Purification and Biochemical Characterization of the F1-ATPase from Acidithiobacillus ferrooxidans NASF-1 and Analysis of the atp Operon

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ATPase was purified 51-fold from a chemoaerotrophic, obligately acidophilic iron-oxidizing bacterium, Acidithiobacillus ferrooxidans NASF-1. The purified ATPase showed the typical subunit pattern of the F1-ATPase on a polyacrylamide gel containing sodium dodecyl sulfate, with 5 subunits of apparent molecular masses of 55, 50, 33, 20, and 18 kDa. The enzyme hydrolyzed ATP, GTP, and ITP, but neither UTP nor ADP. The Km value for ATP was 1.8 μM. ATPase activity was optimum at pH 8.5 at 45°C, and was activated by sulfate. Azide strongly inhibited the enzyme activity, whereas the enzyme was relatively resistant to vanadate, nitrate, and N,N-dicyclohexylcarbodiimide. The genes encoding the subunits for the F1F0-ATPase from Acidithiobacillus ferrooxidans NASF-1 were cloned as three overlapping fragments by PCR cloning and sequenced. The molecular masses of the α, β, γ, δ, and ε subunits of the F1 portion were deduced from the amino acid sequences to be 55.5, 50.5, 33.1, 19.2, and 15.1 kDa, respectively.

Key words: Acidithiobacillus ferrooxidans; acidophile; F1F0-ATPase; atp operon

ATP synthesis in bacteria, mitochondria, and chloroplast is catalyzed by membrane-bound F1F0-ATPase, fueled by an electrochemical gradient of proton or, in some cases, sodium ion.1–3 From prokaryotes to eukaryotes, the F1F0-ATPase has a large number of subunits and high structural complexity. It is composed of two major portions: a cytoplasmic F1 portion (αβγδε) including catalytic sites for ATP synthesis/hydrolysis and a membrane-embedded F0 portion (αβγδε) constituting a proton channel. These two portions are structurally connected by two stalks, a central stalk constituted by γ and ε subunits and an outer stalk constituted by δ and β subunits linking αβγδε to the a subunit. Recent studies indicate that the F1F0 complex works through a rotational catalysis.4–7 The proton or sodium ion flux across the membrane induces rotation of the rotor ring (c10–14). The rotation of the asymmetrically bent γ subunit within the central cavity of the αβγδε cylinder elicits periodic structural changes in the catalytic β subunits, which are instrumental in ATP synthesis. Acidithiobacillus ferrooxidans (formerly Thiobacillus ferrooxidans) is a chemoaerotrophic, obligately acidophilic bacterium obtaining energy for growth and CO2 fixation from the oxidation of ferrous iron and/or reduced inorganic sulfur compounds, and having an optimum growth pH at 2–3.8,9 Although enzymes in periplasm and outer membrane from this bacterium have an optimum pH around 3, the cytoplasmic pH is thought to be 6–7.10 Thus the bacterium has a large ΔpH gradient between cytoplasm and growth-medium. Nevertheless, ATP synthesis of this bacterium has been known to be catalyzed by an ATPase fueled by a proton gradient generated during electron transfer from Fe2+ to O2.11,12 The existence of F-type ATPase in A. ferrooxidans has been shown by experiments complementing unc mutants of Escherichia coli.13 Clones containing five genes encoding the subunits of the F1 portion and two genes encoding the subunits (b and c) of the F0 portion have been isolated, and the nucleotide sequences of the genes have been determined.13 The five subunits of the F1 portion of Acidithiobacillus ferrooxidans ATPase formed a functional association with the subunits of the F0 portion of Escherichia coli ATPase. However, a clone that was introduced genes for the c and b subunits of A. ferrooxidans ATPase did not complement the corresponding E. coli unc mutants. These results suggest that A. ferrooxidans possesses F-type ATPase, and that subunits of the F0 portion of A. ferrooxidans ATPase are different from those of E. coli ATPase. The characteristics of ATPase partially purified from Thiobacillus ferrooxidans have been reported by Adapoe and Silver.14 Although the ATPase of this bacterium is thought to play a critical role in the conversion of energy from iron or sulfur oxidation and in the transport of ions, no additional information on the characteristics of the

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ATPase from this bacterium is available. In this report we describe some additional characteristics of an ATPase partially purified from *A. ferrooxidans* NASF-1 cells and an analysis of genes encoding *F*-ATPase of *A. ferrooxidans* NASF-1.

