Crystal Structures and Site-directed Mutagenesis of a Mycothiol-dependent Enzyme Reveal a Novel Folding and Molecular Basis for Mycothiol-mediated Maleylpyruvate Isomerization*

Received for publication, November 6, 2006, and in revised form, March 30, 2007 Published, JBC Papers in Press, April 11, 2007, DOI 10.1074/jbc.M610347200

Rui Wang1,2, Ya-Jie Yin1,2, Feng Wang2, Mei Li3, Jie Feng4, Hong-Mei Zhang4, Ji-Ping Zhang4, Shuang-Jiang Liu4, and Wen-Rui Chang5

From the 1National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, the 2State Key Laboratory of Microbial Resource, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100080, and the 3Graduate University of Chinese Academy of Sciences, Beijing 100049, China

Mycothiol (MSH) is the major low molecular mass thiol in many Gram-positive bacteria such as Mycobacterium tuberculosis and Corynebacterium glutamicum. The physiological roles of MSH are believed to be equivalent to those of GSH in Gram-negative bacteria, but current knowledge of MSH is limited to detoxification of algalating chemicals and protection from host cell defense/killing systems. Recently, an MSH-dependent maleylpyruvate isomerase (MDMPI) was discovered from C. glutamicum, and this isomerase represents one example of many putative MSH-dependent enzymes that take MSH as cofactor. In this report, fourteen mutants of MDMPI were generated. The wild type and mutant (H52A) MDMPIs were crystallized and their structures were solved at 1.75 and 2.05 Å resolution, respectively. The crystal structures reveal that this enzyme contains a divalent metal-binding domain and a C-terminal domain possessing a novel folding pattern (αβαβαβα fold). The divalent metal-binding site is composed of residues His52, Glu144, and His148 and is located at the bottom of a surface pocket. Combining the structural and site-directed mutagenesis studies, it is proposed that this surface pocket including the metal ion and MSH moiety formed the putative catalytic center.

Mycothiol, also known as MSH and chemically 1D-myoinositol-2-(N-acetyl-L-cysteinyl)-amido-2-deoxy-α-D-glucopyranoside (1–4), is the major low molecular mass thiol in many groups of Gram-positive bacteria such as coryneform bacteria, mycobacteria, and streptomyces (5, 6). These bacteria synthesize MSH but lack GSH molecule that plays important roles in many physiological processes. It is believed that MSH functions similarly to GSH in many microbial activities (6). However, the understanding of MSH physiological function was limited to detoxification of reactive oxygen/alkalating species and to protection of pathogens such as Mycobacterium tuberculosis from host cell defense systems (1, 7, 8). Very recently, a novel physiological role of MSH in assimilation of aromatic compounds was described and an MSH-dependent maleylpyruvate isomerase (MDMPI) was identified in Corynebacterium glutamicum (9). This MDMPI catalyzes the conversion of maleylpyruvate (substrate) to fumarylpyruvate (product) (Fig. 1).

BLAST searches with MDMPI sequence against GenBankTM and other protein data bases revealed that MDMPI is not homologous to any functionally identified proteins but showed significant identities (27–36%) to a range of conserved hypothetical proteins from the genomes of Streptomyces coelicolor, Streptomyces avermitilis, Propionibacterium acnes, and Nocardia farcinica (10) (Fig. 2A). Earlier research on mycothiol biochemistry and biosynthesis has been conducted with mycobacteria such as M. tuberculosis, with the ultimate aim of defining new targets against tuberculosis, the resurgence of which has been a growing health concern in both developed and developing nations. The understanding of MSH-dependent enzyme structure is the key to the characterization of MSH-dependent enzymes that are helpful for the development of new drugs against diseases caused by Mycobacterium spp.

To elucidate the catalytic mechanism of MDMPI and to provide clues to find more MSH-dependent enzymes, we have determined the crystal structure of MDMPI from C. glutamicum at a resolution of 1.75 Å. The structure reveals that the MDMPI contains two domains: A metal binding domain binds a divalent metal cofactor (Zn2+) and an extended domain con-

* This work was supported by National Natural Sciences Foundation of China and Knowledge Innovation Program of the Chinese Academy of Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 US.C. Section 1734 solely to indicate this fact.

