Determinants of the Dual Cofactor Specificity and Substrate Cooperativity of the Human Mitochondrial NAD(P)\(^+\)-dependent Malic Enzyme

**FUNCTIONAL ROLES OF GLUTAMINE 362**

Received for publication, April 11, 2006, and in revised form, June 2, 2006. Published, JBC Papers in Press, June 6, 2006, DOI 10.1074/jbc.M603451200

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The human mitochondrial NAD(P)\(^+\)-dependent malic enzyme (m-NAD-ME) is a malic enzyme isoform with dual cofactor specificity and substrate binding cooperativity. Previous kinetic studies have suggested that Lys\(^{362}\) in the pigeon cytosolic NADP\(^+\)-dependent malic enzyme has remarkable effects on the binding of NAD\(^+\) to the enzyme and on the catalytic power of the enzyme (Kuo, C. C., Tsai, L. C., Chin, T. Y., Chang, G.-G., and Chou, W. Y. (2000) Biochem. Biophys. Res. Commun. 270, 821–825). In this study, we investigate the important role of Gln\(^{362}\) in the transformation of cofactor specificity from NAD\(^+\) to NADP\(^+\) in human m-NAD-ME. Our kinetic data clearly indicate that the Q362K mutant shifted its cofactor preference from NAD\(^+\) to NADP\(^+\). The \(K_m(NADP)\) and \(k_{cat}(NADP)\) values for this mutant were reduced by 4–6-fold and increased by 5–10-fold, respectively, compared with those for the wild-type enzyme. Furthermore, up to a 2-fold reduction in \(K_m(NADP)/K_m(NAD)\) and elevation of \(k_{cat}(NADP)/k_{cat}(NAD)\) were observed for the Q362K enzyme. Mutation of Gln\(^{362}\) to Ala or Asn did not shift its cofactor preference. The \(K_m(NADP)/K_m(NAD)\) and \(k_{cat}(NADP)/k_{cat}(NAD)\) values for Q362A and Q362N were comparable with those for the wild-type enzyme. The \(\Delta G\) values for Q362A and Q362N with either NAD\(^+\) or NADP\(^+\) were positive, indicating that substitution of Gln with Ala or Asn at position 362 brings about unfavorable cofactor binding at the active site and thus significantly reduces the catalytic efficiency. Our data also indicate that the cooperative binding of malate became insignificant in human m-NAD-ME upon mutation of Gln\(^{362}\) to Lys because the sigmoidal phenomenon appearing in the wild-type enzyme was much less obvious that that in Q362K. Therefore, mutation of Gln\(^{362}\) to Lys in human m-NAD-ME alters its kinetic properties of cofactor preference, malate binding cooperativity, and allosteric regulation by fumarate. However, the other Gln\(^{362}\) mutants, Q362A and Q362N, have conserved malate binding cooperativity and NAD\(^+\) specificity. In this study, we provide clear evidence that the single mutation of Gln\(^{362}\) to Lys in human m-NAD-ME changes it to an NADP\(^+\)-dependent enzyme, which is characteristic because it is non-allosteric, non-cooperative, and NADP\(^+\)-specific.

Malic enzymes are a newly discovered family of oxidative decarboxylases that catalyze the conversion of the substrate l-malate to CO\(_2\) and pyruvate, with concomitant reduction of NAD(P)\(^+\) to NAD(P)H (1–5). Divalent metal ion (Mn\(^{2+}\) or Mg\(^{2+}\)) is required for the enzyme-catalyzed reaction. These enzymes exist ubiquitously in nature, with conserved sequences and similar overall structural topology among different species (6–10). In mammalian cells, according to their cofactor specificity, three isoforms of malic enzyme have been recognized: cytosolic NADP\(^+\)-dependent (c-NADP-ME) (11, 12), mitochondrial NADP\(^+\)-dependent (13), and mitochondrial NAD(P)\(^+\)-dependent (m-NAD-ME) (3, 14). The third isoform, m-NAD-ME, can utilize both NAD\(^+\) and NADP\(^+\) as a cofactor, but it prefers NAD\(^+\) under physiological conditions (3). Via the NADH and pyruvate products, human m-NAD-ME may play an important role in the metabolism of glutamine in fast growing tissues and tumors (3, 14–21).