**Materials and Methods**

*Microorganisms, plasmid, media, and cultivation.* *A. ferrooxidans* NASF-1 was used in this study and cultured in iron medium, as described previously.\(^{15}\) *E. coli* Top10 was cultured in LB (Luria-Bertani) medium. An LB agar plate containing 50 μg/ml kanamycin was used for selection and isolation of transformants. Plasmid pCR-XL-TOPO vector (Invirtogen, Carlsbad, CA.) was used to clone *atp* genes amplified by PCR.

*Solubilization and purification of ATPase.* Intact cells were prepared as described previously.\(^{15}\) The intact cells were suspended in buffer A [50 mM Tris–SO\(_4\) (pH 8.0) containing 10 mM MgSO\(_4\) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], and were broken by passing them through a French press cell at 1500 kg/cm\(^2\). The homogenate was centrifuged at 12,000 × g for 10 min to obtain cell-free extract. The cell-free extract was further centrifuged at 105,000 × g for 60 min. The pellet was washed with buffer A and used as a membrane fraction. To extract ATPase from the membrane, membrane vesicles (20 mg/ml) were homogenized with buffer B [50 mM Tris–SO\(_4\) (pH 8.0) containing 10 mM MgSO\(_4\), 0.5 mM PMSF, 200 mM Na\(_2\)SO\(_4\) and 0.5% (w/v) n-Dodecyl-β-D-maltopyranoside (DM)]. After 1 h of incubation with constant stirring at 4 °C, the nonsolubilized material was removed by ultracentrifugation (105,000 × g for 60 min). The solubilized ATPase in the supernatant was loaded onto a Butyl-Toyopearl column (1.6 × 10 cm) equilibrated with buffer B. The column was successively washed with 50 ml of buffer B, 50 ml of buffer A containing 100 mM Na\(_2\)SO\(_4\) and 0.5% DM, and 50 ml of buffer A containing 0.5% DM. ATPase activity was eluted at the final washing step. The active fraction was loaded onto a DEAE-Toyopearl column (1.6 × 2 cm) equilibrated with buffer A containing 0.5% DM. The column was successively washed with 20 ml of buffer A, 20 ml of buffer containing 50 mM Na\(_2\)SO\(_4\), 20 ml of buffer A containing 100 mM Na\(_2\)SO\(_4\), and finally 20 ml of buffer A containing 300 mM Na\(_2\)SO\(_4\). ATPase activity was eluted at 100 mM Na\(_2\)SO\(_4\). The active fraction was concentrated by Centricut U-10 (Kurarbo Biomedical, Osaka, Japan). The concentrated solution was applied to Native polyacrylamide gel electrophoresis (Native-PAGE) using 7.5% (w/v) polyacrylamide gel, as described by Davis.\(^{16}\) The gel was overlayed with 1% (w/v) Tween 20 was used as a running buffer for electrophoresis. After electrophoresis, proteins in the gels were stained with silver and with the ATPase activity.\(^{17}\) The gel piece involving ATPase activity was excised from the gel and homogenized in buffer A containing 0.5% DM to extract the ATPase protein. The extracted solution was concentrated by Centricut U-10.

**ATPase activity measurement.** ATPase activity was measured at 40 °C for 10 min in a mixture containing 100 mM Tris–SO\(_4\) (pH 8.5), 5 mM MgSO\(_4\), 5% (v/v) glycerol, 10 mM Na\(_2\)SO\(_4\), and 0.02% DM. The reaction was initiated by adding 3 mM ATP (final concentration), and stopped with 7% trichloroacetic acid (final concentration). ATPase activity was calculated using a colorimetric assay that measures the amount of inorganic phosphate (Pi) liberated from ATP.\(^{18}\) When the effect of inhibitors was tested, membrane or purified ATPase was incubated with inhibitors at 40 °C for 10 min before analysis. To determine the optimal temperature for ATPase activity, the reaction mixture containing membrane or purified ATPase was preincubated in the absence of ATP at each temperature for 10 min, and then ATP (3 mM) was added. The mixture was incubated at the same temperature for 10 min. One unit of ATPase activity was defined as the amount of enzyme that liberated 1 μmol of Pi per min.

