Dual roles of Lys$^{57}$ at the dimer interface of human mitochondrial NAD(P)$^{+}$-dependent malic enzyme

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Human m-NAD(P)-ME [mitochondrial NAD(P)$^{+}$-dependent ME (malic enzyme)] is a homotetramer, which is allosterically activated by the binding of fumarate. The fumarate-binding site is located at the dimer interface of the NAD(P)-ME. In the present study, we decipher the functional role of the residue Lys$^{57}$, which resides at the fumarate-binding site and dimer interface, and thus may be involved in the allosteric regulation and subunit–subunit interaction of the enzyme. In the present study, Lys$^{57}$ is replaced with alanine, cysteine, serine and arginine residues. Site-directed mutagenesis and kinetic analysis strongly suggest that Lys$^{57}$ is important for the fumarate-induced activation and quaternary structural organization of the enzyme. Lys$^{57}$ mutant enzymes demonstrate a reduction of $K_{d}$ and an elevation of $k_{cat}$ following induction by fumarate binding, and also display a much higher maximal activation threshold than WT (wild-type), indicating that these Lys$^{57}$ mutant enzymes have lower affinity for the effector fumarate. Furthermore, mutation of Lys$^{57}$ in m-NAD(P)-ME causes the enzyme to become less active and lose co-operativity. It also increased $K_{d}$.mutant and decreased $k_{cat}$ values, indicating that the catalytic power of these mutant enzymes was significantly impaired following mutation of Lys$^{57}$. Analytical ultracentrifugation analysis demonstrates that the K57A, K57S and K57C mutant enzymes dissociate predominantly into dimers, with some monomers present, whereas the K57R mutant forms a mixture of dimers and tetramers, with a small amount of the enzyme in monomeric form. The dimeric form of these Lys$^{57}$ mutants, however, cannot be reconstituted into tetramers with the addition of fumarate. Modelling structures of the Lys$^{57}$ mutant enzymes show that the hydrogen bond network in the dimer interface where Lys$^{57}$ resides may be reduced compared with WT. Although the fumarate-induced activation effects are partially maintained in these Lys$^{57}$ mutant enzymes, the mutant enzymes cannot be reconstituted into tetramers through fumarate binding and cannot recover their full enzymatic activity. In the present study, we demonstrate that the Lys$^{57}$ residue plays dual functional roles in the structural integrity of the allosteric site and in the subunit–subunit interaction at the dimer interface of human m-NAD(P)-ME.

Key words: allosteric regulation, analytical ultracentrifugation, malic enzyme, mutagenesis.

INTRODUCTION

ME (malic enzyme) is an important enzyme that catalyses the oxidative decarboxylation of malate to yield CO$_2$ and pyruvate with the concomitant production of NAD(P)H [1,2]. A divalent metal ion (Mn$^{2+}$ or Mg$^{2+}$) is required for this enzymatic reaction. These enzymes are highly conserved between species with regard to protein sequences and structural topology [2–5]. In mammals, MEs can be divided into three isoforms according to their cofactor preference. They are c-NADP-ME (cytosolic NADP$^{+}$-dependent ME) [5–7], m-NADP-ME (mitochondrial NADP$^{+}$-dependent ME) [8], and m-NAD(P)-ME [mitochondrial NAD(P)$^{+}$-dependent ME] [9–13]. m-NAD(P)-ME has dual coenzyme specificity, utilizing both NAD$^{+}$ and NADP$^{+}$ as coenzymes, although the preferred coenzyme under physiological conditions is NAD$^{+}$ [2,14]. The physiological role of c-NADP-ME and m-NADP-ME isoforms is mainly to produce NADPH for fatty acid biosynthesis. The m-NADP-ME isoform primarily generates NADH as the reducing equivalent in energy production and this enzyme may be associated with rapid tumour growth through the NADH and pyruvate products in glutaminolysis [15–19].

