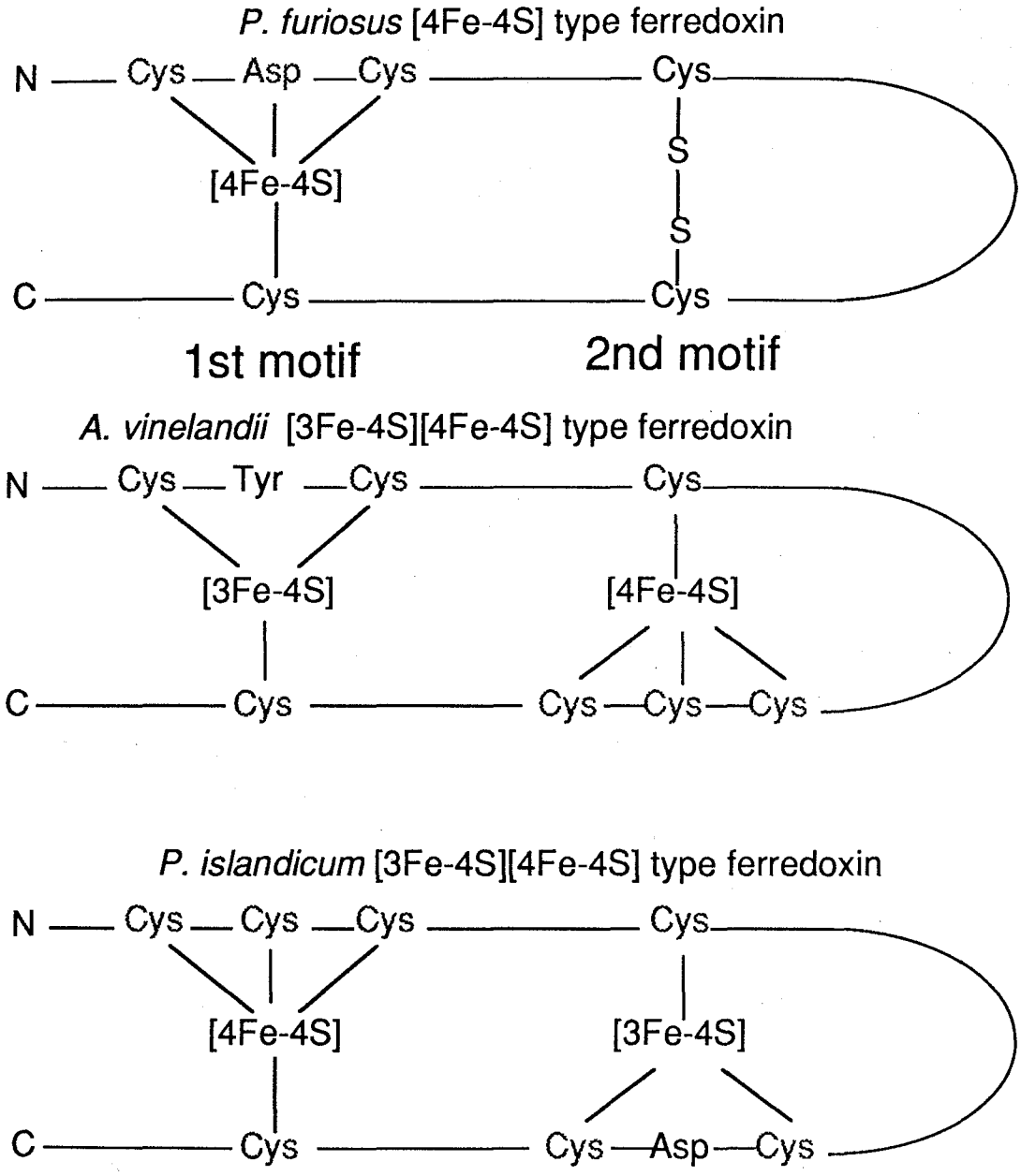

Fig. 2-12. Schematic model of possible ligand residues of bacterial-type ferredoxins. Cysteine or aspartate residues for Fe-S cluster are indicated by *gray letters*.

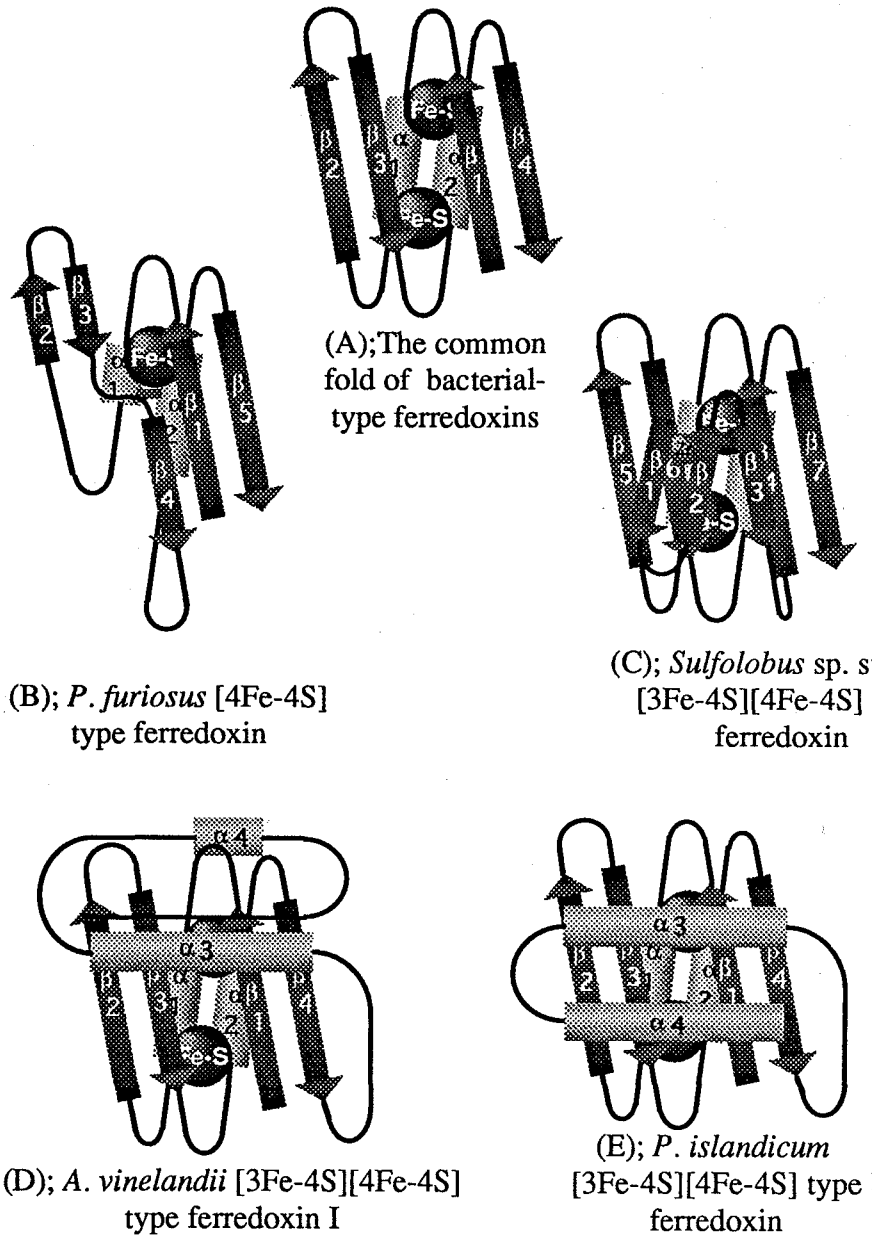

Fig. 2-13. Schematic structure of bacterial-type ferredoxins. The Fe-S clusters, α-helices and β-strands are shown by balls, cylinders and arrows.

α-helixes. Interestingly, many bacterial-type ferredoxins which have been determined by crystal structure or NMR spectral analysis enhance the stability of the two β-sheets in various way. In the [3Fe-4S] monocluster ferredoxin from *P. furiosus*, a triple-stranded antiparallel β-sheet is formed instead of the usual double-stranded antiparallel β-sheet (Fig. 2-13(B)) (35). *Sulfolobus* sp. strain 7 ferredoxin has N-terminal extension and zinc ion located between the core fold and N-terminal extension, and connects the β-sheet in the N-terminal extension and the central β-sheet in the core fold through the zinc ligation (Fig. 2-13(C)) (11,12). Dicluster-type *A. vinelandii* Fd I has a C-terminal extension containing an α-helix, and the carboxyl oxygens of ^{71}Asn in the middle of the α-helix make hydrogen bonds with backbone oxygens and nitrogens of the β-strands of both two β-sheets to link together (Fig. 2-13(D)) (36). Although the structure and function of C-terminal extension of *P. islandicum* ferredoxin has not been determined, it may play an important role in enhancing the thermostability of bacterial-type ferredoxin molecules like C-terminal extension of *A. vinelandii* Fd I (Fig. 2-13(E)).

Phylogenetic Tree of Archaeal Ferredoxins

In the present study, I determined the amino acid sequence of *P. islandicum* ferredoxin and then investigated the phylogenetical relationship between bacterial ferredoxins. Figure 2-14 shows the phylogenetic tree of ferredoxins determined by the parsimony and neighbor-joining methods. The ferredoxins can be divided into two distinct subdivisions, a di-cluster type and mono-cluster type. On the basis of the biochemical studies, the *P. islandicum* ferredoxin can be classified as a di-cluster type ferredoxin. However, the ferredoxin is included in neither di-cluster type nor mono-cluster type in the phylogenetic tree as shown in Figure 2-14. Therefore, the heat-stable *P. islandicum* ferredoxin may be a novel iron-sulfur protein that has not been found in Archaea and Bacteria.