Distinctive from the other two mammalian isoforms, m-NAD-ME has a complex regulatory system that controls its catalytic activity (22–24). It displays cooperative behavior with respect to the substrate l-malate, and the enzyme activity can be allosterically activated by fumarate (19, 22–28). Furthermore, previous studies have suggested that the inhibitory effect of ATP may operate through an allosteric mechanism (19, 22, 26, 29, 30), and the allosteric properties of m-NAD-ME imply its specific role in the pathways of malate and glutamine oxidation in tumor mitochondria (18–22, 25). However, site-directed mutagenesis and kinetic studies have demonstrated that ATP may actually act as an active-site inhibitor rather than an allosteric inhibitor (30, 39).

The crystal structures of malic enzymes reveal that the enzyme is a homotetramer with a dimer of dimers quaternary structure. These structural data establish malic enzymes as a new class of...
Malic enzymes have high amino acid sequence conservation but distinct cofactor specificities. Previous kinetic studies have suggested that Lys\(^{362}\) in pigeon c-NADP-ME has noteworthy impact on the binding of NADP\(^+\) to the enzyme as well as a considerable effect on the catalytic power of the enzyme. The structure of pigeon c-NADP-ME reveals that the 2'-'phosphate of NADP\(^+\) is placed on the surface of the enzyme and interacts with Ser\(^{346}\) and the ammonium side chain of Lys\(^{362}\) (8). Sequence alignments of malic enzymes revealed that Lys\(^{362}\) is completely conserved among NADP\(^+\)-dependent malic enzymes, but in NAD\(^+\) dependent malic enzymes, residue 362 is glutamine instead (Fig. 1B). This prompted us to examine the effect of mutation of Gln\(^{362}\) to Lys on the cofactor specificity of human m-NAD-ME.

In this study, we provide detailed kinetic evidence delineating the determinants of the dual cofactor specificity and substrate cooperativity of human m-NAD-ME. Site-directed mutagenesis was used to identify the significance of Gln\(^{362}\) in the cofactor specificity, substrate cooperativity, and catalytic activity of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Malic Enzymes**—The detailed expression and purification steps for human m-NAD-ME were described previously (3, 32). Briefly, m-NAD-ME was cloned into the expression vector pRH281 and transformed into *Escherichia coli* BL21 cells for overexpression of the enzyme under the control of the inducible trp promoter system (3). The overexpressed enzyme was purified by anion exchange DEAE-Sepharose chromatography (Amersham Biosciences), followed by ATP-agarose affinity chromatography (Sigma). After purification, the enzyme was buffer-exchanged and concentrated in 30 mM Tris-HCl (pH 7.4) using Amicon Ultra-15 centrifugal filter devices (Millipore Corp.) with a molecular mass cutoff at 30 kDa. The enzyme purity was examined by SDS-PAGE, and the protein concentrations were determined by the method of Bradford (35).
Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange kit (Stratagene). The purified DNA of human m-NAD-ME was used as template, and the desired primers were used to mutate the Gln362 codon to the desired primers were used to mutate the Gln362 codon to the Ala, Lys, and Asn codons using Photolink DNA polymerase, which replicated both plasmid strands with high fidelity in the PCR. The primers containing the mutated site were 25–45-mers, which were needed for specific binding of template DNA. The synthetic oligonucleotides used as mutagenic primers were 5'-GCAAATAGATGTTAGCTGACATTTCACCTCAC-TCAGC-3' for Q362A, 5'-GCAAATAGATGTTAGCTGACATTTCACCTCACCTCACACCA-3' for Q362K, and 5'-GCAAATAGATGTTAGCTGACATTTCACCTCACCTCACACCA-3' for Q362N (with the mutation positions underlined and in boldface). After 16–18 temperature cycles, mutated plasmids including staggered nicks were produced. The PCR products were then treated with DpnI to digest the human wild-type m-NAD-ME templates. Finally, the nicked DNAs containing the desired mutations were transformed into the Escherichia coli XL-1 strain, and their DNA sequences were checked by autosequencing.