**Preparation of DNA probes and southern hybridization.* Genomic DNA (gDNA) from NASF-1 cells was prepared with phenol/chloroform/isoamylalcohol, as described previously.\(^{15}\) The primers used to produce a probe for the *atpD* gene (Fig. 1), PAF03 (5'-AAAGGCGGTTAAGGTCGTTCTCT-3') and PAR02 (5'-GACAATTTCATCCATGCCAAGTGG-3'), were designed based on the amino acid sequences conserved in β subunits of five bacteria, *A. ferrooxidans* ATCC 33020 (accession no. M81087), *E. coli* (M25464), *Enterococcus faecalis* (M90060), *Streptococcus mutans* (U31170), and *Desulfovibrio vulgaris* (AB022018). La Tag polymerase (TaKaRa Bio, Ohtsu, Japan) was used to amplify the DNA fragment. To produce a probe for the *atpH* gene (Fig. 1), primers, PADF02 (5'-GATCCGCGATGTACCGGCCG-3') and PARR01 (5'-TTAGCTTCGCAAGGGTTCCGGG-3'), were designed based on the partial nucleotide sequence of the *atpH* gene determined in this study. The PCR-amplified DNA fragments were purified, sequenced, and labeled with digoxigenin (DIG), as described previously.\(^{15}\) Southern blotting analysis was performed to hybridize the gDNA digested with restriction enzymes and DNA fragments amplified by PCR, as described previously.\(^{15}\) The sequences were determined as described previously.\(^{15}\)

**Analysis of the atp operon of *A. ferrooxidans* NASF-1.** PCR cloning was employed to determine the nucleotide sequences of *atp* genes encoding *F*-ATPase of *A. ferrooxidans* NASF-1. Primer pairs, C1 (5'-GATCGCGATGTACCGGCCG-3') and LAR01 (5'-AACAATTCATCCATGCCAAGTGG-3')
GGCAATGAT-3'), PABF01 (5'-CGCGGGCTGACC-GTCACCTCGT-3') and TAIL3 (5'-WGTGNAGWAN-CANAGA-3'), and PAAF01 (5'-TGCACCGGCAGA-GACAATGCA-3') and LADR02 (5'-CTCGAGCGCG-GACTGCACAACCGTCTTCTG-3'), were used to amplify the atp genes, as shown in Fig. 1. Each PCR reaction was separately carried out twice. Each sequence was determined using two clones separately obtained from the PCR reactions.

Database analysis. The sequence data of genes for F$_1$F$_0$-ATPase from A. ferrooxidans ATCC 23270 were obtained from the Institute for Genomic Research (http://www.tigr.org). Blast searches and CLUSTALW analyses were carried out through the web sites at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and at the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/search/clustalw-j.html) respectively.

Nucleotide sequence accession number. The DNA sequence encoding the ATPase of A. ferrooxidans NASF-1 has been submitted to the DNA Data Bank of Japan under accession no. AB206839.

Results and Discussion

Properties of membrane-bound ATPase of A. ferrooxidans NASF-1

Properties of ATPase activity in membrane from A. ferrooxidans NASF-1 were initially examined, and are summarized in Fig. 2. ATPase activity showed the pH optimum at 8.5 (Fig. 2A). Some ATPases are known as an Na$^+$-dependent ATPase specifically activated by Na$^+$ or Li$^+$. Hence the effect of NaCl on the activity was examined. Although the optimum pH did not change in the presence of NaCl, it inhibited ATPase activity (Fig. 2A). The results indicated that A. ferrooxidans NASF-1 cells did not possess Na$^+$-dependent ATPases. Since a distinctive pH-peak was not detected at around 5 or 6, ATPase activity was measured at pH 8.5. The optimum temperature for membrane-bound ATPase activity was 45 °C (Fig. 2B). Sulfite has been known to stimulate the activity of chloroplast F-type ATPase, archaebacterial ATPase, and yeast vacuolar ATPase. The activity of membrane-bound ATPase from A. ferrooxidans NASF-1 was stimulated by sulfite, whereas sulfate slightly inhibited the activity (Fig. 2C). The optimum pH measured in the presence of 10 mM sulfite did not change (data not shown).
Table 1. Purification of ATPase from the Membrane of A. ferrooxidans NASF-1