The m-NAD(P)-ME isoform is distinct from the other two mammalian isoforms in that it is a regulatory enzyme with a multifaceted control system to moderate its catalytic activity [20–22]. The enzyme displays a positive co-operative manner of binding the substrate L-malate, and it can be allosterically activated by fumarate binding [14,16,21,23]. Furthermore, it is inhibited by ATP through an active-site competition mechanism [24–26]. This ATP inhibition may have evolved through the particular role of the enzyme in the pathways of malate and glutamine oxidation in tumour mitochondria [27,28].

The crystal structures of MEs reveal that they have a quaternary structure of a dimer of dimers (Figure 1A), and suggest that these enzymes may be a new class of oxidative decarboxylases with a novel backbone structure [2,4,29–33]. The structures of human m-NAD(P)-ME reveal that there are two regulatory sites in addition to the active site (Figure 1A) [2,21,22,24]. One, called the exo-site, is situated at the tetramer interface, and is occupied by an NAD or ATP molecule. The other, located at the dimer interface, is occupied by fumarate [21]. In Ascaris suum m-NAD(P)-ME, a separate allosteric site is also found at the dimer interface [23,34,35]. Figure 1(B) shows the fumarate-binding site at the dimer interface. In the structure, fumarate interacts directly with Arg67 and Arg91. Mutation analysis confirmed that Arg67 and Arg91 are crucial for fumarate activation. However, both Arg67 and Arg91 are also conserved among non-allosteric ME isoforms. Therefore additional factors may participate in the mechanism of fumarate activation control. Besides interacting with fumarate, Arg67 is also ion-paired with Glu99. Earlier studies have shown that the E59L mutation totally abolishes the fumarate-induced
Figure 1  Fumarate-binding site of human m-NAD(P)-ME

(A) Tetramer of human m-NAD(P)-ME (PDB code 1PJ3). The active site in each subunit, the exo-site in the tetramer interface and the fumarate-binding site in the dimer interface are indicated as a ball-and-stick model. NAD$^+$ in the active site is coloured in blue and that in the exo-site is coloured in light blue; fumarate is coloured in yellow. (B) Lys$^57$ in the fumarate-binding pocket and in the dimer interface. Both (A) and (B) were produced using PyMOL (DeLano Scientific; http://pymol.sourceforge.net/). (C) Multiple sequence alignments of three clusters of ME isoforms around the fumarate-binding region in the dimer interface. Amino acid sequences of MEs were obtained by BLAST [45], and alignments were generated by ClustalW [46]. (C) was generated using the BioEdit sequence alignment editor program [47].

enzyme activation, and demonstrated the remarkable effects of Glu$^{59}$ on the allosteric activation of the enzyme [21].

Our previous work suggests that the electrostatic balance in the fumarate-binding pocket may be a crucial factor governing the regulatory mechanism of fumarate-induced activation [17]. In the present study, we further examine a cationic residue Lys$^{57}$, which is ion-paired with Glu$^{59}$ (Figure 1B). Both Lys$^{57}$ and Glu$^{59}$ uniquely exist in the m-NAD(P)-ME isoform, but not in the non-allosteric isoforms. Thus we investigated the functional role of Lys$^{57}$ within the allosteric site. Also of interest is the fact that Lys$^{57}$ is situated at the dimer interface. The side chain of Lys$^{57}$ is not only ion-paired with Glu$^{59}$ but also hydrogen-bonded with the Pro$^{216}$ and Tyr$^{218}$ residues from another subunit. Therefore we also examined the possible role of Lys$^{57}$ in subunit–subunit interactions. Based on multiple sequence alignments, Lys$^{57}$ is substituted to cysteine and serine residues, found in c-NADP-ME and m-NADP-ME respectively (Figure 1C). The K57A and K57R mutations are designed to examine the effect of positive charge on this residue. Detailed kinetic and analytical ultracentrifugation analysis shows that the Lys$^{57}$-involved hydrogen-bond network at the dimer interface plays dual functional roles in the allosteric regulation and subunit–subunit interaction of the human m-NAD(P)-ME.

