Crystal structure of lipoate-protein ligase A from Escherichia coli: Determination of the lipoic acid-binding site

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Crystal Structure of Lipoate-Protein Ligase A from Escherichia coli

DETERMINATION OF THE LIPOIC ACID-BINDING SITE*

Lipoate-protein ligase A (LplA) catalyzes the formation of lipoyl-AMP from lipoate and ATP and then transfers the lipoyl moiety to a specific lysine residue on the acyltransferase subunit of α-ketoacid dehydrogenase complexes and on H-protein of the glycine cleavage system. The lysyllysine arm plays a pivotal role in the complexes by shuttling the reaction intermediate and reducing equivalents between the active sites of the components of the complex. We have determined the X-ray crystal structures of Escherichia coli LplA alone and in a complex with lipoic acid at 2.4 and 2.9 Å resolution, respectively. The structure of LplA consists of a large N-terminal domain and a small C-terminal domain. The structure identifies the substrate binding pocket at the interface between the two domains. Lipoic acid is bound in a hydrophobic cavity in the N-terminal domain through hydrophobic interactions and a weak hydrogen bond between the carboxyl group of lipoic acid and the Ser-72 or Arg-140 residue of LplA. No large conformational change was observed in the main chain structure upon the binding of lipoic acid.

Lipoic acid is a prosthetic group of acyltransferase (E2) subunit of the pyruvate, α-ketoglutarate, and branched-chain α-ketoacid dehydrogenase complexes and of H-protein of the glycine cleavage system (1–4). It attaches to a specific lysine residue on the proteins via an amide linkage between the ε-amino group of the lysine residue and the carboxyl group of lipoic acid. In the reaction sequence of the complexes, the lypoalline arm shuttles the reaction intermediates and reducing equivalents between the active sites of the components of the complexes.

The attachment of lipoic acid to the proteins occurs by two-step reactions in which a lipoyl-AMP intermediate is formed from lipoic acid and ATP, and pyrophosphate is released in the initial activation reaction (Reaction 1).

\[
\text{Lipoate + ATP} \rightarrow \text{lipoyl-AMP} + \text{PP}_i
\]

REACTION 1

The lipoyl moiety of the intermediate is then transferred to apoproteins in the second transfer reaction, yielding the lipoylated protein and AMP (Reaction 2).

\[
\text{Lipoyl-AMP + apoprotein} \rightarrow \text{lipoylated protein + AMP}
\]

REACTION 2

The abbreviations used are: LplA, lipoate-protein ligase A; Se-LplA, selenomethionine-substituted LplA; APMSF, (p-amidinophenyl) methanesulfonyl fluoride hydrochloride; DTT, dithiothreitol; r.m.s., root mean square.
EXPERIMENTAL PROCEDURES

Construction of the Expression Vector Containing lplA Gene—DNA manipulations were accomplished by the standard techniques (12). The gene encoding LplA was amplified by a PCR from the E. coli BL21(DE3) gene using a pair of oligonucleotides, 5'-AGGGTACCATATGGTCCATCATACGTCCTGACTGCA-3' (Ndel, underlined), Met (boldface letters), and nucleotides 86–104 (5)) and 5'-AGGGATCCTACCTACGCGCCGCGCAT-3' (BamHI (underlined), Stop codon (boldface letters), and nucleotides 1096–1079) designed on the basis of the lplA sequence. The PCR product was inserted into the pET-3a expression vector (13).

The bacterial cells were harvested by centrifugation. The bacterial cells were suspended in 40 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 40 mM (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (APMSF), and 10 μg/ml leupeptin and lysed by sonication. The cell extract was digested with NdeI and BamHI and the pair of the PCR products, the resultant plasmid was transformed into E. coli expression strain BL21(DE3)pLysS or B834(DE3)pLysS to obtain native LplA or selenomethionine-substituted LplA (Se-LplA), respectively. Cells were cultured in M9ZB (13) or Doublé and Carter medium supplemented with selenomethionine (30 mg/liter) (14) containing 25 μg/ml ampicillin, 25 μg/ml chloramphenicol, and 25 μl isopropyl-β-D-thiogalactopyranoside at 30 °C for 22 h. The bacterial cells were harvested by centrifugation.