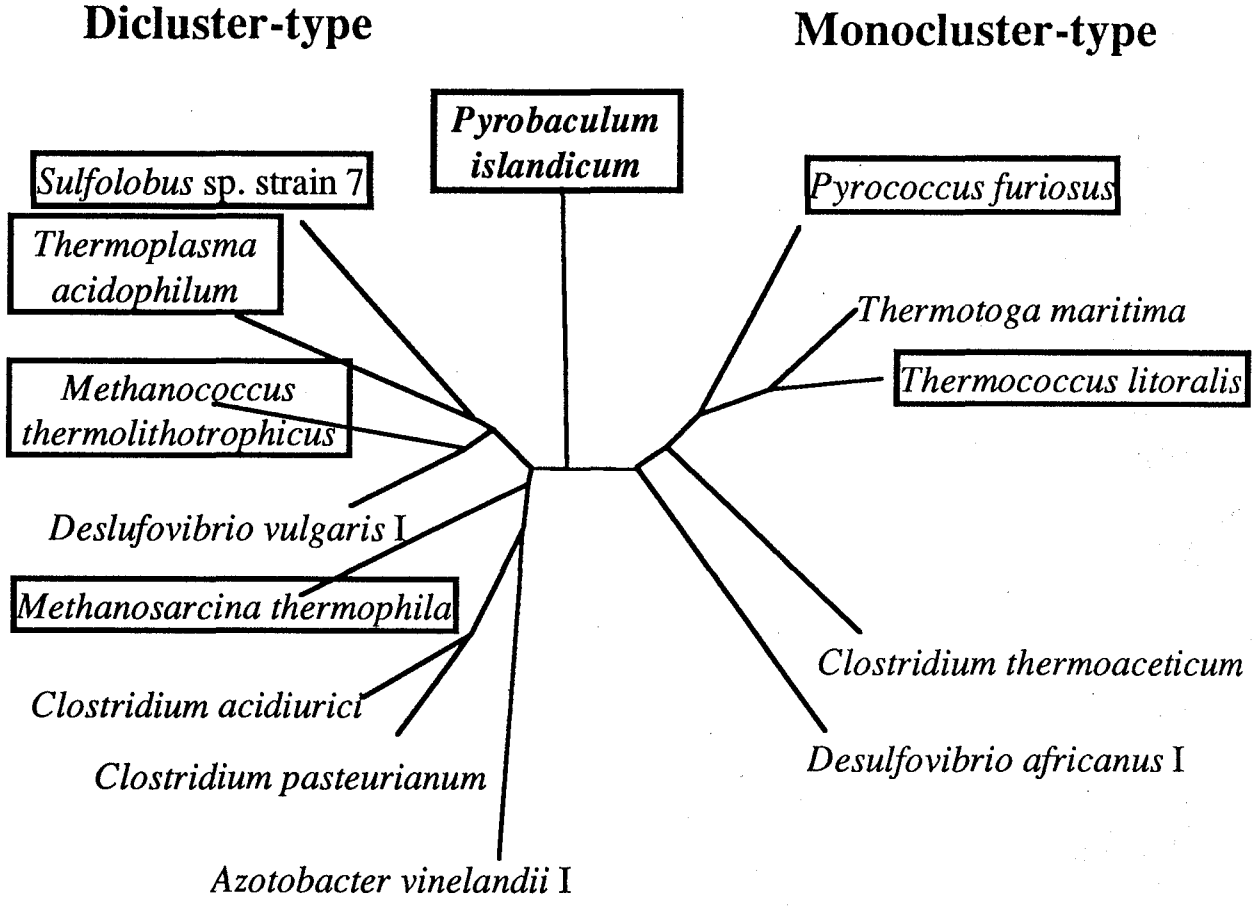

Fig. 2-14. A phylogenetic tree of bacterial-type ferredoxins from archaea and bacteria. The branch lengths were calculated by the parsimony and neighbor-joining method on the basis of amino acid sequences of ferredoxins. The *boxed* microorganisms are archaea.

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CHAPTER 3

Purification and Characterization of Cytochrome *b*-559 from Hyperthermophilic Archaeon *Pyrobaculum islandicum*

Introduction

Hyperthermophilic archaeon, *Pyrococcus furiosus*, has a novel fermentative pathway. Recently, the electron transfer system between glycolytic pathway and sulfur reduction or hydrogen production in the archaeon has been proposed by many researchers as follows; glycolytic pathway→Fd→Fd/NADP→oxidoreductase(Sulfide dehydrogenase?)→(NADP)NADP/H^+→Hydrogenase→H_2 (or H_2S) (1-5) (Fig. 3-1(A)). In the glycolytic pathway of the archaeon, the ferredoxin seems to serve as an electron carrier.

Hyperthermophilic archaeons, *Pyrodictium brockii* and *Pyrobaculum islandicum* are considered to produce ATP by respiration (6). *P. brockii* grows chemolithotrophically (7,8) and utilizes H_2 and elemental sulfur as respiratory substrate and terminal electron acceptor, respectively (9-11). Recently, Pihl *et al.* have reported that a quinone and *c*-type cytochromes are present in the membranes of *P. brockii* and proposed the primitive electron transport chain in the archaeon (12) (Fig. 3-1(B)). On the other hand, *P. islandicum* is a facultative chemoautotrophic archaeon. Although elemental sulfur is strictly required as terminal electron acceptor for autotrophic growth as same as in *P. brockii*, *P. islandicum* can utilize elemental sulfur, thiosulfate, sulfite, L(-)cystine and oxidized glutathione as terminal electron acceptor under heterotrophic growth condition (13).

In the present study, I purified the membrane-bound cytochrome *b*-559 and investigated the anaerobic respiratory chain for *P. islandicum*.

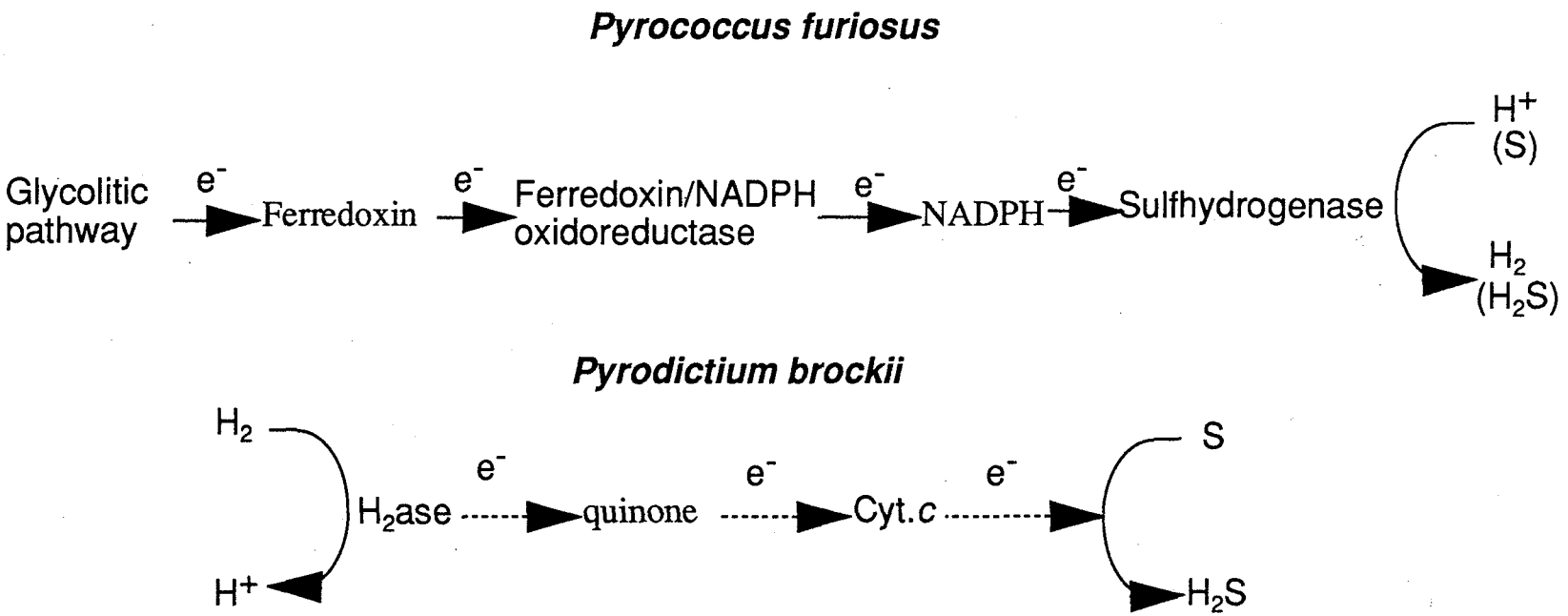

Fig. 3-1. Proposals of the electron transfer systems of *Pyrococcus furiosus* (1-5) and *Pyrodictium brockii* (12).

Materials and Methods

Preparation of Soluble and Membrane Fraction

Soluble and membrane fractions were prepared under the strict anaerobic conditions as follows. Frozen cells were suspended in 10 mM Tris-HCl buffer (pH 8.0) which was degassed and N_2-flushed, and disrupted with a French pressure cell at 1,100 kg/cm^2, 4°C. After the unbroken cells were removed by centrifugation at 10,000 x *g* for 15 min, the supernatant obtained was degassed and flushed with N_2, and further centrifuged at 104,000 x *g* for 1 h. The degassed and N_2-flushed supernatant was used as soluble fraction. The precipitate was suspended in 10 mM Tris-HCl buffer (pH 8.0), degassed and flushed with N_2, and used as membrane fraction.