MATERIALS AND METHODS

Preparation of recombinant MEs

Detailed expression and purification protocols for human m-NAD(P)-ME have been described previously [4,9,29]. Briefly, the m-NAD(P)-ME was cloned into the expression vector (pRH281) under an inducible trp promoter system. The ampicillin-resistant vector was transformed into *Escherichia coli* BL21 cells for enzyme overexpression. Expression of m-NAD(P)-ME was induced with 50 μg/ml IAA (β-indole-3-acetic acid) and the cells were incubated at 25 °C overnight. After induction by IAA, cells were centrifuged at 6000 g at 4 °C for 15 min. The supernatant was removed and the cell pellets were resuspended in...
buffer A [3 mM MgCl₂, 1 mM MnCl₂, 2 mM 2-mercaptoethanol, 0.2 mM EDTA and 30 mM Tris/Cl (pH 7.4)]. After sonication to lyse the cells, the lysate was centrifuged (15 000 g at 4 °C for 20 min) and the supernatant was collected for further purification. An anionic exchange, DEAE–Sepharose (Amersham Biosciences) column, followed by an ATP–agarose affinity chromatography (Sigma) column was utilized in the enzyme purification. The DEAE–Sepharose was equilibrated with buffer A and the supernatant was then added to the column. The lysate–DEAE was washed in a stepwise procedure (buffer A with steps of 20, 40, 60 and 80 mM NaCl) to minimize the association of unwanted proteins. Finally, human m-NAD(P)-ME was eluted with buffer B (buffer A with 100 mM NaCl). After dialysis with buffer A, the enzyme was then loaded into the ATP–agarose affinity chromatography, which was pre-equilibrated with buffer A. The enzyme was finally eluted using buffer C (buffer A with 4 mM NAD⁺). The purified enzyme was then buffer-exchanged and concentrated in 30 mM Tris/HCl (pH 7.4) with 2 mM 2-mercaptoethanol by a centrifugal filter device (Amicon Ultra-15, Millipore) with a molecular mass cut-off of 30 kDa. The enzyme purity was examined by SDS/PAGE, and the protein concentrations were estimated using the Bradford method [36].

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the QuikChange® kit (Stratagene). The purified DNA of human m-NAD(P)-ME was used as a template, and the primers with desired codon changes were employed to change Lys⁵⁷ to an alanine, cysteine, serine or arginine residue using a high-fidelity Pfu DNA polymerase in the PCR reaction. Primers spanning the mutation site were 25–45-mers, which is considered necessary for specific binding of template DNA. The synthetic oligonucleotides used in the site-directed mutagenesis experiments were: 5'-TTCTAGAGACTTCTACTTCCCGCCATAGAGACACAAAGATATTC-3' for K57A, 5'-TTCTAGAGACTTCTACCTCCCCCACATAGAGACACAAAGATATTC-3' for K57C, 5'-TTCTAGAGACTTCTACCTCCCGGTATAGAGACACAAAGATATTC-3' for K57S and 5'-TTCTAGAGACTTCTACCTCCCGGTATAGAGACACAAAGATATTC-3' for K57R. The constructs were ligated into pET30 which was transformed into E. coli XL-1 cells. All mutation sites were verified by automated sequencing.

Enzyme kinetic analysis

The enzymatic reaction of ME was measured by recording the NADH production. The reaction cocktail was composed of 50 mM Tris/HCl (pH 7.4), 40 mM malate, 2.0 mM NAD⁺ and 10 mM MgCl₂ in a total volume of 1 ml in the absence or presence of fumarate. The absorbance at 340 nm at 30 °C was immediately recorded after the enzyme was added to the reaction mixture and monitored continuously in a Beckman DU 7500 spectrophotometer. A molar absorption coefficient of 6.22 mM⁻¹ cm⁻¹ for NADH was employed to calculate the initial velocities and k₉₄ values. Apparent Michaelis constants of the substrate and coenzymes were determined by changing the concentration of one substrate (or coenzyme) in the region of its K₉ value and, at the same time, keeping the other components constant under the saturation condition.