Purification of the Recombinant LplA—Both LplA and Se-LplA were purified to homogeneity by the following method. E. coli cells were resuspended in 40 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 40 mM (p-amidinophenyl) methanesulfonyl fluoride hydrochloride (APMSF), and 10 μg/ml leupeptin and lysed by sonication. The cell extract was obtained by centrifugation at 105,000 × g for 1 h and applied onto a hydroxyapatite column (3 × 6 cm) equilibrated with 3 mM potassium phosphate buffer, pH 7.4. LplA was eluted with 4 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM DTT and 10 μM APMSF and further purified by a HiPrep 16/10 DEAE column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.5 mM DTT and 10 μM APMSF. Proteins retained were eluted with a linear gradient of 0–0.2 M NaCl in the equilibration buffer.

Mutation of LplA—Mutations of S72A and R140A were introduced using an In-Fusion PCR Cloning kit (BD Biosciences Clontech). For the S72A mutation, PCRs were carried out employing pET-LPLA as a template and primer 1 (5’-AAGGAGATATACATATGTCCACAT-3’) and primer 2 (5’-GCCACCCTGCATGCGCCGGCCGCAG-3’) (nucleotides 307–278; modified bases are shown in boldface letters) to amplify the N-terminal half and primer 3 (5’-GCCACCCTGCATGCGCCGGCCGCAG-3’) and primer 2 (5’-GCCACCCTGCATGCGCCGGCCGCAG-3’) (nucleotides 293–326)) and primer 4 (5’-GTTAGCGCCCGGATCTAC-3’) (pET-3a sequence (underlined) and then nucleotides 83–97 (5’)) to amplify the N-terminal half and primer 3 (5’-GCCACCCTGCATGCGCCGGCCGCAG-3’) (nucleotides 307–278; modified bases are shown in boldface letters)) to amplify the N-terminal half and primer 3 (5’-GCCACCCTGCATGCGCCGGCCGCAG-3’) (nucleotides 293–326)) and primer 4 (5’-GTTAGCGCCCGGATCTAC-3’) (pET-3a sequence (underlined) and nucleotides 1099–1096)) to amplify the C-terminal half. For the R140A mutation, primer 1 and primer 2 (5’-GGTTCGCATAGGCGCGGCGGTGTTAGCGCCCGGATCTAC-3’) (pET-3a sequence (underlined) and nucleotides 1099–1096)) to amplify the C-terminal half. For the R140A mutation, primer 1 and primer 2 (5’-GGTTCGCATAGGCGCGGCGGTGTTAGCGCCCGGATCTAC-3’) (pET-3a sequence (underlined) and nucleotides 1099–1096)) to amplify the C-terminal half.
except that *E. coli* cells were grown in the medium without an addition of lipoic acid (15).

**Crystallization**—Crystallization was carried out at 20 °C by the hanging drop-vapor diffusion method using a protein solution dialyzed against 5 mM HEPES-Na, pH 7.5, and 3 mM DTT. Se-LplA was crystallized in a droplet containing a 1:1 ratio of protein solution at 3 mg/ml and reservoir solutions of 12.5–14.5% ethylene glycol and 21% glycerol. Crystals of Se-LplA/lipoate complex was grown with a modified protein solution at 5 mg/ml and a reservoir solution containing 3 mM R-(+) lipoate.

**Data Collection**—All X-ray data were collected at 100 K on the beamline BL44XU at SPring-8 (Hyogo, Japan). The structure of Se-LplA was solved using Se-SAD methods (16). Before data collection, wavelength was calibrated by fluorescence scan of the Se-LplA crystal, and a wavelength was selected for data collection corresponding to the white line (peak, λ = 0.9796 Å). Data were collected in a 180° sweep in 1° oscillation steps, using an imaging plate detector, and then integrated and scaled with the programs MOSFLM (17) and SCALA (18). The data collection for crystal of Se-LplA/lipoate complex was carried out with a wavelength of 0.9215 Å sweeping 90° in 1° oscillation steps. The crystal containing lipoate was very weak against radiation damage; therefore, two data sets had to be merged to get enough data for the structure determination. Each data set was indexed and integrated with DENZO and then scaled and merged with SCALEPACK (19). The LplA crystals belong to space group C222₁, with unit cell dimensions of *a* = 81.6 Å, *b* = 112.1 Å, and *c* = 289.2 Å for Se-LplA crystal used for structure determination.