Hydrogenase Assay

All hydrogenase activities were measured spectroscopically under strict anaerobic condition using Thunberg type cuvette. H_2-benzyl(methyl) viologen oxidoreductase activity was measured by the method of Kemner *et. al* (14) with slight modifications. The reaction mixture contained 80 mM KPi buffer (pH 7.5), 1 mM benzyl(methyl) viologen, and small amounts of dithionite in a volume of 4.0 ml. The reduction of benzyl(methyl) viologen was followed spectroscopically by the increase in the absorbance at 580(600) nm (ε_{mM} = 7.8 $mM^{-1}\cdot cm^{-1}$) with time at 95 °C. The reaction was started by addition of enzyme. One unit of activity was defined as 1 μmol of H_2 consumed per min.

H_2-NAD(P)$^+$, ferredoxin or decylbenzoquinone oxidoreductase activity was measured by the method as follows. The reaction mixture contained 80 mM KPi buffer (pH 7.5), 0.95 mM NAD(P)$^+$, and 0.05 mM NAD(P)H, ferredoxin (0.30 mg), or 100 μM decylbenzoquinone in a volume of 4.0 ml. The reduction of NAD(P)$^+$ was followed spectroscopically by the increase in the absorbance at 340 nm (ε_{mM} =6.2 $mM^{-1}\cdot cm^{-1}$) with time at 80 °C. The reduction of ferredoxin or decylbenzoquinone was followed spectroscopically by the decrease in the absorbance at 400 nm (ε_{mM} =28.8 $mM^{-1}\cdot cm^{-1}$) or 278 nm (ε_{mM} =14.7 $mM^{-1}\cdot cm^{-1}$), respectively, with time at 80 °C. The reaction was started by addition of soluble (0.121 mg) or membrane (0.044 mg) fraction.

Sulfur Reductase Activity

Sulfur reductase activity was determined under strict anaerobic condition by the method of Ma *et. al* (4) with slight modifications. The main chamber of Thunberg tube contained 2 ml of 80 mM KPi buffer (pH 7.5), 0.1 g of elemental sulfur (powder). After the sample in the side arm were mixed with the reaction buffer, it was incubated at 95°C for 2 h. The content of sulfide in the reaction mixture was determined by methylene blue formation (15). One unit of sulfur reductase activity was defined as 1 μmol of H_2S produced per min.

Ferredoxin Dependent Cytochrome b-559 Reduction

Reduced ferredoxin was prepared with a *Sulfolobus* sp. strain 7 2-oxoacid:ferredoxin oxidoreductase generating system (16). Ferredoxin dependent cytochrome *b*-559 reduction was determined by the method as follows. All experiments were performed at 70 °C under strict anaerobic condition using a Thunberg type cuvette. The reaction mixture contained 40 mM KPi buffer (pH 6.8), 2 mM 2-oxoglutarate, 100 μM coenzyme A, and 0.49 mg of partially purified cytochrome *b*-559 in a volume of 4.0 ml. The reaction was started by addition of the purified 2-oxoacid:ferredoxin oxidoreductase and *P. islandicum* ferredoxin. The reduction of cytochrome *b*-559 was followed spectroscopically by the increase in difference of the absorbance at 426 nm *minus* at 410 nm. *Sulfolobus* sp. strain 7 2-oxoacid:ferredoxin oxidoreductase was kindly gifted by Dr. Iwasaki, T.

Redox Titration

Redox titrations were performed by the method of Dutton (17) using Thunberg-type cuvettes. 10 μM of phenazine methosulfate (PMS), 2,3,5,6,-tetramethyl-*p*-phenylenediamine (DAD), ferrocyanide, methyl viologen, pyocyanin and anthraquinone-2-sulfonate were used as redox mediators in 10 mM sodium phosphate buffer, pH 7.0. The absorbance difference at (559 nm-(575 nm-550 nm)/2) was monitored as a function of the redox potential. Horse cytochrome *c* was used as a standard; its midpoint redox potential is +255 mV.

Physical Measurements

Heme *b* was extracted from the membrane fraction or purified cytochrome *b*-559 with acetone-HCl by the method of Jacobs *et. al* (18) as

modified by Kühn *et. al* (19). The heme *b* concentration of the samples was determined by the pyridine hemochrome spectra (ε_{mM} = 27.7 mM^{-1} cm^{-1}). The qualitative analyses of the hemes were carried out by a reverse-phase HPLC taken after acetone-HCl extraction, with the following standards: heme *b* extracted from hemoglobin; heme *o* extracted from *Bacillus* YN-2000 membranes.

Protein content was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Bovine serum albumin was used as a standard.

Spectrophotometric measurements were performed with a Shimadzu MPS-2000 spectrophotometer and Hitachi 220A with 1-cm light path cuvettes. The metal content was determined by inductively coupled Plasma Spectrometer SPS 1500 VR (Seiko Instruments Inc.).

Reagents

CM-cellulose and Sephadex G-75 were purchased from Pharmacia Fine Chemicals (Sweden). DEAE-Toyopearl were purchased from Tosoh Corporation (Tokyo, Japan). Molecular weight maker for SDS-polyacrylamide gel electrophoresis was purchased from Fluka AG (Switzerland).

Results and Discussion

Hydrogen-Dependent Sulfur Reduction by P. islandicum

Figure 3-2 shows hydrogen-dependent sulfur reduction by the intact cells which were cultivated with sulfur and by the membranes or soluble fractions prepared from the cells. The soluble fraction did not show any production of sulfide. Therefore, these results suggest that an electron transfer system from hydrogen to sulfur is mediated only with the respiratory components in the membranes.

Glucose-Dependent Sulfur Reduction

Figure 3-3 shows glucose-dependent sulfur reduction by the membranes or soluble fractions prepared from the cells which were cultivated with sulfur. Although the only soluble fraction or the membrane fraction did not show any production of sulfide, the mixture of the fractions showed glucose-dependent sulfur reduction. These results suggest that glycolytic pathway and sulfur reduction is coupled each other and the electron transfer from glucose to sulfur is consisted with both membrane and soluble fraction.

Purification and Characterization of Membrane-Bound Hydrogenase of P. islandicum

The membrane fraction prepared from *P. islandicum* showed benzyl viologen dependent hydrogenase activities at 95 °C with 56.8 U/mg protein. Figure 3-4 shows the effects of temperature on the H_2-BV oxidoreductase activity of the membrane fraction. The activity was increased with the temperature of the reaction mixture. As oxygen was not effective in the H_2-BV oxidoreductase activity of the membrane fraction, the purification was performed under aerobic condition at room temperature.

The membrane fraction was suspended in 70 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100. The suspension was gentry stirred for 1 h at 4°C, and then centrifuged at 104,000 x *g* for 1 h. The supernatant thus obtained was dialyzed against 200 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% Triton X-100 for 4 h. The dialyzed solution was charged on a DEAE-Toyopearl column (2.6 x 12 cm) equilibrated with the same buffer as used for dialysis. After the column was washed with 200 ml of the same buffer, the hydrogenase was eluted with a linear gradient

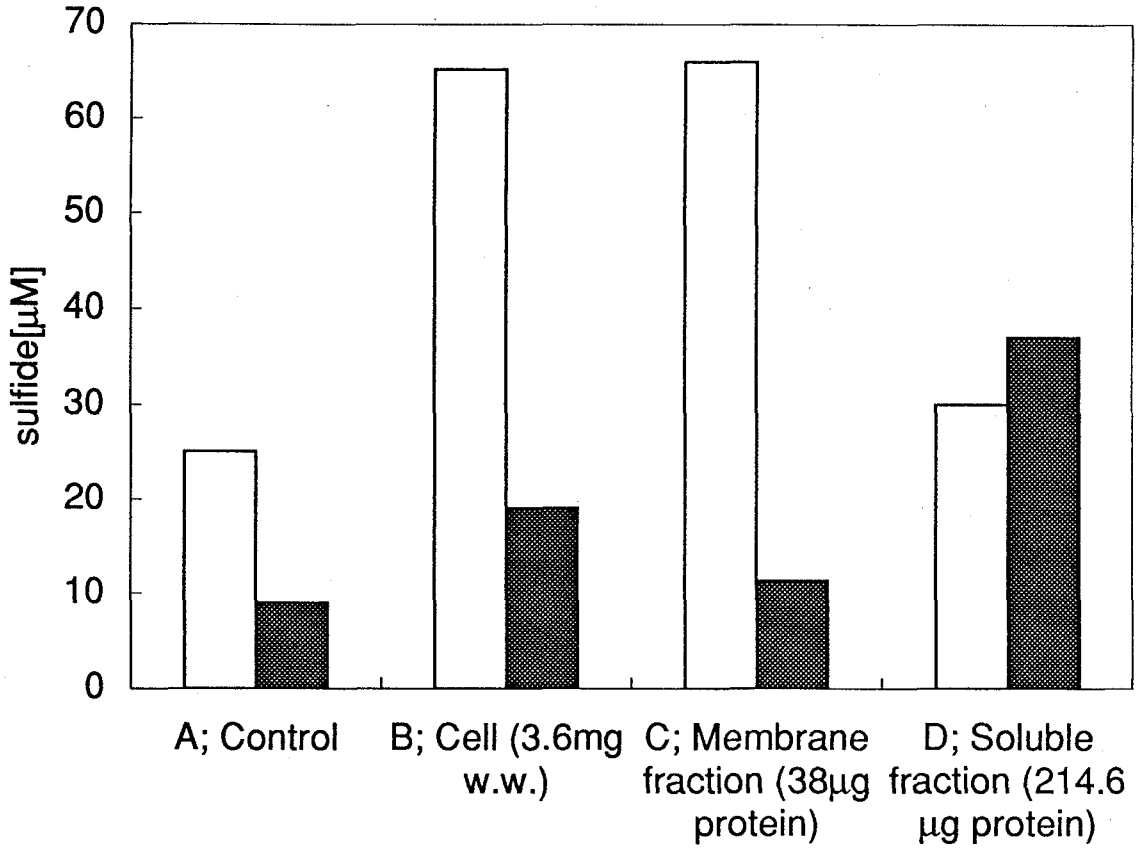

Fig. 3-2. Hydrogen-dependent sulfide production by sulfur-grown cell or membranes of *P. islandicum* . Reaction mixture contains 50 mM KPi buffer (pH 6.5) and 0.02% Tween-20 in the presence (*open box*) or absence (*close box*) of sulfur under 100% H_2 atmosphere as a gas phase. A; no enzymatic sulfide production; B, sulfide production by cells (3.6 mg) wet weight; C, sulfide production by membrane fraction (38 μg protein); D, sulfide production by soluble fraction (214.6 μg protein).