Enzyme Kinetic Analysis—The enzyme reaction was measured by the production of NADH or NADPH. The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 10 mM malate (pH 7.4), various concentrations of NAD+ or NADP+, and 10 mM MgCl2 in a total volume of 1 ml. The absorbance at 340 nm at 30 °C was instantly recorded after the enzyme was added to the reaction mixture and monitored continuously in a Beckman DU 7500 spectrophotometer. In this process, 1 unit of enzyme is defined as the enzyme amount that catalyzes the production of 1 μmol of NAD(P)H/min. An absorption coefficient of 6.22 mm−1 for NAD(P)H was used in the calculations. Apparent Michaelis constants of the substrate and cofactors were determined by varying the concentration of one substrate (or cofactor) around its Km while leaving the other components unvaried at the saturated concentrations. The ATP inhibition experiment for malic enzyme activity was carried out at a series of ATP concentrations from 0 to 3 mM at different concentrations of NAD+. The total set of data was globally fitted to the following equation, which expresses a competitive inhibition pattern: 

\[ v = \frac{V_{\text{max}}[S]/[S]}{K_m + ([ATP]/K_{i(\text{ATP})})} \]

where \( v \) is the observed initial velocity, \( V_{\text{max}} \) is the maximum rate of the reaction, \( K_m \) is the Michaelis constant for the substrate, and \( K_{i(\text{ATP})} \) is the inhibition constant for ATP.

The sigmoidal curves of [malate] versus initial rates were fitted to the Hill equation, and data were further analyzed to calculate the \( K_{O.5} \) value, the substrate concentration at half-maximal velocity, and the Hill coefficient (h), which is a measure of the degree of cooperativity: 

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

All data fitting work was performed with the SigmaPlot 8.0 program (Jandel, San Rafael, CA).

### RESULTS

Expression, Purification, and Characterization of Recombinant Human m-NAD-ME—The recombinant malic enzymes were successfully expressed and purified to apparent homogeneity (data not shown). The amounts of purified protein were 5.1, 1.7, 5.9, and 1.9 mg from 250 ml of cell lysate, and the specific activities of these enzymes were 45.2, 4.1, 1.8, and 3.8 units/mg for the wild-type (WT), Q362A, Q362K, and Q362N enzymes, respectively. The results indicate that the WT and Gln362 mutant enzymes were significantly overexpressed. The much lower specific activities of these Gln362 mutant enzymes imply that Gln362 may play an important role in the catalytic mechanism of the malic enzyme.

The kinetic parameters for the recombinant malic enzymes utilizing NAD+ or NADP+ as the cofactor were determined in the absence or presence of fumarate (Table 1). The initial velocities measured in various concentrations of NAD+ or NADP+ followed hyperbolic kinetics. In the presence of fumarate, the \( K_m([\text{NAD}] \text{ or NADP}) \) and \( K_{m([\text{NAD}] \text{ or NADP})} \) values for the WT and Gln362 mutant enzymes were reduced, and the \( k_{cat} \) values for these enzymes were elevated, indicating that these mutant enzymes can still be allosterically activated by fumarate. The \( K_m([\text{NAD}] \text{ or NADP}) \) values for the Gln362 mutants were increased by ~2–4-fold compared with that for the WT enzyme, but the \( K_{m([\text{NAD}] \text{ or NADP})} \) values increased slightly for Q362A and Q362N and notably decreased for Q362K. Furthermore, the \( K_m([\text{NAD}] \text{ or NADP}) \) values for the WT, Q362A, and Q362N enzymes were larger than the \( K_m([\text{NAD}] \text{ or NADP}) \) values; however, the opposite results were found for the Q362K enzyme. Although fumarate always decreased the \( K_m \) values for these enzymes, it seemed to be less effective in reduction of the \( K_{m([\text{NAD}] \text{ or NADP})} \) values. The decrease in the \( K_{m([\text{NAD}] \text{ or NADP})} \) values for these enzymes by fumarate was ~1.6–3.6-fold, but the decrease in the \( K_{m([\text{NAD}] \text{ or NADP})} \) values was ~1.2–1.9-fold.