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (U)*</th>
<th>Sp Ac (U/mg)</th>
<th>Purification (fold)</th>
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<tbody>
<tr>
<td>Membrane</td>
<td>410</td>
<td>275</td>
<td>0.67</td>
<td>1</td>
</tr>
<tr>
<td>Extraction</td>
<td>76</td>
<td>71</td>
<td>0.93</td>
<td>1.4</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>4.3</td>
<td>24</td>
<td>5.64</td>
<td>8.4</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>0.79</td>
<td>13</td>
<td>16.43</td>
<td>24.5</td>
</tr>
<tr>
<td>Gel-cut (protein 2)</td>
<td>0.018</td>
<td>0.61</td>
<td>34.12</td>
<td>50.9</td>
</tr>
</tbody>
</table>

*One unit equaled 1 μmol of inorganic phosphate produced per min.

Fig. 3. Polyacrylamide Gel Electrophoreses of ATPase from A. ferrooxidans NASF-1.
A. Native-PAGE of the active fraction from DEAE-Toyopearl chromatography. Two protein bands (1 and 2) are indicated by arrowheads. Gel was stained with silver. B, SDS–PAGE of the protein (protein 2) having a higher mobility on Native-PAGE (A) and extracted from the gel. The gel was stained with silver. M; molecular weight standard in kDa.

Properties of F1-ATPase purified from A. ferrooxidans NASF-1

The purified enzyme was remarkably stable at 4°C in 50 mM Tris–SO₄ buffer (pH 8.5); approximately 95% of the activity was retained after 8 d (data not shown). The optimum pH for ATPase activity was 8.5, and ATPase activity decreased markedly below 7.0 or above 9.5 (Fig. 4A). The optimum pH for T. ferrooxidans TM ATPase activity has been reported to be 9–10. The optimum temperature for A. ferrooxidans NASF-1 ATPase activity was 45°C (Fig. 4B). ATPase activity was stimulated approximately 4-fold by sulfate at a concentration of 50 mM, whereas sulfate slightly inhibited it (Fig. 4C). Although a sulfite-dependent ATPase from Thiobacillus thiooxidans has been reported, its TM ATPase activity was not affected by sulfate ion. It has been reported that activation of chloroplast ATPase activity by sulfite parallels the release of tightly bound ADP from a catalytic site. In Rhodobacter capsulatus, the sulfite-activated state is unable to translocate protons across the membrane or to promote chemiosmotic ATP synthesis, and the mechanism of sulfite activation probably involves two binding sites, with which phosphate binding can interfere.

In Paracoccus denitrificans, sulfite functioned as a non-essential activator that slightly modified the affinity for ATP and increased the Vₘₐₓ. Since sulfite is an intermediate of sulfur oxidation in A. ferrooxidans, activation of ATPase activity by sulfite ion appeared to be possible in A. ferrooxidans cell. Mg²⁺ was the most effective ion for ATPase activity. In T. ferrooxidans TM, Mn²⁺, Ca²⁺, and, to a lesser extent, Co²⁺ and Zn²⁺, have been reported to replace Mg²⁺ for ATPase activity. Approximately 20% of A. ferrooxidans NASF-1 ATPase activity detected in a complete reaction mixture containing 5 mM MgSO₄ was detected when a reaction mixture containing 5 mM MnSO₄ or CaSO₄ was used (Fig. 4D). Chloride ion showed an inhibitory effect on ATPase activity. Fe²⁺,
Fe³⁺, Ca²⁺, and Na⁺ at a concentration of 1 mM did not affect ATPase activity measured in the presence of 5 mM MgSO₄, whereas Ni²⁺, Zn²⁺, Cu²⁺, and Mn²⁺ at a concentration of 1 mM reduced ATPase activity measured in the presence of 5 mM MgSO₄ by 47, 80, 99, and 50%, respectively.