1 The online version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

2 The atomic coordinates and structure factors (code 2NSF and 2NSG) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

3 These two authors contributed equally to this work. R. W. contributed to the crystallization and structure determination and analysis, and Y.-J. Y. contributed to the mutagenesis and enzymatic activity determination.

4 To whom correspondence may be addressed: National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Rd., Chaoyang District, Beijing 100101, China. Tel.: 86-10-64888512; Fax: 86-10-64889867; E-mail: wrchang@sun5.ibp.ac.cn.

5 To whom correspondence may be addressed: Institute of Microbiology, Chinese Academy of Sciences, ZhongGuanCun, Haidian, Beijing 100080, China. Tel.: 86-10-62527118; Fax: 86-10-62652317; E-mail: liuisj@sun.im.ac.cn.

6 The abbreviations used are: MSH, mycothiol; MDMPI, MSH-dependent maleylpyruvate isomerase.
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Figure 1. Isomerization of maleylpyruvate into fumarylpyruvate catalyzed by mycothiol-dependent maleylpyruvate isomerase. Intermediates (i) and (ii) have not been experimentally confirmed.

Bacterial Strains, Plasmids, and Culture Conditions—Bacterial strains and plasmids used in this study are listed in supplemental Table S1. Escherichia coli strains were grown aerobically on a rotary shaker (150 rpm) at 37 °C in LB broth or on LB plate with 1.5% (w/v) agar. When needed, kanamycin at 50 μg ml⁻¹ for E. coli was used.

Expression and Purification of MDMPI and Its Mutants—Various derivatives of plasmid pET28a (supplemental Table S1) that harbor MDMPI or its mutants were constructed by methods described previously (10). Recombinant proteins were expressed in E. coli BL21 (DE3) cells by inducing with 0.5 mM isopropyl-β-D-galactopyranoside when the culture reached an A₆₀₀ of 0.6–0.8. The culture was harvested after growing for additional 8 h at 25 °C. Protein was purified using a nickel affinity column and a Superdex 75 gel-filtration column (GE Healthcare). The prepared protein sample was checked for purity by SDS-PAGE and by gel-filtration chromatography, which showed only one protein of 28 kDa corresponding to the molecular mass of MDMPI and its mutants. After changing buffer to 10 mM Tris (pH 8.0) by repeatedly concentration and dilution using Amicon Ultra-4 concentrators (molecular weight cutoff 5000), the purified protein was finally concentrated to 17 mg/ml and stored at −80 °C.

Crystallization and Data Collection—The hanging-drop vapor-diffusion method was used for crystallization. The drop was formed by mixing 1 μl of protein solution (17 mg/ml) with an equal volume of reservoir solution containing 1.2 M (NH₄)₂SO₄, 12% glycerol, and 0.1 M Tris (pH 8.5). A 1.75 Å resolution native data set was collected utilizing Rigaku RAXIS IV image plate detector at Institute of Biophysics (Chinese Academy of Sciences). A bromide derivative was prepared by soaking the crystal in cryoprotecting solution containing 1.2 M (NH₄)₂SO₄, 25% glycerol, 1 M NaBr, and 0.1 M Tris (pH 8.5) for 10 min. Single-wavelength anomalous dispersion data were collected at the Beijing Synchrotron Radiation Facility at the peak wavelength of bromine (0.920 Å) with 1° oscillation and an exposure of 15 s per image. A total of 600° of data were collected to a resolution of 1.95 Å with an average of 20-fold redundancy.

The crystallization of mutant H52A was performed similarly to that of native MDMPI. The drops were composed of 1 μl of protein solution (15 mg/ml) and 1 μl of reservoir solution containing 1.4 M (NH₄)₂SO₄, 11% glycerol, and 0.1 M Tris (pH 8.5). A 2.05 Å resolution data set for this mutant was collected at the Institute of Biophysics (Chinese Academy of Sciences).

All the diffraction data (Table 1) were integrated using DENZO and scaled using SCALEPACK (11).