The \( k_{cat} \) values for the WT and Gln362 mutant enzymes with NADP+ as the cofactor were much less than those with NAD+ as the cofactor except for the Q362K enzyme. The \( k_{cat([\text{NAD}] \text{ or NADP})} \) for Q362K was ~2-fold larger than the \( k_{cat([\text{NAD}] \text{ or NADP})} \) for the enzyme, indicating that the Q362K enzyme favors NADP+ as the cofactor. The \( k_{cat([\text{NAD}] \text{ or NADP})} \) and \( k_{cat([\text{NAD}] \text{ or NADP})} \) values for the Gln362 mutants were decreased by ~2–7- and ~4–19-fold, respectively, compared with those for the WT enzyme, suggesting that this amino acid residue has its
specific role in the catalytic mechanism of the malic enzyme. Similar to the $k_{\text{cat(NAD)}}$ and $k_{\text{cat(NAD)}}/K_{m(NAD)}$ values for Q362A and Q362N, the $k_{\text{cat(NADP)}}$ and $k_{\text{cat(NADP)}}/K_{m(NADP)}$ values for these two mutants were not less than those for the WT enzyme. However, the $k_{\text{cat(NADP)}}$ for the Q362K enzyme was increased by ~5–10-fold in the absence or presence of fumarate, respectively, compared with that for the WT enzyme. Furthermore, the $k_{\text{cat(NADP)}}/K_{m(NADP)}$ values for Q362K were ~27–35-fold higher than those for the WT enzyme. Fumarate increased the $k_{\text{cat(NAD)}}$ and $k_{\text{cat(NADP)}}$ values for these enzymes, although its effect was more pronounced in increasing the $k_{\text{cat(NADP)}}$ for Q362K. Fumarate increased the $k_{\text{cat(NAD)}}$ and $k_{\text{cat(NADP)}}$ values for these enzymes by ~1.5–2.0-fold; but for Q362K, the $k_{\text{cat(NADP)}}$ was only slightly increased by 1.1-fold.

**Cooperative Effect of Malate on Wild-type and Mutant m-NAD-MEs—**The initial velocities of m-NAD-ME measured in various concentrations of malate with either NAD$^+$ or NADP$^+$ as the cofactor demonstrated sigmoidal kinetics (Fig. 2, closed circles), which implies cooperative malate binding. Fumarate completely abolished this cooperative effect of malate. At a saturated concentration of fumarate (3 mM), which can fully activated malic enzyme activities, the curve changed from sigmoidal to hyperbolic (Fig. 2, closed triangles). For the WT (Fig. 2, A and B), Q362A (Fig. 2, C and D), and Q362N (Fig. 2, G and H) enzymes, the initial velocities at various malate concentrations in the absence of fumarate showed obvious sigmoidal kinetics, and this phenomenon was diminished after addition of fumarate. These results show that mutation of Gln$^{362}$ to Ala or Asn did not change the cooperative effect of malate binding to the enzyme and that this cooperative effect could still be abolished by fumarate. For the Q362K enzyme, the cooperative effect of malate with NAD$^+$ as the cofactor was not significant (Fig. 2F), and in contrast to the WT enzyme, both cooperativity and fumarate activation were not obvious in the Q362K enzyme with NADP$^+$ as the cofactor (Fig. 2F), suggesting that the mutant enzyme is less sensitive to fumarate with NADP$^+$ as the cofactor.

Table 2 summarizes the results obtained from analysis of the sigmoidal curves fitted by the Hill equation. For the WT enzyme, the $K_{0.5,\text{malate(NAD)}}$ and $K_{0.5,\text{malate(NADP)}}$ values, corresponding to half-saturation for i-malate with NAD$^+$ or NADP$^+$ as the cofactor, were similar, and both were reduced by fumarate. This suggests that the cooperative effect of malate is independent of cofactor specificity. The Hill coefficient ($h$) with either NAD$^+$ or NADP$^+$ ($h_{\text{NAD}}$ and $h_{\text{NADP}}$, respectively) was