The relative activity of phosphate hydrolysis from various nucleotide phosphates was measured. *A. ferrooxidans* NASF-1 ATPase appeared to be specific to purine nucleotides, such as ATP, GTP, and ITP. When GTP and ITP were used as substrates, 120 and 33% of ATPase activity was detected, respectively. UTP and ADP were not hydrolyzed by the enzyme. *T. ferrooxidans* TM ATPase has been reported to use ITP as a better substrate than ATP.¹⁴) Since we did not use the ATP regeneration system for the measurement of ATPase activity, a correct $K_m$ value for ATP was not determined. An apparent $K_m$ of the enzyme for ATP was, however, estimated to be 1.8 mM at pH 8.5 at 40°C. A $K_m$ value of 7.75 mM for ATP has been reported in *T. ferrooxidans* TM ATPase.¹⁴)

The sensitivity of purified *A. ferrooxidans* NASF-1 ATPase activity to some of the specific inhibitors was examined and compared with activity in the absence of the inhibitors. Azide (2 mM), known as an inhibitor of F-type ATPase, showed a significant inhibitory effect (91% inhibition) on the activity. Vanadate (1 mM), an inhibitor of ion-translocating ATPase, and NaNO₃ (20 mM), an inhibitor of V-type ATPase, reduced *A. ferrooxidans* NASF-1 ATPase activity by 2 and 22%, respectively, whereas $N,N'$-dicyclohexylcarbodi-imide (DCCD, 0.1 mM) increased ATPase activity by 20%. ATPase activity was scarcely inhibited by DCCD at concentrations of up to 1 mM. Since the ATPase purified from *A. ferrooxidans* NASF-1 did not have the Fₗ portion of FₗFₒ-ATPase and DCCD binds to sites on the c subunit, the effect of DCCD on purified *A. ferrooxidans* NASF-1 ATPase activity appeared to be reasonable. The reason for slight activation of *A. ferrooxidans* NASF-1 ATPase activity by DCCD at 0.1 mM is unclear. *T. ferrooxidans* Fₗ/Fₒ-E. coli Fₗ/Fₒ hybrid ATPase expressed in E. coli was also more resistant to inhibitors DCCD and azide than E. coli Fₗ/Fₒ-ATPase, suggested both poor coupling and reduced subunit co-operativity.¹³) The results obtained in this study using specific inhibitors indicate that the ATPase purified from *A. ferrooxidans* NASF-1 cells was an Fₗ-ATPase. The biochemical properties of ATPases from *A. ferrooxidans* NASF-1 and other *Thiobacillus* strains are summarized in Table 2.

Although *A. ferrooxidans* is an acidophile and grows optimally within a pH range 1.5–3.5, the cytoplasmic pH of the cells is maintained near neutral (pH 6–7).¹⁰) Therefore, this bacterium must possess some mechanism to maintain neutral cytoplasmic pH. We expected that ATPase from *A. ferrooxidans* NASF-1 would show optimum pH for activity at around 5, because ATPase of an acidothermophilic archaeabacterium, *Sulfolobus acidocaldarius*, has done so.²⁵) *A. ferrooxidans* NASF-1 ATPase activity unexpectedly showed a pH optimum at alkaline pH (8.5), similarly to *T. ferrooxidans* TM.
ATPase activity (pH 9–10).\textsuperscript{14} The pH optima for the activity of ATPases from \textit{A. ferrooxidans} strains might indicate that the ATPases function exclusively to synthesize ATP using the proton gradient formed during iron or sulfur oxidation, not to hydrolyze ATP, because the cytoplasmic pH is thought to be 6–7 and ATPase activity was very low under the physiological condition (Fig. 4A). Although we tried to purify an ATPase in the form of F$_1$F$_0$-complex from \textit{A. ferrooxidans} NASF-1 cells, the ATPase purified from the membrane did not contain the F$_0$-portion. Hence, we could not measure ATP synthase activity. Isolation of ATPase as the F$_1$F$_0$-ATPase of \textit{A. ferrooxidans} NASF-1 was not conserved in the c subunit of the \textit{A. ferrooxidans} NASF-1 and ATCC 23270, although the positions of the amino acid sequences of the ATPases from both \textit{A. ferrooxidans} NASF-1 and \textit{A. ferrooxidans} ATCC 23270 were closely related to the molecular masses deduced from the amino acid sequences of the F$_1$-ATPase subunits. The Walker-A motif (\textit{\beta}$149\textit{GGAGVGKT}$\gamma$156 in \textit{E. coli} ATPase numbering),\textsuperscript{31} the Walker-B motif (\textit{\beta}$239\textit{LLFVD}$\gamma$243),\textsuperscript{31} the nucleotide binding region sequence (\alpha$169\textit{GDRQ}$\gamma$176), the \gamma M23 residue necessary for the coupling of proton conduction with ATP synthesis,\textsuperscript{32–34} residues (\alpha$541$, \beta$381$, and \gamma$87$) involved in the rotation mechanism,\textsuperscript{32,35} the residue (\textit{\beta}R36) necessary for the retention of coupling,\textsuperscript{36} and the sequence (c$141\textit{RQP}$c$143$) connecting two helical region involved in the rotation mechanism,\textsuperscript{32,35} was conserved in the amino acid sequences of the ATPases from both \textit{A. ferrooxidans} NASF-1 and ATCC 23270, although the positions and the sequences were slightly different from those of \textit{E. coli} ATPase.