Phasing, Structural Determination, Refinement, and Analysis—Five bromide heavy atom sites in the asymmetric unit were determined using PHENIX via the single-wavelength anomalous dispersion method (12, 13). After phase improvement an initial model was built using PHENIX with the final figure of merit 0.65 for eight bromide sites. ARP/wARP was used to automated model rebuilding with the sequence of MDMPI (14, 15). The model was subsequently subjected to annealing refinement by CNS (16) using 50-1.75 Å data, and the traditional crystallographic refinement was carried out using CNS (16). After several cycles of refinement and model rebuilding a final model with an R factor of 0.21 and an R_free factor of 0.22 was obtained, which consisted of 240 residues and 272 water molecules. We modeled Zn²⁺ into the density and the B-factor of Zn²⁺ has been refined to 26. Crystallographic statistics showed the quality of the overall structure was very good. The N-terminal His-tag fragment was missed in the electron density map.

Contains totally new folding. The site-directed mutagenesis reveals 9 of the 14 conserved amino acid residues are essential for activity. One mutant (H52A) was crystallized. By combination of structural and biochemical results, a putative catalytic center is proposed. So far as we know this is the first structure of an MSH-dependent enzyme, as well as an enzyme involved in geniticate/3-hydroxybenzoate assimilation that is widely existed in bacteria and participate in microbial geocycling of elements of aromatic compounds in nature.
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TABLE 1
Data collection, phasing, and refinement statistics for MDMPI and H52A structures

|                    | Native       | Br-MDMPI    | H52A        |
|--------------------|--------------|-------------|-------------|
| Data collection    |              |             |             |
| Wavelength (Å)     | 1.5418       | 0.92 (peak) | 1.5418      |
| Space group        | P3,21        | P3,21       | P3,21       |
| Cell dimensions    |              |             |             |
| a, b, c (Å)        | 67.3, 67.3, 128.2 | 67.1, 67.1, 128.1 | 66.6, 66.6, 129.4 |
| a, b, c (%)        | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0 |
| Resolution (Å)     | 1.75         | 1.95        | 2.05        |
| Rmerge (%)         | 4.4 (44.2)   | 6.9 (47.8)  | 10.1 (42.3) |
| Rfactors (Å²)      | 47.5 (5.7)   | 49.9 (8.5)  | 24.3 (7.8)  |
| Completeness (%)   | 100.0 (99.9) | 100.0 (100.0)| 100.0 (99.9)|

Refinement

|                    | Native       | Br-MDMPI    | H52A        |
|--------------------|--------------|-------------|-------------|
| Non-hydrogen atoms | 2146         | 2148        |             |
| Protein            | 240          | 240         |             |
| Water              | 272          | 274         |             |
| Rwork (%)          | 20.9         | 19.9        |             |
| Rpvalue (%)        | 21.9         | 22.2        |             |
| B-Factors (Å²)     |              |             |             |
| Protein            | 24.1         | 25.0        |             |
| Water              | 30.2         | 30.5        |             |
| Root mean square deviation from ideality |              |             |             |
| Bond lengths (Å)   | 0.005        | 0.006       |             |
| Bond angles (degree) | 1.27        | 1.24        |             |
| Ramachandran analysis (%) | 96.6     | 96.2        |             |
| Most-favored regions (%) | 2.9       | 3.4         |             |
| Additional favored regions (%) | 0.5      | 0.5         |             |

RESULTS AND DISCUSSION

Overall Structure of the MDMPI—The MDMPI of C. glutamicum was purified from recombinant E. coli and its crystal structure was resolved at 1.75 Å. The MDMPI is a monomer and belongs to α/β folding type (Figs. 2B and Fig. 3). Two domains were clearly observed: An N-terminal domain largely composed of α-helices and a C-terminal domain characterized by shorter stretches of α-helices and β-sheets. The core of the N-terminal domain consists of five long (≈20 residues) α-helices (α1, α2, α3, α4, and α5). Also included in the core domain is a metal ion (Fig. 3). The C-terminal domain shows a novel fold with the pattern of αβαβαβ fold, which contains three long loops, three α-helices (α7, α8, and α9), and three β-strands (β3, β4, and β5). It is noteworthy that 42% of the amino acid residues in the C-terminal domain are located at the three long loops, with the longest loop containing 20 residues (residues 221–241) and are not part of the core C-terminal domain structure (Fig. 3).