The sigmoidal curves of [malate] against initial velocity were fitted into the Hill equation. Further analysis revealed the K₉,₅ value, the substrate concentration at half-maximal velocity, and the Hill coefficient (h), both of which were utilized to evaluate the degree of co-operativity (eqn 1):

\[
v = \frac{V_{\text{max}}[\text{malate}]^h}{K_9^h + [\text{malate}]^h}
\]

All data-fitting work was carried out using the Sigma Plot 10.0 program (Jandel).

**Quaternary structure determination by analytical ultracentrifugation**

Sedimentation velocity experiments were executed using a Beckman Optima XL-A analytical ultracentrifuge. Sample (400 μl) and buffer (420 μl) solutions were loaded into the double sector centrepiece individually and were housed in a Beckman An-50 Ti rotor. Experiments were carried out at 20 °C and a rotor speed of 42 000 rev./min was used. Protein samples were monitored by UV absorbance at 280 nm in a continuous mode with a time interval of 480 s and a step size of 0.002 cm. Numerous scans at different time points were fitted to a continuous size-distribution model by the program SEDFIT [37–41]. All size distributions were resolved on a confidence level of P = 0.95, a best-fitted average anhydro fraction ratio (f/f₀), and a resolution N of 200 sedimentation coefficients between 0.1 and 20.0 S.

Molecular modelling

The template used for the homology modelling was the crystal structure of human m-NAD(P)-ME in a pentameric complex with its natural substrate pyruvate, its cofactors NAD⁺, Mn⁺ and the allosteric activator fumarate (pdb ID: 1PJ3) [33]. The tetrameric models of WT (wild-type) ME and four mutants (K57A, K57C, K57S and K57R) were created using MODELLER version 9.5 [42]. From 100 models generated from each modelling process, we selected five models with the best molpdp (molecular probability density function) scores. We then submitted the models to the PISA (protein interfaces, surfaces and assembly) service at the European Bioinformatics Institute (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) [43] to measure the tetramer interface area and the number of potential hydrogen bonds across the interface.

**RESULTS**

**Activating effect of fumarate on the human WT and Lys⁵⁷ mutant m-NAD(P)-ME**

The initial velocity of m-NAD(P)-ME measured under various concentrations of fumarate displayed a hyperbolic kinetic pattern (Figure 2). The WT enzyme reached a maximal activation of approx. 1.6-fold at the saturated fumarate concentration (Figure 2, ●), with an apparent activation constant (K₉) of 0.20 ± 0.03 mM (Table 1). The apparent K₉ values of K57R, K57A and K57S mutant enzymes were higher than that of WT by approx. 10-fold (Table 1). The enzyme activity of K57R can be stimulated by fumarate with an apparent K₉ value of 2.8 ± 0.4 mM (Table 1), and its maximal enzyme activity was recovered to approx. 60 % of WT (Figure 2, ■). The K57A and K57S enzymes had similar titration curves with fumarate (Figure 2, ○ and Δ respectively). The apparent K₉ values of K57A and K57S were 3.36 ± 0.73 mM and 2.36 ± 0.27 mM respectively (Table 1), and their enzyme activity with saturated fumarate was restored to approx. half of...
parameters of WT and Lys57 mutant enzymes, determined with or without fumarate concentration. Table 1 shows the kinetic parameters of these mutant enzymes were determined using the respective fumarate-induced activation threshold than WT. These kinetic results, when taken together, implied that not only the allosteric, but also the catalytic site of the enzyme, were affected by mutation of Lys57, thus these Lys57 mutants exhibited a less active enzyme form.