Proton-binding known to occur at site c$\delta 61$ in \textit{E. coli} ATPase\textsuperscript{7} was not conserved in the c subunit of the ATPases from \textit{A. ferrooxidans} NASF-1, ATCC 23270, or ATCC 33020. Glutamate residue instead of aspartate

### Table 2. Comparison of Properties of ATPase from \textit{A. ferrooxidans} NASF-1 with Those from Other \textit{Thiobacillus} Strains

<table>
<thead>
<tr>
<th>Source</th>
<th>\textit{A. ferrooxidans} NASF-1</th>
<th>\textit{T. ferrooxidans} TM</th>
<th>\textit{T. thiooxidans}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>This study</td>
<td>14</td>
<td>26, 27</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>8.5</td>
<td>9–10</td>
<td>7.5–8.0</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
<td>45</td>
<td>ND$^a$</td>
<td>ND</td>
</tr>
<tr>
<td>Substrate specificity</td>
<td>GTP &gt; ATP &gt; ITP</td>
<td>ITP &gt; GTP &gt; ATP</td>
<td>NT$^b$</td>
</tr>
<tr>
<td>$K_a$ for ATP (mM)</td>
<td>1.8</td>
<td>7.75</td>
<td>0.7</td>
</tr>
<tr>
<td>Requirement of cation</td>
<td>Mg$^{2+}$ &gt; Ca$^{2+}$ &gt; Mn$^{2+}$</td>
<td>Mg$^{2+}$ &gt; Mn$^{2+}$ &gt; Ca$^{2+}$</td>
<td>Mg$^{2+}$ &gt; Ca$^{2+}$</td>
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<tr>
<td>Activation by SO$_4^{2-}$</td>
<td>+</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Inhibition by Azide</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Inhibition by nitrate and vanadate</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$not determined, $^b$not tested.

\textbf{Analysis of F$_1$F$_0$-ATPase operon from \textit{A. ferrooxidans} NASF-1}

The nucleotide sequence of the operon encoding F$_1$F$_0$-ATPase of \textit{A. ferrooxidans} ATCC 33020 has been partially determined by complementing \textit{E. coli} F$_1$ mutants.\textsuperscript{13} The sequence data for \textit{A. ferrooxidans} ATCC 23270 is also available from the Institute for Genomic Research (http://www.tigr.org). Although, in autotrophic bacteria such as the cyanobacteria, the genes for the F$_0$ subunits are located on a locus distinctly separated from the F$_1$ cluster,\textsuperscript{30} the \textit{atp} operon of \textit{A. ferrooxidans} has been known to exist as a single copy in the genome and to be arranged in a manner most like that of the \gamma-proteobacteria such as \textit{E. coli}.\textsuperscript{13} PCR cloning was employed to obtain information on the gene structure of the \textit{atp} operon from \textit{A. ferrooxidans} NASF-1.