**TABLE 2**

|                  | $K_{0.5,\text{malate(NAD)}}$ | $K_{0.5,\text{malate(NADP)}}$ | $b_{\text{NAD}}$ | $b_{\text{NADP}}$ | $K_{\text{cat(NAD)}}$ | $K_{\text{cat(NADP)}}$ |
|------------------|-----------------------------|-----------------------------|------------------|------------------|-------------------|-------------------|
| Wild-type (−)    | 12.84 ± 0.48                | 15.50 ± 0.78                | 1.93 ± 0.10      | 1.94 ± 0.14      | 0.30 ± 0.04       | 0.33 ± 0.03       |
| Wild-type (+)    | 3.26 ± 0.50                 | 4.31 ± 0.66                 | 1.04 ± 0.12      | 1.15 ± 0.13      | 0.27 ± 0.06       | 0.32 ± 0.07       |
| Q362A (−)        | 15.87 ± 0.66                | 12.60 ± 0.72                | 3.89 ± 0.18      | 2.98 ± 0.12      | 0.17 ± 0.01       | 0.32 ± 0.02       |
| Q362A (+)        | 3.91 ± 1.54                 | 5.25 ± 0.39                 | 1.61 ± 0.21      | 1.08 ± 0.06      | 0.27 ± 0.06       | 0.33 ± 0.07       |
| Q362K (−)        | 11.85 ± 0.94                | 6.83 ± 0.33                 | 1.57 ± 0.14      | 1.34 ± 0.08      | 0.17 ± 0.01       | 0.32 ± 0.02       |
| Q362K (+)        | 5.09 ± 1.42                 | 3.44 ± 0.39                 | 1.00 ± 0.18      | 1.06 ± 0.10      | 0.33 ± 0.06       | 0.57 ± 0.07       |
| Q362N (−)        | 16.77 ± 1.63                | 10.48 ± 0.67                | 1.91 ± 0.25      | 2.04 ± 0.20      | 0.33 ± 0.06       | 0.57 ± 0.07       |
| Q362N (+)        | 4.79 ± 0.63                 | 5.48 ± 0.51                 | 1.06 ± 0.10      | 1.16 ± 0.08      | 0.33 ± 0.06       | 0.57 ± 0.07       |

**Figure 2. Cooperative effect of malate on the human mitochondrial malic enzyme.** The assay mixture contained 10 mM MgCl$_2$, 1 mM NAD$^+$ or NADP$^+$, and various concentrations of malate in a 50 mM Tris-HCl (pH 7.4) buffer system in the absence or presence of fumarate. The fumarate concentrations used in the experiments were 0 mM (closed circles), 0.2 mM (open circles), and 3 mM (closed triangles). A, C, E, and G show the titration curves with NAD$^+$ as the cofactor, whereas B, D, F, and H show those with NADP$^+$ as the cofactor. A and B, the WT enzyme; C and D, the Q362A enzyme; E and F, the Q362K enzyme; G and H, the Q362N enzyme.

**Figure 3. Inhibitory effect of ATP on the human mitochondrial malic enzyme.** ATP inhibition of the WT and Gln$^{362}$ mutant enzymes was assayed using NAD$^+$ (A) or NADP$^+$ (B) as the cofactor. The assay mixture contained 15 mM malate, 10 mM MgCl$_2$, and 1 mM NAD$^+$ or NADP$^+$ in a 50 mM Tris-HCl (pH 7.4) buffer system. The ATP concentration ranged from 0 to 3 mM.
enzymes were at the same level with NADP.
Residual enzyme activities of the WT, Q362A, and Q362K treatments almost overlapped with that for the WT enzyme. The h values for the WT and Gln362 mutant enzymes were reduced almost to 1 in the presence of fumarate were reduced to similar levels for the WT and mutant enzymes. The h values for Q362K were 1.57 and 1.34, respectively, which are smaller than those for the WT enzyme, suggesting that the cooperative effect of malate binding is insignificant in this mutant enzyme. The h values for Q362A (h = 2.39) was larger than that for the WT enzyme, but the h values for Q362K was slightly smaller (h = 1.75). The h values for Q362N were 1.91 and 2.04, respectively, similar to those for the WT enzyme. The h values for the WT and Gln362 mutant enzymes were reduced almost to 1 in the presence of fumarate as manifested by the change of the sigmoidal curves to hyperbolic kinetics after fumarate addition (Fig. 2).

Inhibitory Effect of ATP on Wild-type and Gln362 Mutant Malic Enzymes—ATP could effectively inhibit the catalytic activity of human m-NAD-ME. The inhibitory effect of ATP was more pronounced on these Gln362 mutant enzymes with NAD+ as the cofactor (Fig. 3A). Q362K was the most susceptible to ATP inhibition (Fig. 3A, closed triangles). The residual enzyme activities were in the following order: WT, Q362N, Q362A, and Q362K (Fig. 3A). However, ATP inhibition was less obvious with NADP+ as the cofactor (Fig. 3B). The inhibition curves for Q362A and Q362K with increasing ATP concentrations almost overlapped with that for the WT enzyme. The residual enzyme activities of the WT, Q362A, and Q362K enzymes were at the same level with NADP+ (Fig. 3B), but were quite different with NAD+. However, the Q362N enzyme became a little more resistant to ATP inhibition compared with the WT enzyme (Fig. 3B).