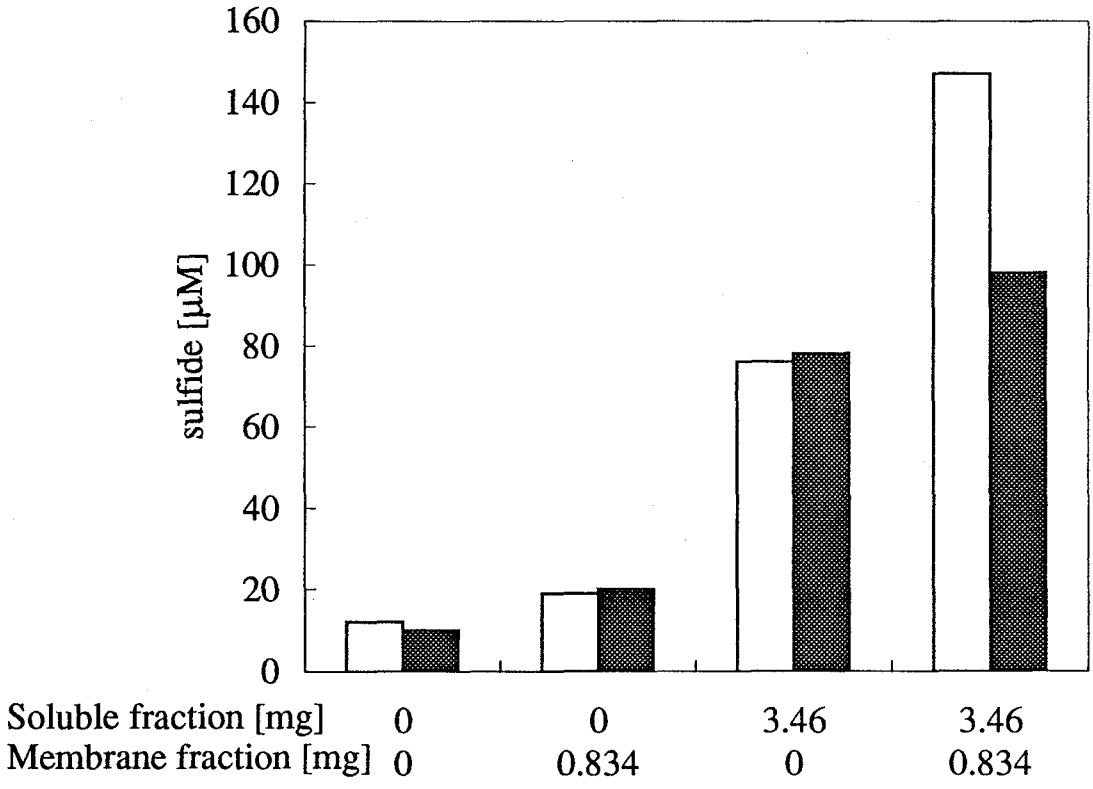

Fig. 3-3. Glucose-dependent sulfide production by soluble fractions or membrane fractions prepared by sulfur-grown cell of *P. islandicum* . Reaction mixture contains 50 mM KPi buffer (pH 6.5), 0.02% Tween-20, 10 mM glucose under anaerobic condition. Lanes: A, no enzymatic sulfide production; B, sulfide production by 0.834 mg of membrane fraction; C, sulfide production by 3.46 mg of soluble fraction; D, sulfide production by 0.834 mg of membrane fraction and 3.46 mg of soluble fraction.

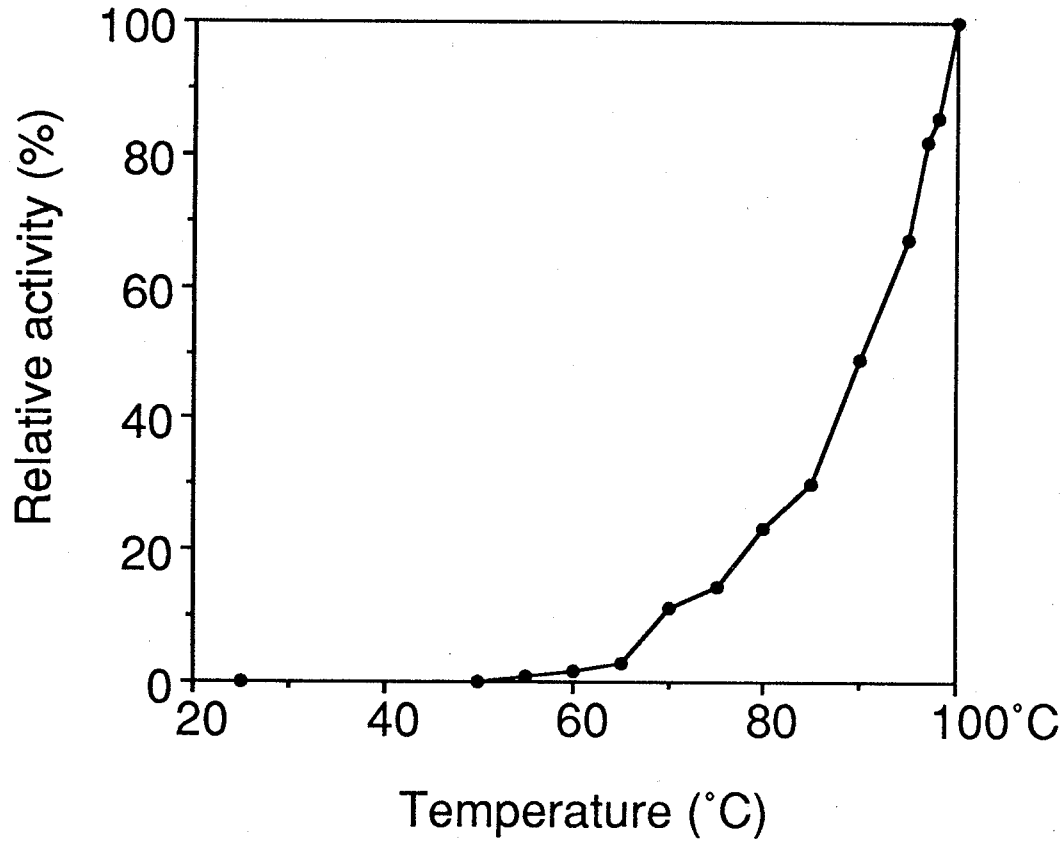

Fig. 3-4. Effect of temperature on the H_2-benzyl viologen oxidoreductase activity of the membrane prepared from *P. islandicum*. H_2-BV oxidoreductase activity was determined at various temperatures by the method as described in Materials and Methods. The reaction mixture contained 80 mM KPi buffer (pH 7.5), 1 mM benzyl viologen and membrane fraction (0.069 mg protein) under 100% H_2 atmosphere as a gas phase.

produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and the buffer containing 0.4 M NaCl. After the hydrogenase fraction were concentrated with ultrafiltration in a Amicon unit (centriflo CF25), the fraction was subjected to gel filtration on a Sephacryl S-300 column (2.4 x 115 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and 200 mM NaCl. To the eluates which contained hydrogenase, ammonium sulfate was added to give a final concentration of 0.68 M, and the suspension was stirred for 30 min. After the resulting solution was centrifuged at 10,000 x *g* for 15 min, the eluates which contained hydrogenase, ammonium sulfate was added to give a final concentration of 1.36 M, and the suspension was stirred for 30 min. Resulting solution was centrifuged at 10,000 x *g* for 15 min. The precipitates obtained were suspended in 1 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 and it were used as the partially purified hydrogenase preparation.

Recently, Pihl *et. al* have proposed the anaerobic respiratory chain of *P. brockii* composed of membrane-bound hydrogenase, quinone and cytochrome *c* (12). In this model, quinone functions as electron acceptor for hydrogenase. Then to identify the physiological electron acceptor for hydrogenase of *P. islandicum*, the effects of respiratory inhibitors on hydrogen-dependent sulfide production by the membranes were analyzed by the method as described in Materials and Methods. In the presence of HOQNO, the hydrogen-dependent sulfide production with the membranes was strongly inhibited. *K*i value for HOQNO was 18 μM (Fig. 3-5). However, the sulfide production was not effected by 1 mM of antimycin A, KCN, abietic acid, rotenone, SHAM and myxothiazol. These results clearly shows that quinone is physiological electron mediator in hydrogen-sulfur electron transport chain. Indeed, the presence of menaquinone in *P. islandicum* has been reported by Tindall (20).