Fumarate was able to partially activate these Lys57 mutant enzymes. In the presence of fumarate, the high \( K_{\text{cat}} / K_{\text{m, malate}} \) values of Lys57 mutant enzymes were reduced and the low \( k_{\text{cat}} \) values were elevated (Table 1). For K57A, K57S and K57R, the \( K_{\text{m, malate}} \) value of K57C mutant. The \( K_{\text{cat}}/K_{\text{m, malate}} \) values of K57C, although reduced approx. 2.5-fold by the addition of fumarate, is still 10-fold higher than WT. Meanwhile, the \( k_{\text{cat}} \) values of these Lys57 mutants, although elevated by the addition of fumarate, were still lower than WT by 1.8–3-fold. The \( k_{\text{cat}}/K_{\text{m, malate}} \) values of the K57A and K57R enzymes were elevated to 16% of WT levels, but the K57C and K57S enzymes were still only 4% of the WT enzyme levels (Table 1), demonstrating that the catalytic efficiencies of these Lys57 mutants was still much less than WT. The key fact from these kinetic data is that the Lys57 mutant enzymes have unusually low \( K_{\text{m, malate}} \) values which can be decreased to a level similar to that of WT by the addition of fumarate; however, the 50% fumarate saturation levels for these mutants is much higher than WT. These kinetic results, when taken together, implied that not only the allosteric, but also the catalytic site of the enzyme, were influenced by the replacement of Lys57. A conformational change which is unfavourable for catalysis and allosteric activation of the enzyme may occur in these Lys57 mutants.

Dependence curve of malate against initial velocity in the presence of fumarate for the human WT and Lys57 mutant m-NAD(P)-ME

The human m-NAD(P)-ME acts in a co-operative fashion upon binding of malate. The initial rates of the WT enzyme measured under various concentrations of malate demonstrated sigmoidal kinetics (Figure 3, ○). The co-operativity of malate binding, however, can be abolished by fumarate. In the presence of fumarate, the curve changed from sigmoidal to hyperbolic (Figure 3, ▼), and the \( h \) value was extensively reduced from 1.8 to 1.0 (Table 1). For these Lys57 mutant enzymes, the co-operativity of malate binding seen in WT almost fully vanished (Figures 3B–3E, ▼), indicating that these mutants had become non-co-operative.

<table>
<thead>
<tr>
<th>WT/mutant</th>
<th>Fumarate</th>
<th>( K_{5.5, \text{malate}} ) (mM)</th>
<th>( K_{\text{m, NAD}} ) (mM)</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( k_{\text{cat}}/K_{0.5, \text{malate}} ) (mM ( \text{h} \cdot \text{s}^{-1} ))</th>
<th>Fold increase in ( k_{\text{cat}}/K_{0.5, \text{malate}} ) ( K_{\text{m, NAD}} ) (mM ( \text{h} \cdot \text{s}^{-1} ))</th>
<th>( h )</th>
<th>( K_{\text{cat}, \text{app}} ) (mM)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>−</td>
<td>12.0 ± 1.8</td>
<td>3.4 ± 0.6</td>
<td>1.2 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>1.57 ± 0.30</td>
<td>5.1 ± 1.2</td>
<td>9.3 ± 2.0</td>
<td>4.6 ± 1.2</td>
<td>3.0 ± 1.2</td>
<td>5.0 ± 1.2</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>K57A</td>
<td>−</td>
<td>211.7 ± 35.8</td>
<td>1.44 ± 0.15</td>
<td>0.19 ± 0.04</td>
<td>0.015</td>
<td>1.0 ± 0.2</td>
<td>3.36 ± 0.73</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>8.6 ± 0.6</td>
<td>1.14 ± 0.15</td>
<td>1.1 ± 0.1</td>
<td>0.16</td>
<td>1.1 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>K57C</td>
<td>−</td>
<td>78.9 ± 9.3</td>
<td>7.51 ± 2.08</td>
<td>0.09 ± 0.3</td>
<td>0.008</td>
<td>1.0 ± 0.1</td>
<td>16.8 ± 5.1</td>
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</tr>
<tr>
<td>+</td>
<td></td>
<td>32.0 ± 1.5</td>
<td>0.63 ± 0.14</td>
<td>0.1 ± 0.1</td>
<td>0.04</td>
<td>1.0 ± 0.1</td>
<td>2.36 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>K57S</td>
<td>−</td>
<td>173.0 ± 13.7</td>
<td>3.05 ± 0.24</td>
<td>0.11 ± 0.01</td>
<td>0.009</td>
<td>1.1 ± 0.1</td>
<td>2.82 ± 0.40</td>
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</tr>
<tr>
<td>+</td>
<td></td>
<td>9.7 ± 0.5</td>
<td>1.62 ± 0.38</td>
<td>0.6 ± 1.5</td>
<td>0.04</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>K57R</td>
<td>−</td>
<td>93.7 ± 12.3</td>
<td>4.09 ± 0.73</td>
<td>0.18 ± 0.04</td>
<td>0.015</td>
<td>1.1 ± 0.1</td>
<td>2.36 ± 0.27</td>
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<tr>
<td>+</td>
<td></td>
<td>5.0 ± 0.5</td>
<td>1.29 ± 0.20</td>
<td>0.11 ± 0.4</td>
<td>0.17</td>
<td>1.1 ± 0.2</td>
<td>0.20 ± 0.03</td>
<td></td>
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*Values derived from Figure 2.
†Values derived from Figure 1.