The C-terminal Domain Represents a Novel Folding Pattern and Is Necessary for Enzymatic Activity—The folding topology of the C-terminal domain of MDMPI is novel. Structural similarity searches of the C-terminal domain utilizing the DALI server (20) showed few matches with significant Z score and small root mean square deviation values. The best match was a putative sterol carrier protein (PDB code 1WFR) with Z value of 3.0. The 67 residues of this protein share 15% sequence identity with the C-terminal domain of MDMPI and can be superimposed to this domain with a root mean square deviation of 3.3 Å.

The C-terminal domain is necessary for the enzyme activity and structural stability. When the C-terminal domain was deleted, the truncated MDMPI did not show any enzyme activity (Table 2) and existed as inclusion bodies in E. coli cells (data not shown). Furthermore, site-directed mutagenesis revealed
The MDMPI is a Metal-dependent Protein and Contains a Metal-binding Motif, His52-Glu144-His148.

Structural analysis revealed that MDMPI is a metal-dependent protein with the metal binding site located in the N-terminal domain. The metal atom is coordinated by His52 (NE2), Glu144 (OE1), His148 (NE2), two water molecules and one glycerol molecule (Fig. 5A). The x-ray fluorescence scan gave clear absorption jump at the spectrum over K-edge of Zn2+ and Ni2+ (data not shown), which demonstrated that this was a mixture of Zn2+ and Ni2+. Coordination of Zn2+ and Ni2+ indicated that either could be coordinated in a tetragonal bipyramidal geometry (21). Since in the whole expression and purification course, we did not add any zinc-containing reagent, it suggested that Zn2+ was integrated into the metal-ion binding site in vivo. In addition, previous enzyme activity assays showed that Zn2+ (1 mM) stimulated isomerase activity up to 121%, while Ni2+ (1 mM) did not affect activity (9). Therefore, it is deduced that the natural MDMPI contains Zn2+ ion instead of Ni2+ ion. It is further deduced that Zn2+ was partially replaced by Ni2+ during purification of MDMPI with a nickel-affinity chromatography.

The contribution of the three conserved residues, namely His52, Glu144, and His148, to the binding of metal atom was examined by individual mutation of each amino acid residue. Enzyme activity assays indicated that all mutants (H52A,
E144A, and H148A) were inactive. Moreover, the crystal structure of mutant H52A at resolution of 2.05Å revealed that there was no metal ion bound to the protein (although nickel-affinity chromatography was used in the purification process). The metal atom position at the native MDMPI molecule was occupied by one glycerol moiety (Fig. 5B). Compared with the native MDMPI, the mutant MDMPI (H52A) has the following structural changes: 1) the main chain of the loop (amino acid residues 36–45) moved toward the inner; 2) the side chain of Trp44 at the loop is much closer to the former metal binding site; 3) the side chain of Glu144 had double conformations; and 4) conformational change took place at the side chain of Arg82 (Fig. 6). Probably it is the absence of the metal ion that resulted in the significant conformation change around the proposed metal binding region.

Previously, it was proposed that MDMPI represents a novel family of proteins (9), but the structures of this novel family were unknown. According to sequence alignment, the metal binding site, namely residues His52-Glu144-His148, are highly conserved throughout the whole family (Fig. 2A), and these binding residues are located in the surface pocket between the α2 (His52) and α5 (Glu144 and His148) in N-terminal domain (Fig. 5A).

Biochemical and Structural Identification of Catalytic Center—In total fourteen amino acid residues were targeted for mutation (Table 2), according to sequence alignment and the above structure analysis. Some mutant MDMPIs such as H52A, E144A, H148A, D151A, and R222A that completely (100%) lost enzymatic activity were described above. The other mutant MDMPIs include three (H48A, C61A, E85A) that did not lose activity, two that showed significant loss of activity (S78A, 7%; R82A, 30%; activity remained), and four (Y76A, R141A, W44A, N56A) that demonstrated a complete loss of activity (Table 2).