Involvement of Cytochrome b in an anaerobic respiratory chain of P. islandicum

As described in Introduction, *P. islandicum* utilizes three kinds of sulfur compounds, elemental sulfur, sodium sulfite, or sodium thiosulfate as terminal electron acceptor under heterotrophic growth condition. Figure 3-6 shows (Reduced)-(Oxidized) difference spectra of the membrane fractions and the soluble fractions prepared from the cells which were cultivated in the presence of elemental sulfur, sodium sulfite and sodium thiosulfate, respectively. All

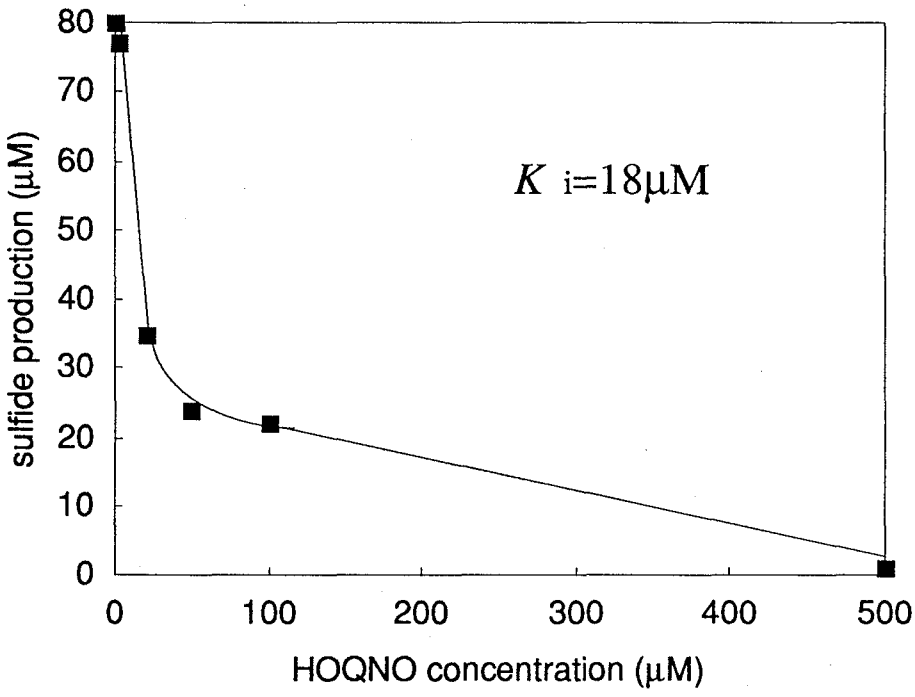

Fig. 3-5. Effect of HOQNO on hydrogen-dependent sulfide production by membrane fraction from sulfur-grown cell. Reaction mixture contains 50 mM KPi buffer (pH 6.5), 0.02% Tween-20 and sulfur under 100% H_2 atmosphere as a gas phase.

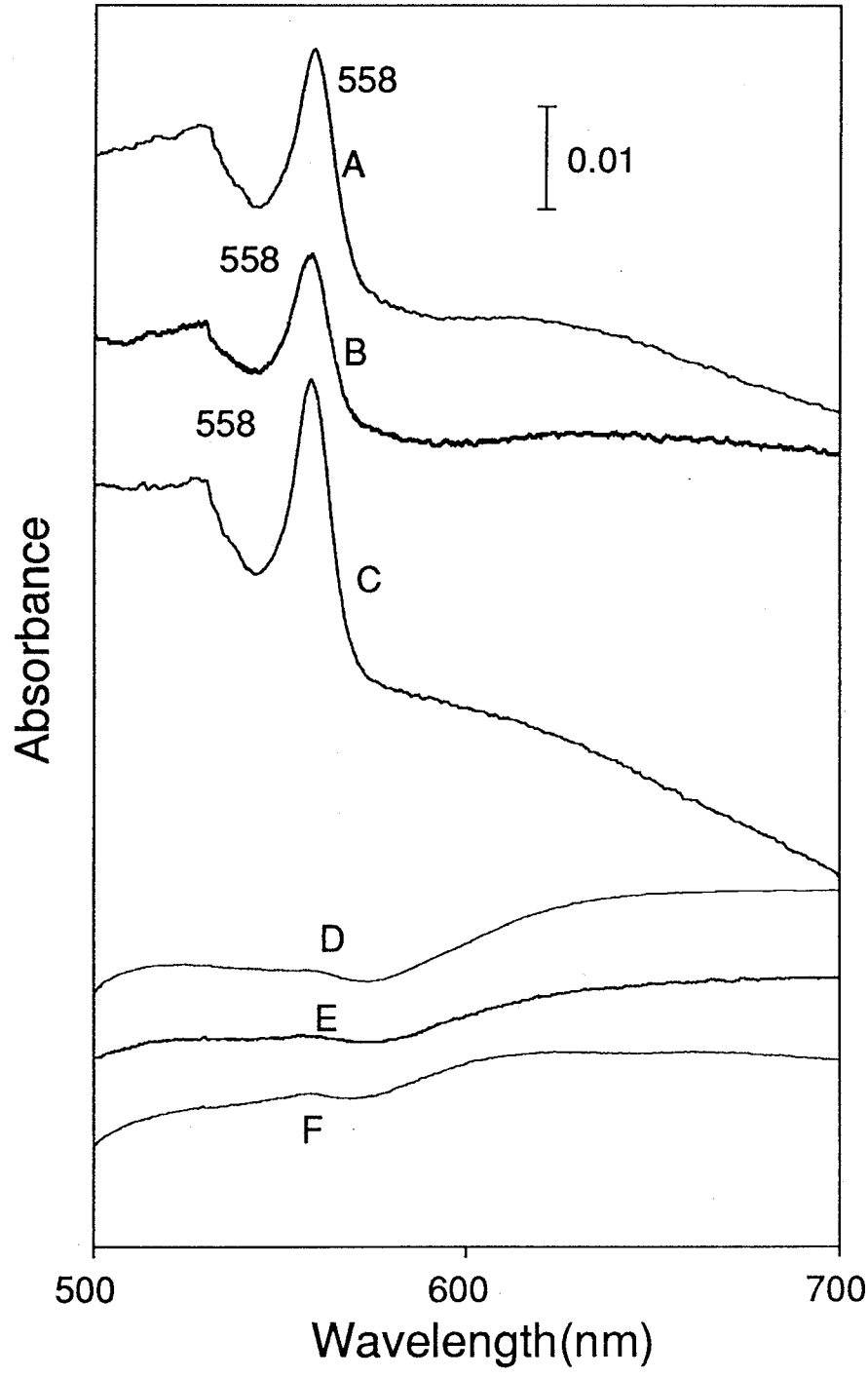

Fig. 3-6. (Dithionite-reduced) *minus* (air-oxidized) difference spectra. (A) Membrane fraction prepared from S^0-grown cells. (B) Membrane fraction prepared from Na_2SO_3-grown cells. (C) Membrane fraction prepared from $Na_2S_2O_3$-grown cells. (D) Soluble fraction prepared from S^0-grown cells. (E) Soluble fraction prepared from Na_2SO_3-grown cells. (F) Soluble fraction prepared from $Na_2S_2O_3$-grown cells.

membranes contained *b*-type cytochromes, while all soluble fractions contain few amounts of *b*-type cytochromes. The contents of *b*-type cytochromes in the membranes were determined to be 39.8 (S^0-grown cells), 28.0 (Na_2SO_3-grown cells), 50.2 ($Na_2S_2O_3$-grown cells) nmol/mg protein on the basis of the pyridine hemochrome spectra and protein contents. The heme contents were much higher than that of the membranes prepared from *P. brockii* (12). Furthermore, the qualitative analyses of the hemes in the solubilized membrane fraction by HPLC analysis was carried out by the method as described in Materials and Methods. Only *b*-type cytochromes were found in the solubilized membrane and *o*-type or *c*-type cytochromes were not found. Neither *b*-type or *c*-type cytochromes were found in the soluble fraction from pyridine hemochrome spectra taken after acetone-HCl extraction.

To elucidate the involvements of cytochromes *b* in the respiratory chain, the reduction of cytochromes *b* with H_2 in the cell-free extract was examined spectroscopically by following the increase in the absorbance at 558 nm. As shown in Fig 3-7(A), cytochromes *b* in the cell free extract which was prepared from the thiosulfate-grown cells were not reduced at 25 °C with H_2, while they were rapidly reduced at 95 °C with H_2. About 74% of cytochromes *b* were reduced with H_2. The reduction rate of cytochromes *b* was not so changed even in the presence of 250 μM of HOQNO. These results suggest that although the cytochrome *b* can accept electrons from H_2 with the membrane-bound hydrogenase, quinones are not involved in electron transfer from H_2 to cytochrome *b*. Furthermore, as shown in Fig 3-7(A), about 42% and 32% of ferrocytochromes *b* in the cell-free extract were reoxidized by addition of sodium thiosulfate and elemental sulfur under anaerobic condition, respectively. However, the ferrocytochromes *b* were not oxidized with elemental sulfur in the presence of 250 μM of HOQNO. These results suggest that *b*-type cytochrome is participated in the anaerobic respiratory chain from H_2 to thiosulfate or elemental sulfur and locate on the substrate side of the electron transport chain with respect to the quinone.