**Structure Determination**—All of the 15 expected selenium sites (for three monomers containing 5 Se-Met residues/asymmetric unit) were found using the heavy atom search program in CNS (20), and the initial phases were calculated with SHARP (21). 2.8 Å resolution maps calculated after density modification by SOLOMON (22) implemented in SHARP was used for determination of molecule mask and noncrystallographic symmetry operators for further density improvement. Solvent flattening, histogram mapping, and noncrystallographic symmetry averaging were applied for phase improvement using DM (23), and the resulting map was used for model building. The structure of Se-LplA/lipoate was solved by the molecular replacement method with MOLREP (24) in the CCP4 suite (25) using Se-LplA structure as a search model.

**Model Building and Refinement**—Model building and refinement for all structures were performed using the programs O (26) and REFMAC5 (27) in CCP4 suite (25), respectively. Refinement for Se-LplA was monitored with the Mac5 software (27, 28) implemented in CNS (20). After precession for 90 s, the reaction was started by the addition of ATP or lipoic acid and carried out for 5 min at 37 °C. The reaction was terminated by heating the mixture at 95 °C for 10 min. The amount of lipoylated H-protein in the mixture was determined by the glycine-CO₂ exchange activity using *E. coli* P-protein as a lipoate acceptor, 30 μM R-(+)-lipoic acid, 2 mM ATP, 2 mM MgCl₂, and appropriate amounts of wild-type or mutant LplA. The protein model of Se-LplA was refined against the 43.6–2.4 Å resolution electron density calculated with the program O (26) using the heavy atom search program in CNS (20), and the initial phases were calculated with SHARP (21). 2.8 Å resolution maps calculated after density modification by SOLOMON (22) implemented in SHARP was used for determination of molecule mask and noncrystallographic symmetry operators for further density improvement. Solvent flattening, histogram mapping, and noncrystallographic symmetry averaging were applied for phase improvement using DM (23), and the resulting map was used for model building. The structure of Se-LplA/lipoate was solved by the molecular replacement method with MOLREP (24) in the CCP4 suite (25) using Se-LplA structure as a search model.

**RESULTS AND DISCUSSION**

**Structure Determination**—Crystallization of *E. coli* LplA was mentioned about 10 years ago (6). The structure had never been determined, presumably because of the difficulty to obtain crystals suitable for the structural analysis. Hexagonal shape crystals of Se-LplA (0.06 × 0.08 × 0.1 mm) without substrate were obtained in 2 weeks by the hanging drop-vapor diffusion method, employing ethylene glycol as a precipi-

**TABLE ONE**

<table>
<thead>
<tr>
<th>Data collection and refinement statistics</th>
<th>Se-LplA</th>
<th>Se-LplA</th>
<th>Se-LplA/lipoate complex</th>
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</thead>
<tbody>
<tr>
<td><strong>Data collection</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>C222₁</td>
<td>C222₁</td>
<td>C222₁</td>
</tr>
<tr>
<td>Cell dimensions (Å)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>a</em></td>
<td>81.6</td>
<td>82.0</td>
<td>83.2</td>
</tr>
<tr>
<td><em>b</em></td>
<td>112.1</td>
<td>112.8</td>
<td>111.6</td>
</tr>
<tr>
<td><em>c</em></td>
<td>289.2</td>
<td>289.4</td>
<td>289.6</td>
</tr>
<tr>
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<td>0.9000</td>
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<tr>
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<tr>
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<tr>
<td>No. of unique reflections</td>
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<td>52,725</td>
<td>29,247</td>
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<tr>
<td>Completeness (%)</td>
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<td>99.4 (99.7)</td>
<td>97.4 (99.0)</td>
</tr>
<tr>
<td>I/σ(I)</td>
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<td>18.0 (2.9)</td>
<td>15.6 (3.8)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>9.9 (66.6)</td>
<td>5.9 (29.7)</td>
<td>12.0 (42.1)</td>
</tr>
</tbody>
</table>