ATP is an active-site inhibitor of m-NAD-ME following a competitive mechanism with respect to NAD+ and malate (29, 30). Our data indicate that the Gln362 mutants were also inhibited by ATP. A double-reciprocal plot demonstrated that all lines intersected at the y axis, indicating simple competitive inhibition patterns (data not shown). The inhibition constants of ATP with respect to NAD+ (KATP(NAD)+) and NADP+ (KATP(NADP)+) were 0.30 and 0.33 mM, respectively (Table 2), indicating that the binding affinity of ATP for the enzyme is not correlated with the cofactor specificity. In contrast, the KATP(NAD)+ values for Q362A and Q362K (0.27 and 0.17 mM, respectively) were smaller than those for the WT enzyme. On the other hand, the KATP(NADP)+ values for the WT, Q362A, and Q362K enzymes were almost identical (0.32–0.33 mM), whereas Q362N had a slightly larger KATP(NADP)+ value (0.57 mM) compared with the WT enzyme. However, Gln362 is not primarily responsible for the binding of ATP in the active site (10, 30, 33); thus, no significant changes were observed in the KATP(NAD)+ values for the WT and Gln362 mutant enzymes. Structural studies have revealed that ATP is bound at the cofactor-binding site, residing in the adenosine diphosphate-binding region (33). The additional phosphate of NADP seems to have no effect on the binding affinity of ATP.

**DISCUSSION**

Malic enzymes have discrete specificities for the dinucleotide cofactor in that some can only use NAD+ as the cofactor, whereas others can only use NADP+. Human m-NAD-ME is one of a small number of malic enzymes that have dual specificity (3). The molecular basis for the cofactor selectivity of these enzymes is still poorly understood.

Previous studies with other enzymes revealed that an Asp residue near the active site is expected to have an NAD+ preference and cannot tolerate the additional phosphate group of NADP+ (36, 37). However, the respective Asp residue, Asp345, is conserved in all malic enzymes. The structures show that Asp345 is pointed away from the ribose and is ion-paired with Arg354 (10). This provides an explanation for the observation that some malic enzymes with a conserved Asp at this position can still use NADP+ as the cofactor. The cofactor-binding regions of human m-NAD-ME and pigeon c-NADP-ME are shown in Fig. 4 (A and B, respectively), with the ionic interaction between Asp345 and Arg354 indicated by green dashed lines.

In c-NADP-ME, Asp345 is ion-paired not only with Arg354 but also with Lys362 (Fig. 4B). The additional salt bridge is not observed in human m-NAD-ME because the corresponding residue at position 362 is glutamine (Fig. 4A). Mutagenesis and kinetic studies have suggested that Lys362 in pigeon c-NADP-ME plays a critical role governing the cofactor specificity (38). Mutation of Lys362 has a tremendous effect on NADP+ binding to c-NADP-ME. Thus, the NADP+ specificity is proposed to be determined by the electrostatic interactions involving the positive charge of the side chain Lys362 (Fig. 4B).

This important residue is replaced by glutamine in human m-NAD-ME. The most important result of this work is our demonstration that the Gln362 mutants of human m-NAD-ME shift their cofactor specificity from NAD+ to NADP+.

*Cofactor Preference Shifting of the Human Q362K Enzyme—* Our kinetic data clearly indicate that the Q362K mutant enzyme shifted its cofactor preference from NAD+ to NADP+. The Km(NADP) and Kcat(NADP) values for this mutant were reduced by 4–6-fold and increased by 5–10-fold, respectively, compared with those for the WT enzyme. Furthermore, up to a 2-fold reduction in Km(NADP)/Km(NAD) and elevation of kcat(NADP)/kcat(NAD) were observed for the Q362K enzyme (Table 1). To evaluate the contribution of a side chain to substrate specificity in the WT enzyme relative to its change in a mutant, we further calculated the free

**FIGURE 4.** Active-site pockets with bound NAD+ or NADP+ and LIGPLOT of the human mitochondrial malic enzyme. A and B, the NAD-binding pocket of human m-NAD-ME (Protein Data Bank code 1PI3) and the NADP-binding pocket of pigeon c-NADP-ME (Protein Data Bank code 1GQ2), respectively, with interaction of residue 362. The green dashed lines (generated by Swiss-Pdb Viewer) (42) represent the hydrogen bonds between the amino acid residues and NAD+ or NADP+. C, the ligand interactions of Gln362 shown as a LIGPLOT diagram (43). The thick bonds are Gln362; the thin bonds are the hydrogen-bonded residues; and the green dashed lines correspond to the hydrogen bonds. Spoked arcs represent hydrophobic contacts.
energy change (ΔG) for the Gln362 mutant enzymes according to the following equation: ΔG = -RT ln((k_{cat(mut)})/K_m(mut))/(k_{cat(WT)}/K_m(WT))). The k_{cat}/K_m values and the ΔG values with NAD+ or NADP+ in the malic enzyme reaction are shown in Table 3.