The whole nucleotide sequence of the operon of the F$_1$F$_0$-ATPase genes from \textit{A. ferrooxidans} NASF-1 was determined by sequencing the 4.3 kb, 2.3 kb, and 6.0 kb PCR-products, as shown in Fig. 1. The 4.3 kb fragment contained the entire \textit{atpA} (\alpha subunit) and \textit{atpG} (\gamma subunit) genes and partial \textit{atpH} (\delta subunit) and \textit{atpD} (\beta subunit) genes of the F$_1$ portion. The 2.3 kb fragment contained the entire \textit{atpD} (\beta subunit) and \textit{atpC} (\epsilon subunit) genes of the F$_1$ portion. The 6.0 kb fragment contained the \textit{atpl}, B, E, F, and H genes encoding the i, a, c, b, and \delta subunits of F$_1$F$_0$-ATPase, respectively. The \textit{atp} operon from \textit{A. ferrooxidans} NASF-1 consisted of 8,663 bp. The molecular weights estimated based on the deduced amino acid sequences of the i, a, c, b, \delta, \alpha, \gamma, \beta, and \epsilon subunits of \textit{A. ferrooxidans} NASF-1 F$_1$F$_0$-ATPase were 13.5, 27.5, 8.9, 17.8, 19.2, 55.3, 33.1, 50.5, and 15.1 kDa, respectively. The apparent molecular masses (55, 50, 33, 20, and 18 kDa) for the subunits of purified \textit{A. ferrooxidans} NASF-1 ATPase were closely related to the molecular masses deduced from the amino acid sequences of the F$_1$-ATPase subunits. The Walker-A motif (\textit{\beta}$149\textit{GGAGVGKT}$\gamma$156$ in \textit{E. coli} ATPase numbering),\textsuperscript{31} the Walker-B motif (\textit{\beta}$239\textit{LLFVD}$\gamma$243$),\textsuperscript{31} the nucleotide binding region sequence (\alpha$169\textit{GDRQ}$\gamma$176$), the \gamma M23 residue necessary for the coupling of proton conduction with ATP synthesis,\textsuperscript{32–34} residues (\alpha$541$, \beta$381$, and \gamma$87$) involved in the rotation mechanism,\textsuperscript{32,35} the residue (\textit{\beta}R36) necessary for the retention of coupling,\textsuperscript{36} and the sequence (c$141\textit{RQP}$c$143$) connecting two helical region involved in the rotation mechanism,\textsuperscript{32,35} was conserved in the amino acid sequences of the ATPases from both \textit{A. ferrooxidans} NASF-1 and ATCC 23270, although the positions and the sequences were slightly different from those of \textit{E. coli} ATPase.

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residue was deduced at position 63 of the c subunit in A. ferrooxidans ATPases, such that it appeared possible that carboxyl residue might play a role similar to that of aspartate residue in E. coli ATPase. The essential residue ar210 was postulated to interact with cd61 during protonation/deprotonation in E. coli ATPase.38 Since the N-terminal of the a subunit of A. ferrooxidans ATPases is approximately 20 residues shorter than that of E. coli ATPase, the residue corresponding to ar210 in E. coli ATPase was conserved at position 188 in the a subunit of ATPases of A. ferrooxidans ATCC 33020. The atpE gene (encoding the c subunit) from A. ferrooxidans ATCC 33020 did not complement the E. coli uncE mutant.13 Although the necessity of co-expression of the atpE (c subunit) and atpB (a subunit) genes in the uncE mutant and the toxicity of the c subunit of A. ferrooxidans ATCC 33020 in E. coli cells have been pointed out,13 the shorter N-terminal of the a subunit of A. ferrooxidans ATPase and the change of glutamine residue to aspartate residue in the c subunit of the A. ferrooxidans ATPase might result in the failure of complementation of the E. coli uncE mutant. Since the genes for the F1F0-ATPase of A. ferrooxidans are available, it is possible and would be interesting to complement the E. coli F0 mutant by using the genes.

Acknowledgment

Preliminary sequence data for A. ferrooxidans ATCC 23270 was obtained from the Institute for Genomic Research (http://www.tigr.org).

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