**Structure refinement**

- Resolution (Å): 43.6–2.4; 66.7–2.9
- No. of reflections: 49,805 / 27,666
- Rmerge (%): 17.1 / 23.3
- I/σ(I): 18.6 / 27.1
- Rfree (%): 112.1

**Atoms per asymmetric unit**

- Protein: 7895
- Lipoic acid: 24
- Water: 530

**Ramachandran plot**

- Most favored (%): 89.9
- Additional allowed (%): 7.7
- Generously allowed (%) 0.3

**Data collection and refinement statistics**

- *a*: The numbers in parentheses represent statistics in the highest resolution shell.
- *b*: Rmerge = Σ ||Fo| - |Fc||/Σ |Fo|, where (Fo) is the mean intensity of symmetry-equivalent reflections.
- *c*: R = Σ ||Fo| - |Fc|| / Σ |Fo|, where Fo and Fc are the observed and calculated structure factors for data used for refinement, respectively.
- *d*: Rfree = Σ ||Fo| - |Fc|| / Σ |Fo| for 5% of the data not used at any stage of structural refinement.
Crystal Structure of E. coli Lipoate-Protein Ligase A

FIGURE 2. The molecular architecture of LplA. A, ribbon representation of three LplA molecules (MolA, MolB, and MolC) in the asymmetric unit drawn by tube mode. The figure was generated using MOLSCRIPT (39) and RASTER3D (40).

The phase problem for LplA was solved by the SAD method, and the initial model could be built into the electron density. The final structure was refined using a 2.4 Å resolution data set, with cell dimensions of $a = 82.0$ Å, $b = 112.8$ Å, $c = 289.4$ Å. Results of the X-ray data collection and refinement statistics are summarized in TABLE ONE. Although slight structural changes are found among these molecules (MolA-MolB, r.m.s. deviation 0.57 Å; MolA-MolC, r.m.s. deviation 0.70 Å; MolB-MolC, r.m.s. deviation 0.86 Å), overall fold shows almost identical structures except for some loop regions. N and C indicate the N and C termini, respectively. C, the stereo view of the native Se-LplA structure (MolC). The protein is shown as a ribbon representation with blue coloring at the N terminus along the rainbow colors to red at the C terminus. The lipoic acid molecule of MolA of the complex structure was superimposed and shown in a ball-and-stick mode. The figure was generated using MOLSCRIPT (39) and RASTER3D (40).

Overall Structure of LplA Molecule—Fig. 2C shows a Co trace of LplA (MolC). LplA consists of a large N-terminal domain (residues 1–244) and a small C-terminal domain (residues 253–337) connected by a single stretch of the polypeptide (residues 245–252). The N-terminal domain comprises two $\beta$-sheets, a large mixed $\beta$-sheet consisting of six strands ($\beta_1$, $\beta_2$, $\beta_3$, $\beta_4$, $\beta_5$, and $\beta_6$) and a small mixed $\beta$-sheet consisting of three strands ($\beta_7$–$\beta_9$), with seven $\alpha$-helices ($\alpha_1$–$\alpha_7$) and a 3$_{10}$-helix (3$_{10}$1) surrounding the $\beta$-sheets. The C-terminal domain consists of three $\alpha$-helices ($\alpha_8$–$\alpha_{10}$), two 3$_{10}$-helices (3$_{10}$2 and 3$_{10}$3), and a $\beta$-sheet comprising three strands ($\beta_1$–$\beta_3$). An open and solvent-exposed cleft is formed between the two domains. Fig. 1 shows the location of the secondary structure elements for the E. coli LplA, together with the amino acid sequence alignment of E. coli and putative Streptococcus pneumoniae LplA (30), human lipoate transferase, and E. coli LipB. Amino acid residues well conserved among four proteins and between LplA and lipoate transferase line inside of the cleft (on $\alpha_1$, $\beta_2$, to $\beta_5$, $\beta_6$, and $\beta_{10}$). These results suggest that the cleft is a substrate-binding pocket.