Figure 3-7(B) shows the reduction of cytochromes *b* in the cell-free extract with NADH and oxidation of ferrocytochromes *b* with sodium thiosulfate, elemental sulfur, and sodium sulfite. Although cytochromes *b* was slowly reduced with NADH at 25°C, the reduction rate was greatly accelerated when the temperature of the reaction mixture was increased to 95°C. After the

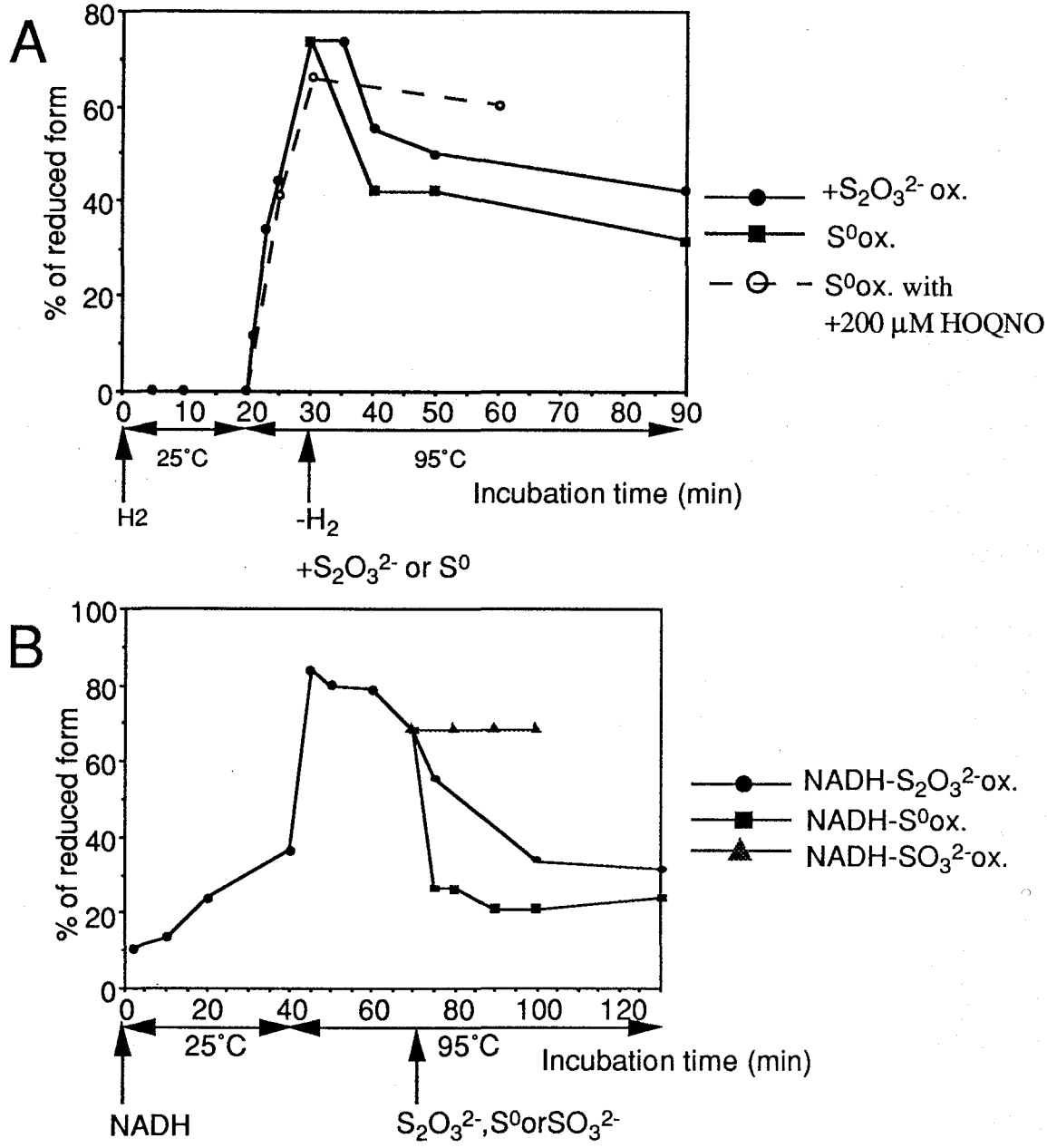

Fig. 3-7. Reduction of cytochromes *b* in the cell free extract with H_2 or NADH and reoxidation of ferrocytochrome *b*with S^0, SO_3^{2-} or $S_2O_3^{2-}$. Cell free extract was prepared from *P. islandicum* by the method as described in Materials and Methods. The contents of ferrocytochromes *b* in the cell free extract was determined spectroscopically by the absorbance at 558 nm. The full reduced form of cytochrome *b* was prepared by addition of $Na_2S_2O_4$. (A) H_2 and (B) NADH were utilized as electron donor, respectively. 2.5% Elemental sulfur, 30 mM sodium thiosulfate and 30 mM sodium sulfite were added to the reaction mixture, respectively, and the oxidation of cytochrome *b* was followed with time. Other experimental conditions were described in Materials and Methods.

reaction mixture was incubated at 95°C for 30 min in the presence of NADH, elemental sulfur, sodium thiosulfate and sodium sulfite were added to the reaction mixture, respectively. The rapid oxidation of ferrocytochromes *b* was observed when elemental sulfur or sodium thiosulfate was used as oxidant. Sodium sulfite did not oxidize ferrocytochromes *b* in the cell free extract. These results suggest that cytochrome *b*-559 may be participated in the respiratory chain from NADH to thiosulfate or elemental sulfur.

Purification and Characterization of Cytochrome b-559

Because oxygen was not effective in the absorption spectrum of cytochromes *b* in the membrane fraction, the cytochrome *b*-559 has been purified from *P. islandicum* under aerobic conditions. The membrane fraction was suspended in 70 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1% SM-1080. The suspension was gentry stirred for 1 h at 4°C, and then centrifuged at 104,000 x *g* for 1 h. The supernatant thus obtained was dialyzed against 200 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.5% SM-1080 for 4 h. The dialyzed solution was charged on a DEAE-Toyopearl column (2.6 x 12 cm) equilibrated with the same buffer as used for dialysis. Most of cytochrome *b* passed through the column. To the eluates which contained cytochrome *b*, ammonium sulfate was added to give a final concentration of 2.06 M, and the suspension was stirred for 30 min. After the resulting solution was centrifuged at 10,000 x *g* for 15 min, the supernatant was charged on a Butyl-Toyopearl column (1.0 x 3 cm) equilibrated with the 10 mM Tris-HCl buffer, pH 8.0, containing 2.06 M ammonium sulfate and 0.5% SM-1080. Cytochrome *b* was eluted with a linear gradient produced from 120 ml each of 10 mM Tris-HCl buffer, pH 8.0 and 0.5% SM-1080, containing 2.06 M ammonium sulfate and the buffer containing 1.35 M ammonium sulfate. The following steps were repeated for three times. The eluates which contained cytochrome *b* were concentrated by ultrafiltration in a Amicon unit (centriflo CF25), the concentrated fraction was subjected to gel filtration on a Sephacryl S-200 column (2.4 x 115 cm) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% SM-1080 and 200 mM NaCl. The eluate was used as the purified cytochrome *b*-559 preparation. These purification steps are summarized in Figure 3-8.

Figure 3-9 shows the absorption spectra of *P. islandicum* cytochrome *b*-559. It showed absorption peaks at 414 nm in the-oxidized state and 426, 529 and 559 nm in the reduced form which was prepared by addition of

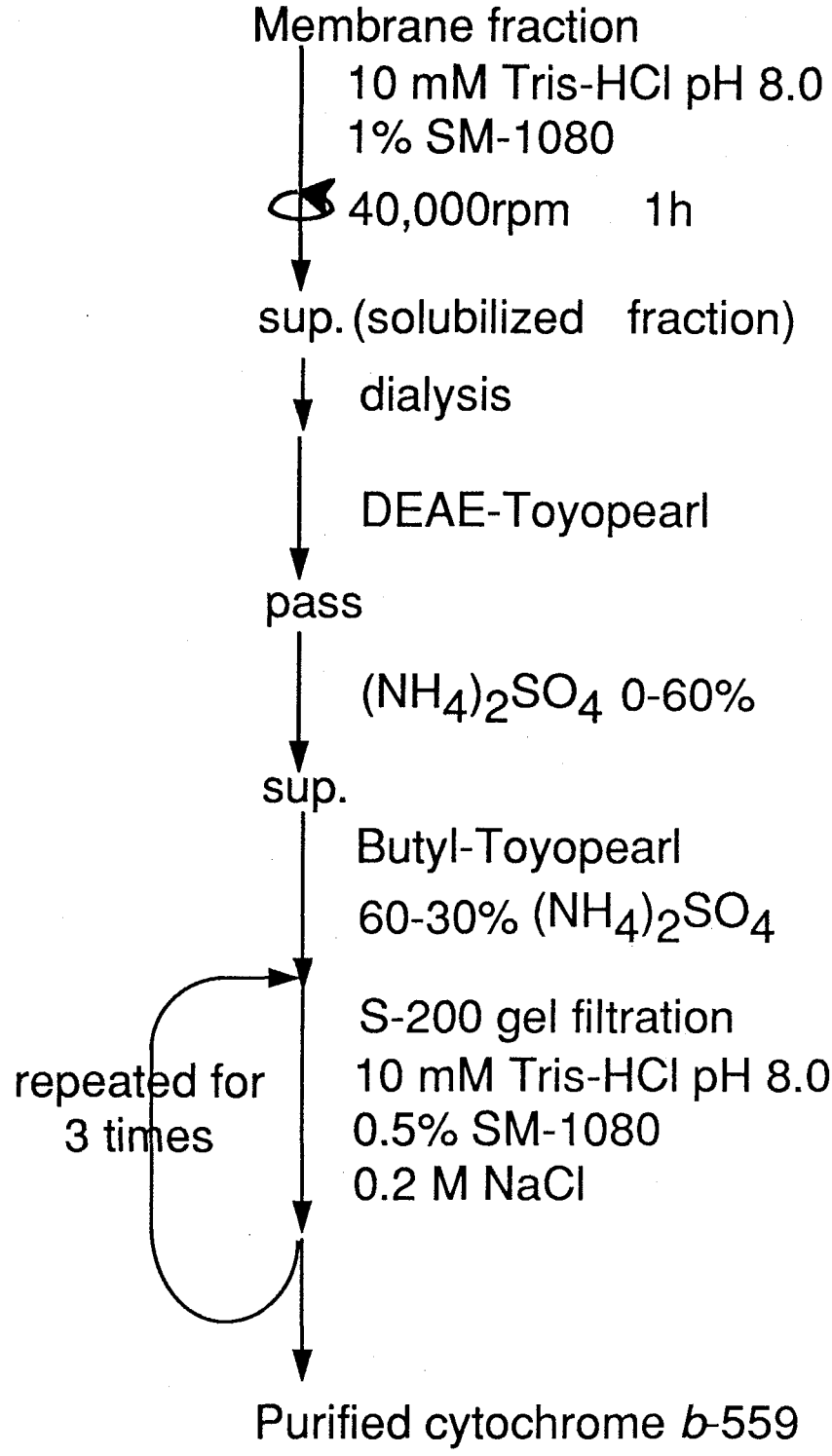

Fig. 3-8. Purification scheme of cytochrome *b*-559 from *P. islandicum*.