**Table 1** Kinetic parameters for the human WT and Lys57 mutant m-NAD(P)-ME

WT, suggesting that they had lower affinity for the effector enzymes had a higher fumarate-induced activation threshold than WT, indicating that these mutants had become non-co-operative.
enzymes. The $h$ value of these mutants was 1.0 both with and without fumarate (Table 1).

In addition to the non-co-operative binding of malate, these Lys$^{57}$ mutant enzymes, in the absence of fumarate, showed very low enzyme activities and required a very high malate concentration to reach their $V_{\text{max}}$ (Figures 3B–3E, ◦). However, in saturated fumarate, these Lys$^{57}$ mutants could still be greatly stimulated by fumarate and required less malate to reach their maximal velocities (Figures 3B–3E, ▼).

**Quaternary structure of the human WT and Lys$^{57}$ mutant m-NAD(P)-ME**

Lys$^{57}$ is located at the dimer interface and is hydrogen-bonded to Pro$^{216}$ and Tyr$^{218}$ from the other subunit. Kinetic analysis indicated that the co-operativity of malate binding was totally lost in these Lys$^{57}$ mutant enzymes. Since the tetramer organization may be involved in the co-operative behaviour of this enzyme [2], we used analytical ultracentrifugation to examine possible changes in the quaternary structure of these Lys$^{57}$ mutants. Figure 4 shows the continuous sedimentation coefficient distribution of WT and Lys$^{57}$ mutants. The sedimentation coefficients of 6.5 S and 9.0 S, corresponding to the molecular masses of 124 and 248 kDa, were the dimeric and tetrameric forms of the protein respectively. The WT m-NAD(P)-ME exists in a dimer–tetramer equilibrium (Figure 4A), and this dimer is possibly an AB dimer rather than an AD dimer, since the contacts in the tetramer interface are weaker than those in the dimer interface (Figure 1A) [2]. In contrast, the K57A, K57C and K57S mutants display predominantly a dimeric quaternary structure with a small amount of monomers, tetramers and polymers (Figures 4B–4D), indicating that the mutation of Lys$^{57}$ leads to a disruption of the quaternary structure at the dimer interface, leading to the dissociation of the enzyme. The dimeric form in the Lys$^{57}$ mutants, however, might be an AD dimer rather than an AB dimer, and the AB dimer seen in WT may be dissociated into monomers in these Lys$^{57}$ mutants. The quaternary structure of the K57R enzyme, similar to that of WT, also exists in a dimer–tetramer equilibrium (Figure 4E), suggesting that the positive-charge on residue 57 may play a role in maintaining the quaternary structure organization in the dimer interface of the enzyme.

Fumarate can assist in reconstituting the dimeric enzymes into tetramers, suggesting that fumarate may be involved in the tetramer reorganization of the enzyme and a function that may facilitate enzyme catalysis [2]. The dimer–tetramer equilibrium of the WT enzyme was shifted following addition of fumarate (Figure 4A, broken line). The K57A, K57C and K57S mutants occurred as dimers, but could not be reconstituted into tetramers like WT (results not shown). Although the K57R enzyme is in dimer–tetramer equilibrium with very few monomers, it could not be reconstituted from dimers into tetramers by addition of fumarate (Figure 4E, broken lines). These results suggest...
that the tetramer organization was truly perturbed in these mutants.