The structure of LplA is similar to that of the putative lipoate-protein ligase from S. pneumoniae (Protein Data Bank accession code 1VQZ), which gives a Z-score of 29.8 for an alignment of 303/326 residue with an r.m.s. deviation of 3.1 Å and 31% sequence identity by the DALI
program (31). The high structural similarity confirmed by superposition of the α-carbon traces of the putative protein and LplA suggests that the putative protein is a homologue of the E. coli lipoate-protein ligase A.

The Lipoic Acid Binding Site—Because lipoic acid is a labile compound, LplA crystals complexed with R-(+)-lipoic acid were obtained by seeding small crystals of the Se-LplA/lipoate complex into a droplet to accelerate the crystallization. Crystals suitable for analysis were obtained within 3 days after setting the droplet. The lipoic acid-bound form was determined at 66.7–2.9 Å resolution. Weak but significant electron density corresponding to lipoic acid was found in MolA and MolB (Fig. 3, A and B). Positions of the lipoic acid in MolA and MolB are slightly different. This may be caused by weak interactions between LplA and lipoic acid or insufficient information from low resolution data. Lipoic acid is located in a hydrophobic core generated by Leu-17, Phe-24, Phe-147, Ala-138, and aliphatic parts of Glu-21 and Ser-72 side chains. Hydrophobic interactions are formed between these residues and the dithiolane ring and the hydrophobic tail of lipoic acid. Leu-17, Ser-72, and Ala-138 are well conserved among LplAs and lipoyltransferase (Fig. 1). The electron density for the carboxyl group is weak relative to that for the hydrophobic moiety. In MolA, the carboxyl group makes a hydrogen bond with the side chain of Ser-72, whereas in MolB, the carboxyl group hydrogen bonds to the side chain of Arg-140 (Fig. 3, C and D). Although the actual binding mode may change somewhat when higher resolution x-ray data are eventually obtained, this complex provides insights into lipoic acid binding to the LplA molecule. Because van der Waals interactions are neither strong nor specific, and a weak hydrogen bond occurs only at the carboxyl group, it may permit LplA to bind not only R-(+)-lipoic acid but also S-(-)-lipoic acid, lipoic acid analogues, and octanoic acid. The binding mode well explains why LplA activates these carboxylic acids and transfers them to apoproteins (6, 7, 32). The reaction rates may be influenced by the slight differences in the van der Waals interactions between LplA and these substrates.

Functional and Structural Similarities to Other Proteins—A systematic analysis using the DALI program (31) identified a number of proteins/domains with significant structural similarities to LplA. The closest of them other than the LplA homologue is BirA, biotin holoenzyme...
synthetase/bio repressor from *E. coli* (Protein Data Bank accession codes 1B1A and 1B1B) (33), which gives a Z-score of 9.4 for an alignment of 139/292 residues with an r.m.s. deviation of 3.5 Å and 12% sequence identity. BirA consists of three domains and functions as a repressor of the biotin biosynthetic operon and as an enzyme that catalyzes the activation and transfer of biotin to a specific lysine residue on the biotindependent carboxylases. The reaction mechanism is remarkably similar to that of LplA. The central domain contributes to the catalysis. Fig. 4 shows a superposition of LplA (residues 1–337) onto BirA (the central and C-terminal domains, residues 68–317) according to the rotation and translation matrices provided by DALI. Several α-helices (α4, α7, and α8) and β-strands (β1, β2, β7, β8, and β10) of LplA are well superimposed on the corresponding secondary structure elements of BirA. In both enzymes, lipoic acid and biotin interact with β-strands in the N-terminal domain and the central domain, respectively, and the substrates are situated in the similar position in the respective enzymes. Functional and structural similarities of these enzymes suggest that evolutionarily they are closely related, although they share only 12% amino acid sequence identity. The absence of a DNA binding domain in the LplA structure may be related to the finding that LplA metabolizes lipoic acid that is exogenously supplied rather than that synthesized endogenously in *E. coli* (5, 7).