Structure modeling indicated that Tyr76, Arg82, Ser78, Arg141, and Glu144 were located at the metal-binding region. Moreover, eight of these amino acid residues, including His52, Trp44, Asn56, Tyr76, Arg82, Arg141, Glu144, and His148, formed a surface pocket (Fig. 7). The metal ion (Zn2⁺) and its binding site (His52, Glu144, and His148) are located at the bottom of the surface pocket. Thus, it is proposed that these highly conserved sites (Trp44, Asn56, Tyr76, Arg82, and Arg141) that surrounding the metal binding site provides the hydrophilic environment for MDMPI catalysis. The surface pocket including the metal atom

### TABLE 2

| MDMPI wild type or mutants | MDMPI activity |
|---------------------------|----------------|
| Wild type                 | 100            |
| N-domain of MDMPI         | 0              |
| W44A                      | 0              |
| H48A                      | 100            |
| H52A                      | 0              |
| N56A                      | 0              |
| C61A                      | 100            |
| Y76A                      | 0              |
| S78A                      | 100            |
| R82A                      | 30             |
| E85A                      | 0              |
| R141A                     | 0              |
| E144A                     | 0              |
| H148A                     | 0              |
| A151A                     | 0              |
| R222A                     | 0              |

*The activity was determined from recombinant E. coli cells that harbor each plasmid (for details, please refer to supplemental Tables S1 and S2) and was induced by isopropyl-1-thio-β-D-galactopyranoside. The wild type MDMPI activity was calculated as 100%.*
Sulfate Ion in the Catalytic Center and Proposed Process for MDMPI-catalyzing Isomerization—A tetrahedral density was observed at this surface pocket. This density was molded as a sulfate ion (Fig. 7). The sulfate ion interacted with Tyr76 and Asn56 and was located near Arg82. These three amino acid residues are conservative in the whole family (Fig. 2A) and are necessary for enzymatic activities, as demonstrated by mutagenesis of these amino acid residues resulting complete loss of enzymatic activity (Table 2). Interestingly, a sulfate ion was also observed in the active site (a deep cavity) of the human GSH-dependent maleylacetoacetate isomerase, and this sulfate ion was proposed to mimicking substrate (maleylacetoacetate) binding (22).

Although the catalytic mechanism of MDMPI is still not clear, this and previous studies have revealed several key molecular elements that are necessary for catalysis, including the metal ion (Zn\(^{2+}\)) and its surrounding amino acid residues (His\(^{52}\), Glu\(^{144}\), and His\(^{148}\)), and the –SH group of the MSH molecule (9). Here, a preliminary and hypothetical process is proposed for description of the MDMPI-catalyzed isomerization (Fig. 1): isomerization of maleylpyruvate is initiated by the attack of –SH of the mycothiol on the α-carbon of the maleylpyruvate molecule, which is catalyzed by the MDMPI (most probably by the Zn\(^{2+}\) ion on the enzyme). This attack results in the formation of MSH-maleylpyruvate conjugate (Fig. 1, step I). Upon conjugation, the resulting single bond would be freely to rotate (Fig. 1, step II). Finally, the MSH moiety is released and the product (fumarylpyruvate) is generated (Fig. 1, step III). Although this proposed mechanism is similar to that proposed for the GSH-dependent maleylacetoacetate isomerase from humans (22), some sharply different properties between MDMPI and GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate isomerase from humans (22), some sharply different properties between MDMPI and GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate were observed. The MDMPI is a metalloprotein but the GSH-dependent maleylacetoacetate were observed.

Acknowledgments—We are grateful to Rui-Fang Wang for protein purification, Yi Han at the Institute of Biophysics (Chinese Academy of Sciences), Yu-Hui Dong and Peng Liu at the Institute of High Energy Physics (Chinese Academy of Sciences) for data collection, and N. Sakabe and A. Iida of the Photon Factory (Tsukuba, Japan) for x-ray fluorescence scanning.
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