**Modelling of the Lys57 mutant m-NAD(P)-ME**

To assess the difference in the dimer interface between the WT and Lys57 mutant structures, we built tetrameric models for K57A, K57C, K57S and K57R mutants by using the comparative modelling software MODELLER [42]. The models were based on the crystal structure of human m-NAD(P)-ME enzyme in a pentanary complex with its natural product pyruvate, its cofactors NAD+, Mn2+ and its allosteric activator fumarate (PDB ID: 1PJ3) [33]. MODELLER employs methods of conjugate gradients and molecular dynamics with simulated annealing to optimize the possible side-chain conformation in the protein–protein interface. We also used a similar procedure to create the WT models. Subsequently, we compared the WT models with the mutant models by calculating the tetramer interface area and the number of potential hydrogen bonds across the interface (Table 2).

**DISCUSSION**

Human m-NAD(P)-ME is characterized as an allosteric enzyme, which differentiates it from the other two isoforms. Fumarate has been recognized as the allosteric activator for human m-NAD(P)-ME by reducing the $K_a$ values of the substrates [13,21,23,28]. Structural analysis indicates that the allosteric site is located at the dimer interface of the enzyme and the binding network of fumarate in this site has been identified [21]. In the fumarate-binding pocket, two arginine residues, Arg57 and Arg91, are the direct ligands for fumarate. An anionic residue, Glu59, which is ion-paired with Arg67 in this site, is also important for the fumarate activation (Figure 5A). Our recent studies have shown that Glu59 is involved, not only in the allosteric regulation, but also the subunit–subunit interactions of the enzyme [44]. An interface cationic residue, Lys57, which is ion-paired with Glu59, also forms a hydrogen bond network with Pro216 and Tyr218 from another subunit (Figure 5A). In the present study, we explored the functional role of Lys57 in the allosteric site and in the dimer interface of the enzyme.

**Table 2** Dimer and tetramer interface of the human WT and Lys57 mutant m-NAD(P)-ME  
Values are means ± S.E.M.

<table>
<thead>
<tr>
<th>WT/mutant</th>
<th>Interface</th>
<th>Interface area (Å²)†</th>
<th>Number of hydrogen bonds</th>
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<tr>
<td>K57A</td>
<td>IAB*</td>
<td>2030.1 ± 44.1</td>
<td>16.8 ± 1.6</td>
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<td></td>
<td>ICD</td>
<td>2072.8 ± 28.3</td>
<td>17.2 ± 1.1</td>
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<td></td>
<td>IAD</td>
<td>899.0 ± 22.8</td>
<td>10.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>862.3 ± 22.8</td>
<td>10.4 ± 0.5</td>
</tr>
<tr>
<td>K57C</td>
<td>IAB*</td>
<td>1984.1 ± 40.2</td>
<td>13.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>ICD</td>
<td>1995.2 ± 22.4</td>
<td>13.0 ± 2.2</td>
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<tr>
<td></td>
<td>IAD</td>
<td>884.1 ± 36.0</td>
<td>9.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>854.6 ± 23.2</td>
<td>9.4 ± 1.5</td>
</tr>
<tr>
<td>K57S</td>
<td>IAB*</td>
<td>2011.5 ± 15.2</td>
<td>15.2 ± 2.2</td>
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<td></td>
<td>ICD</td>
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<tr>
<td></td>
<td>IAD</td>
<td>898.2 ± 17.0</td>
<td>10.2 ± 1.8</td>
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<td>IBC</td>
<td>860.4 ± 9.5</td>
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<td>K57R</td>
<td>IAB*</td>
<td>2016.5 ± 43.1</td>
<td>15.6 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>ICD</td>
<td>1985.9 ± 42.7</td>
<td>14.4 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>IAD</td>
<td>890.5 ± 27.2</td>
<td>10.2 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>IBC</td>
<td>853.2 ± 8.4</td>
<td>9.4 ± 1.1</td>
</tr>
</tbody>
</table>