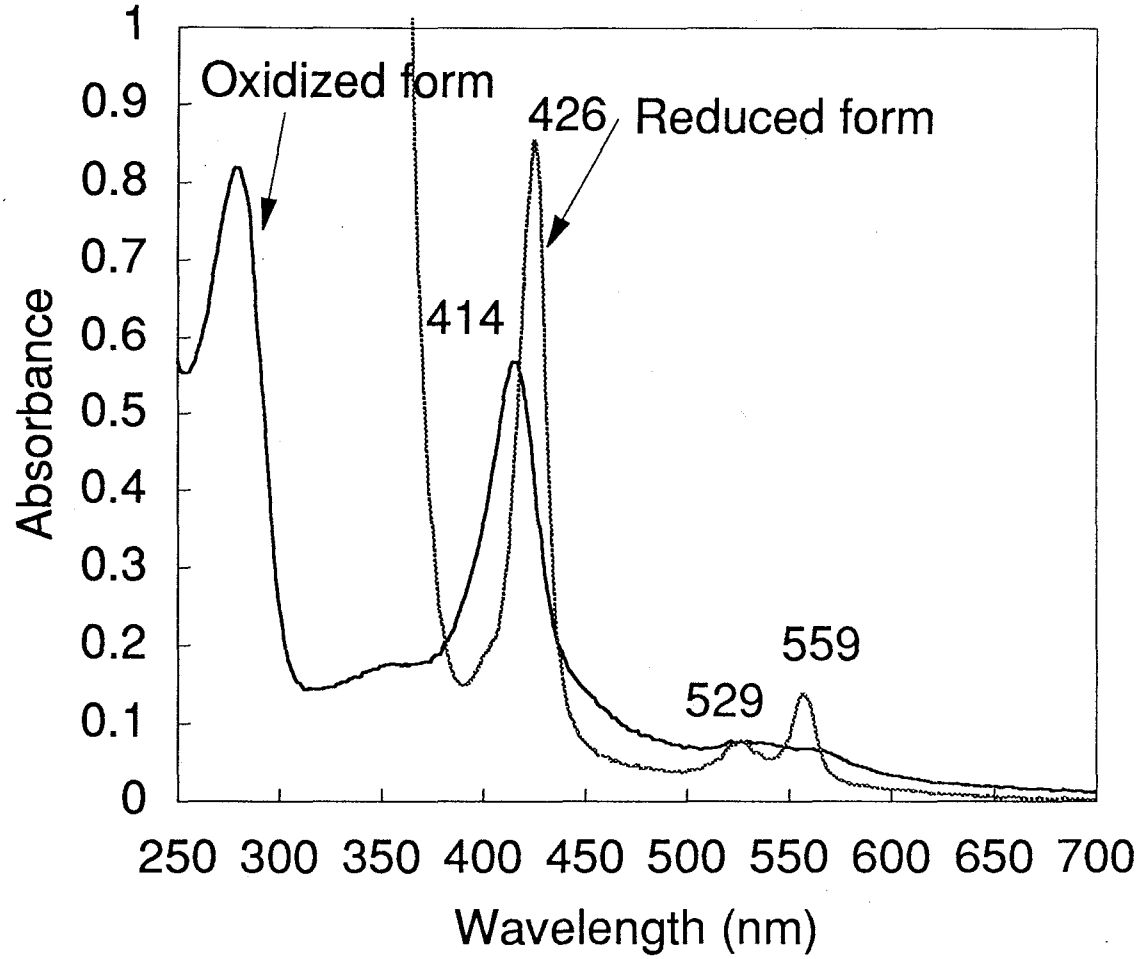

Fig. 3-9. Absorption spectra of *P. islandicum* cytochrome *b* -559.
The cytochrome *b* -559 was dissolved in 10 mM NaPi buffer (pH 7.0)

dithionite. The pyridine ferrohemochrome spectra showed absorption peak at 419, 524, 557 nm (data not shown). The qualitative analyses of the purified cytochrome *b*-559 with a reverse-phase chromatography confirms that the cytochrome contains heme *b* as a prosthetic group.

The midpoint redox potential of cytochrome *b*-559 was determined to be -21 mV at pH 7.0 as shown in Fig. 3-10.

Archaea as *P. furiosus* and *Sulfolobus* sp. strain 7 utilize ferredoxins as electron acceptors for pyruvate: ferredoxin oxidoreductase and 2-oxoglutarate: ferredoxin oxidoreductase in archaeal glycolytic pathway (1,21-23). Recently, Iwasaki *et al.* have been purified 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* sp. strain 7 and proposed the physiological function in the ferredoxin-dependent redox system (16). In the present study, I have tried to reconstitute the electron transport system from 2-oxoglutarate to *P. islandicum* cytochrome *b*-559 with *P. islandicum* ferredoxin and *Sulfolobus* 2-oxoacid:ferredoxin oxidoreductase. As shown in Fig. 3-11, the *P. islandicum* cytochrome *b*-559 was rapidly reduced with *P. islandicum* ferredoxin in the presence of *Sulfolobus* 2-oxoacid: ferredoxin oxidoreductase at 70 °C. About 90% of cytochrome *b*-559 was reduced in 1 min with the electron-donating system which is composed of 2-oxoglutarate, CoA, *Sulfolobus* 2-oxoacid: ferredoxin oxidoreductase and *P. islandicum* ferredoxin. These results suggest that cytochrome *b*-559 is participated in the respiratory chain from ferredoxin to elemental sulfur.

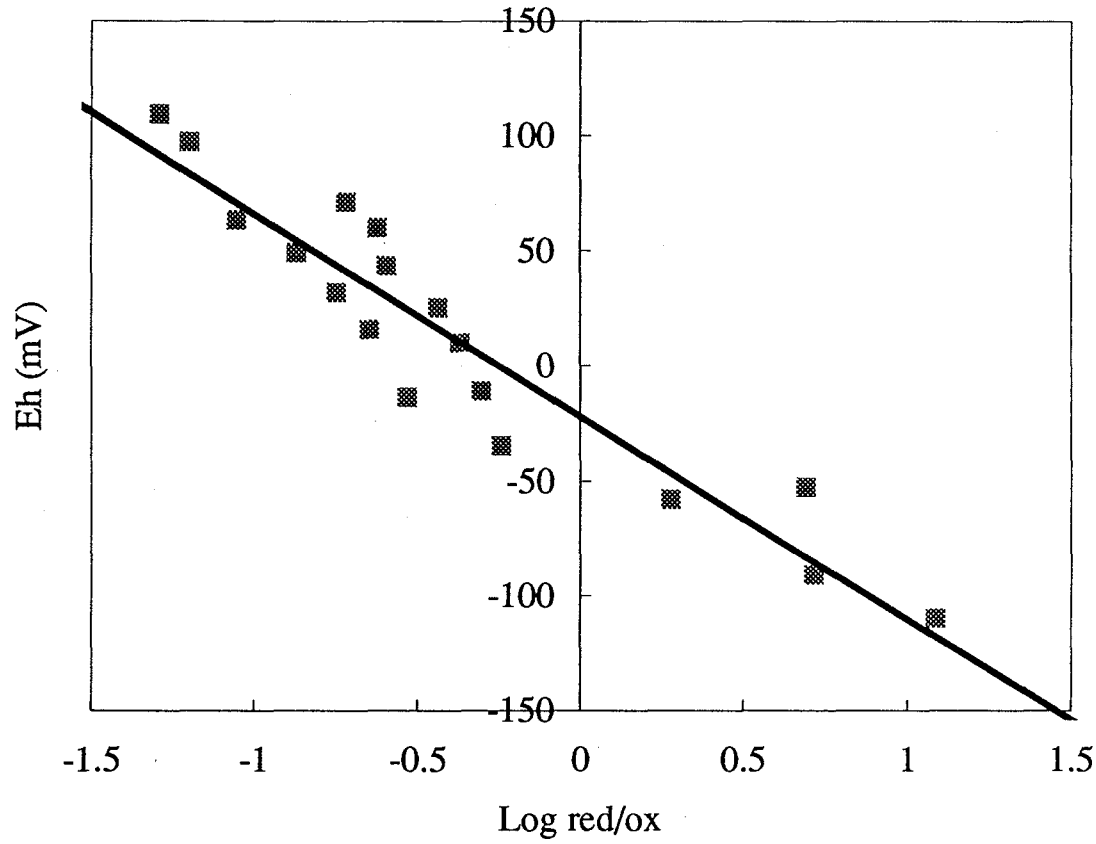

Fig. 3-10. Redox titration of *P. islandicum* cytochrome *b* -559. Cytochrome *b* -559 was dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing the redox mediators as described in Materials and Methods. The experiment was performed anaerobically under an argon atmosphere using a Thunberg-type cuvette. The straight line was drawn according to the Nernst equation (n=1) assuming that the midpoint redox potential at pH 7.0 of cytochrome *b* -559 is -21 mV.