Mechanistic Implication—Ser-72 and Arg-140 residues, respectively, were substituted by Ala to analyze the contribution of the residues to the lipoic acid binding (Fig. 3). Steady state kinetic studies were carried out by varying the concentration of one substrate and keeping the concentration of the other two substrates constant. Apparent kinetic constants are shown in TABLE TWO.

Whereas the S72A mutation results in an about 2-fold increase in $K_{\text{m(app)}}$ for lipoic acid, $K_{\text{m(app)}}$ for ATP is greatly increased by the mutation, indicating the reduced binding affinity for ATP. This result suggests that the hydrogen bond between lipoic acid and Ser-72 may not be so critical to fix lipoic acid at the position determined by the crystal structural analysis. However, the fixation of the carboxyl group to Ser-72 is required for the initiation of the lipoate activation reaction with ATP, or ATP may interact directly with Ser-72.

The R140A mutation results in reduced $V_{\text{max(app)}}$ values, although the values differ depending on the varied substrate because of the usage of the limited concentration of apoH-protein. $K_{\text{m(app)}}$ for lipoic acid is rather decreased. On the other hand, $K_{s(app)}$ for apoH-protein increases by 1 order of magnitude, indicating a reduction of the binding affinity for apoH-protein. In the protein lipoylation reaction, the presence of the Glu residue at the three residues N-terminal side from the Lys residue to be lipoylated in the lipoate acceptor protein is essential (34, 35). Therefore, Arg-140 in LplA may make a salt bridge with the Glu residue in the apoH-protein in the second transfer reaction (Reaction 2). These results of the kinetic analyses support the lipoic acid binding mode shown in Fig. 3 (i.e. lipoic acid is bound at the position by the hydrophobic interactions, and the weak hydrogen bond between the carboxyl group and the Ser-72 or the Arg-140 residue is enough to accommodate lipoic acid).

We attempted to obtain a crystal of the LplA-ATP complex by soaking the native crystal with Mg-ATP and by co-crystallization in the presence of Mg-ATP. However, X-ray analysis of the crystals did not show electron density for ATP clear enough to determine the ATP binding site. The first step of the reaction catalyzed by LplA is thought to
be the nucleophilic attack of the carboxyl oxygen of lipoic acid on the α-phosphorus atom of ATP followed by the release of the pyrophosphate and the formation of a lipoyl-AMP intermediate by analogy to the reaction catalyzed by histidyl-tRNA synthetase (36). The activated carbon atom of the lipoyl-AMP intermediate is then attacked by the non-protonated ε-amino group of the lysine residue of an apoprotein to be lipoylated, producing AMP and the lipoylated protein. To initiate the first reaction, ATP should be anchored near the lipoic acid in LplA, and especially the distance between the α-phosphorus atom of ATP and the carboxyl oxygen atom of lipoic acid should be less than 3 Å.

LplA has a characteristic sequence motif RRSSGGG between positions 69 and 75, which is highly conserved among the proteins listed in Fig. 1. The motif is situated on the loop following the β4-strand (Fig. 2C) close to the bound lipoic acid. In the native LplA structure, the side chain of Arg-70 forms a hydrogen bond network with the main-chain carbonyl groups of Gly-73 and Gly-75 and the side chain of Ser-72, stabilizing the loop structure (Fig. 5). The motif can provide positive charges and a space large enough to accommodate ATP. Therefore, the motif is presumed to be involved in the binding of ATP or the adenylate moiety of the lipoyl-AMP intermediate. Indeed, the mutation of Ser-72 affects the binding affinity for ATP (TABLE TWO). An R72G point mutation may cause the reduction of the lipoylation affinity for ATP (TABLE TWO). An R72G point mutation may cause the reduction of the lipoylation

## Acknowledgments

We thank Prof. Tomitake Tsukihara (Osaka University) for support of this work, Dr. Eiki Yamashita (Osaka University) for help in data collection at the Spring-8 BL44XU and ASTA-Medica (Germany) for the generous gift of R-(+)-lipoate.

## References

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