*For the abbreviation used in this column, IXY means the interface between monomers X and Y in a tetrameric malic enzyme.
††± 1 Å = 0.1 nm.
The Lys^{57}-involved hydrogen-bond network is important for the structural integrity of the allosteric site

Site-directed mutagenesis and kinetic analysis strongly suggest that Lys^{57} has profound effects on the enzymatic allosteric regulation and on the enzyme catalysis. The Lys^{57} mutant enzymes display a much higher threshold for their maximal activation compared with WT, indicating that the conformation of the allosteric site is significantly changed in these mutants and that the binding affinity for fumarate may be significantly decreased. Computer modelling of the Lys^{57} mutants suggests that the hydrogen-bond network in the fumarate-binding site may be partially abolished (Figures 5B–5E) which may lead to conformational changes that are disadvantageous to fumarate-induced activation.

Furthermore, mutation of Lys^{57} in m-NAD(P)-ME causes the enzyme to become a less-active enzyme with a very large $K_{0.5,\text{malate}}$, small $k_{\text{cat}}$ value and low catalytic efficiency ($k_{\text{cat}}/K_{0.5,\text{malate}}K_{\text{m,NAD}}$) (Table 1), implying that the conformation of the active site has been considerably changed. This may result from the loss of the hydrogen-bond network in the fumarate-binding site, which may be detrimental to the active-site conformation of the enzyme. Despite the fact that these structural changes in the Lys^{57} mutants may be partially readjusted by addition of fumarate [which leads to a reduction in $K_{0.5,\text{malate}}$ and an elevation in $k_{\text{cat}}$ values and enzyme activity (Table 1 and Figures 1 and 3)], these Lys^{57} mutants are still less active than the WT enzyme, highlighting the importance of the Lys^{57}-involved binding network. Indeed, mutation of the corresponding residue in c-NADP-ME (S57K mutant) did not cause any change in kinetic properties or in the
quaternary structure organization (results not shown). Thus, Lys\textsuperscript{57} has an unique role in the m-NAD(P)-ME isofom.

The Lys\textsuperscript{57}-involved hydrogen-bond network is important for the subunit–subunit interaction at the dimer interface

In addition to the fumarate-induced activation, Lys\textsuperscript{57} may be involved in the subunit–subunit interactions. Indeed, we have shown that the quaternary structure of Lys\textsuperscript{57} mutants has been significantly changed (Figure 4). Modelling structures of the Lys\textsuperscript{57} mutant enzymes demonstrate that the Lys\textsuperscript{57}-involved subunit–subunit interactions in the dimer interface of the WT enzyme, including the interface area and the number of hydrogen bonds, may be reduced (Table 2 and Figures 5B–5E). Even though the Lys\textsuperscript{57}-involved hydrogen-bond network between subunit A and B was changed, these mutant enzymes still partially retained the ability to be activated by fumarate. Nevertheless, these Lys\textsuperscript{57} mutants cannot be reconstituted into tetramers by the addition of fumarate and cannot recover their full enzymatic activity. Furthermore, perturbation of the tetramer organization between subunits A and B truly caused the enzyme to lose its co-operativity. Conservation of the positive charge on this residue (K57R mutant; Figure 4E) may be helpful in maintaining the quaternary structure of the enzyme, but the co-operativity of the enzyme is still abolished. Thus these Lys\textsuperscript{57} mutants no longer display a sigmoidal kinetic behaviour, the interactions between subunits are perturbed and the co-operativity is abolished. Taken together, these results suggest that the Lys\textsuperscript{57}-involved hydrogen-bond network at the dimer interface of the enzyme is crucial for the quaternary structural organization of the enzyme, and for enzyme regulation.

In conclusion, we suggest that Lys\textsuperscript{57} has dual functional roles in the allosteric site and at the dimer interface of the human m-NAD(P)-ME. Lys\textsuperscript{57}, in the fumarate-binding site, contributes to the conformational integrity of the allosteric site. In addition, at the dimer interface, the Lys\textsuperscript{57}-involved hydrogen-bond network stabilizes the subunit–subunit interaction.
Human mitochondrial malic enzyme


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