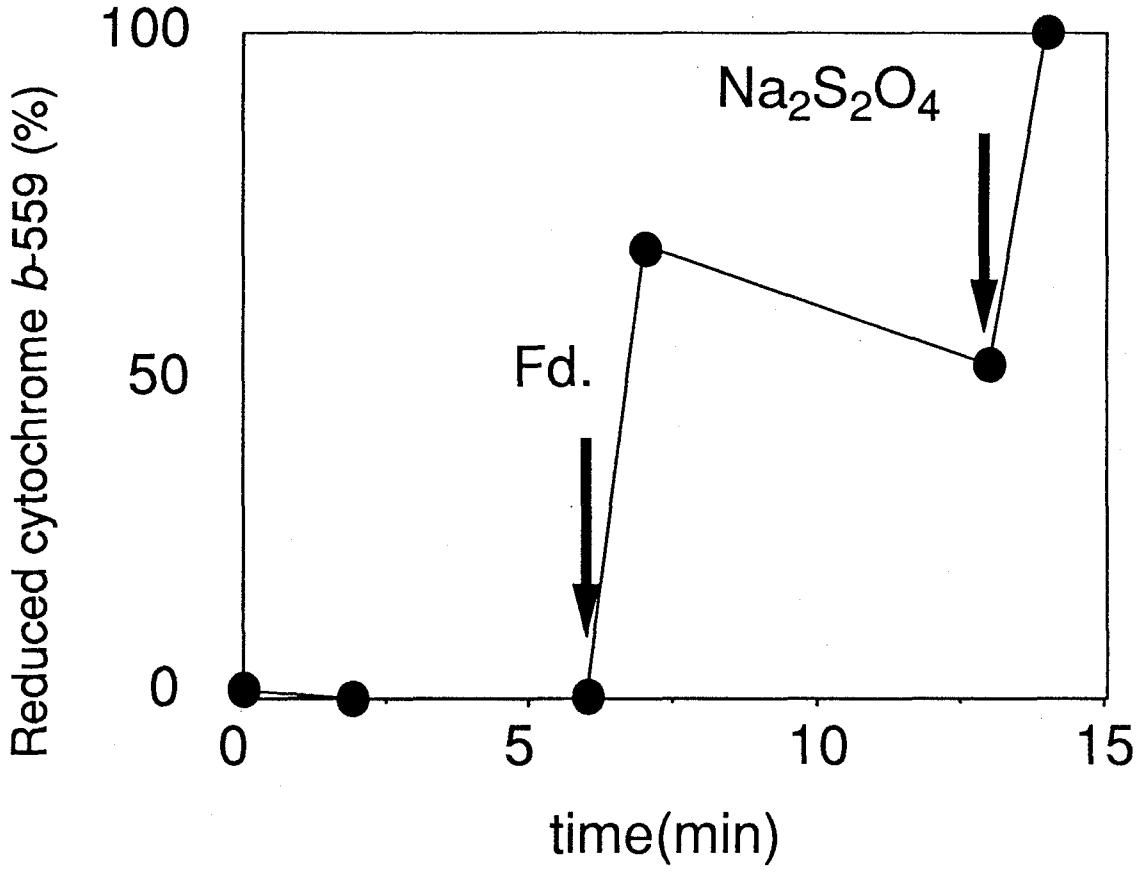

Fig. 3-11. Ferredoxin-dependent anaerobic reduction of cytochrome *b*-559. The reaction mixture contained 40 mM KPi buffer (pH 6.8), 2 mM 2-oxoglutarate, 100 μM coenzyme A, *Sulfolobus* 2-oxoacid: ferredoxin oxidoreductase, and *P. islandicum* cytochrome *b*-559 in a final volume of 4.0 ml. The reaction mixture was kept anaerobically with N_2 at 70 °C. The reaction was started by addition of *P. islandicum* ferredoxin (3.79 μg). (A) 0 min, (B) 30 sec, (C) 6 min.

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CHAPTER 4

Conclusion

In the present study, I have purifed ferredoxin (Chapeter 2) and cytochrome *b*-559 (Chapeter 3) from the hyperthermophilic archaeon *Pyrobaculum islandicum* and investigated their physiological funcitons. The ferredoxin has been shown to contain one [3Fe-4S] and one [4Fe-4S] cluster and to be rapidly reduced by 2-oxoglutarate: ferredoxin oxidoreductase purified from *P.islandicum*. On the otherhand, although I could not purify the *P. islandicum* cytochrome b-559 to an electrophoretically homogenous state, the cytochrome is shown to be only one component with heme in the membrane and be reduced with *P. islandicum* ferredoxin in the presence of *P. islandicum* 2-oxoacid: ferredoxin oxidoreductase at 70 °C. Therefore, it seems likely that the physiological function of the ferredoxin of *Pyrobaculum islandicum* is an electron-sink for the redox enzymes participated in the glycolytic metabolism and to transfer the electrons to cytochrome *b*-559 in the respiratory chain.

P. islandicum contains a membrane-bound ferredoxin oxidase (cytochrome *b*-559), hydrogenase and sulfur reductase (Chapters 2 and 3). Also a succinate dehydrogenase, a 2-oxoacid: ferredoxin oxidoreductase and malate dehydrogenase activity has been found in cell-free extract (Seilig and Schönheit, 1994). From these results, I propose an electron transfer pathway of *P. islandicum* as shown in Fig. 4-1. Cytochrome *b*-559 accepts electrons from the ferredoxin which is reduced by the enzymes participated in citric acid cycle.

However, I have not find the respiratory enzymes with proton-pumping activity in the membanes of *P. islandicum*. Therefore, the mechanism of ATP synthesis at 100°C remains unsolved. It should be studied in future, including heat-stability of the respiratory enzymes and the universal metabolites such as ATP and NADH at 100 °C and over.

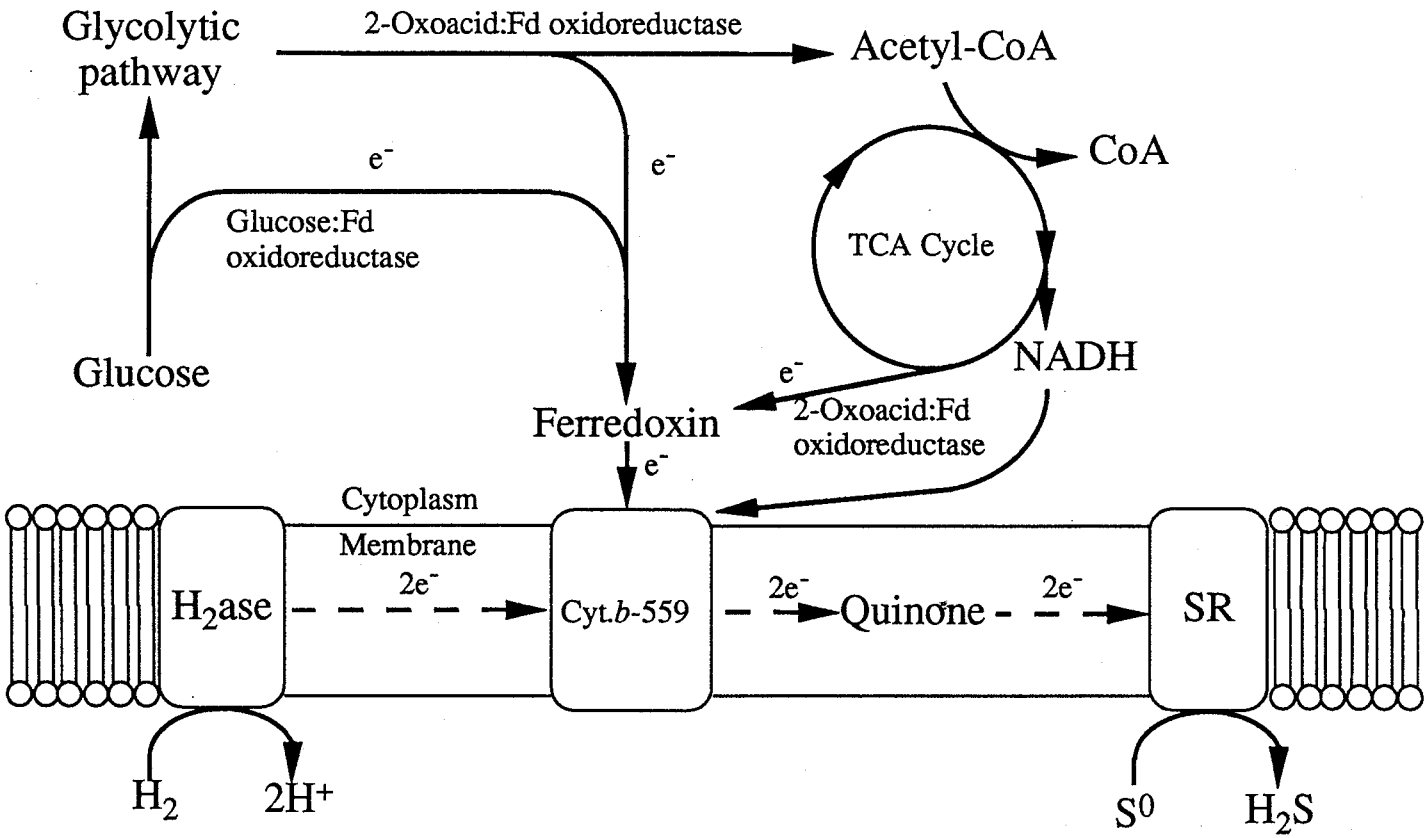

Fig. 4-1. Proposed electron transport chain of *P. islandicum.*

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I want to express my thanks to many laboratory colleagues for their encouragement.

Y. Nakajima

Yasuhiro Nakajima
January, 1998