The ΔG for Q362K with NAD+ was positive (1.16 kcal/mol), whereas that with NADP+ was negative (-2.14 kcal/mol), indicating that substitution of Gln with Lys at residue 362 in human m-NAD-ME increases the binding affinity of NADP+ at the active site and thus significantly improves the catalytic activity with NADP+ as the cofactor. Like pigeon NADP-ME, the Q362K mutant enzyme preferred NADP+ rather than NAD+ as the cofactor in catalysis. Looking at the active-site structure (Fig. 4B), these results are almost certainly due to the formation of an extra salt bridge between Lys362 and Asp345 in the Q362K enzyme. This additional ionic interaction diminishes the repulsion between Asp345 and the phosphate group of NADP+ and thus increases the tolerance of NADP+ docking in the active site, similar to the NADP+-binding mode in pigeon c-NADP-ME (Fig. 4B).

Mutation of Gln362 to Ala or Asn did not shift the cofactor preference from NAD+ to NADP+. The K_m(NADP)/K_m(NAD) and k_{cat(NADP)}/k_{cat(NAD)} values for Q362A and Q362N were comparable to those for the WT enzyme (Table 1). The ΔG values for Q362A and Q362N with either NAD+ or NADP+ were positive, indicating that substitution of glutamine with alanine or asparagine at residue 362 brings about unfavorable effects on cofactor binding at the active site. Furthermore, there was no significant difference between ΔG_{NAD} and ΔG_{NADP} for Q362A, as was also observed for Q362N, supporting the observation that the cofactor preference of these two mutants has not changed, unlike that of Q362K. This might be due to a lack of the extra salt bridge observed between Lys362 and Asp345 in the Q362K enzyme, so the repulsion still exists between Asp345 and NADP+ in the Q362A and Q362N enzymes, and the NAD+ preference is thus conserved in these two mutant enzymes.

Cooperative Effect of Malate Binding and Allosteric Activation of the Gln362 Mutant Enzymes—Our data also point out that the cooperative binding of malate became insignificant in human m-NAD-ME after mutation of Gln362 to Lys, and the sigmoidal phenomenon that appeared in the WT enzyme (Fig. 2, A and B, closed circles) was diminished in Q362K (Fig. 2, E and F, closed circles). The h values for the WT enzyme with either NAD+ or NADP+ as the cofactor were close to 2, whereas those for the Q362K enzyme were 1.57 and 1.34, respectively. This shows that the Q362K enzyme not only shifted its cofactor preference to NADP+ but also reduced its malate binding cooperativity, especially with NADP+ as the cofactor. In addition, the activating effect of fumarate on the Q362K enzyme was less noticeable (Fig. 2F), and the k_{cat} for Q362K was not obviously elevated by addition of fumarate from 58.17 to 66.94 s^{-1}. Therefore, mutation of Gln362 to Lys seems to change the human NAD-ME kinetic properties of cofactor preference, malate binding cooperativity, and allosteric regulation by fumarate. Substitution of Gln with Lys in human m-NAD-ME has thus made the enzyme similar to pigeon c-NADP-ME, which is characterized as non-allosteric, non-cooperative, and NADP+-specific.

However, the kinetic properties of malate binding cooperativity and the NAD+ specificity of the other Gln362 mutants, Q362A and Q362N, are conserved (Fig. 2). Mutation of Gln362 to Ala or Asn did not diminish the sigmoidal binding phenomenon of malate and conserved the activating effect of fumarate, similar to the WT enzyme (Fig. 2). Moreover, the h values for Q362A and Q362N with either NAD+ or NADP+ as the cofactor also approached 2, and these values were reduced almost to 1 in the presence of fumarate, revealing that the Q362A and Q362N enzymes do not have reduced malate binding cooperativity with NAD+ or NADP+ as the cofactor.

Significance of Gln362 in Catalysis of the Malic Enzyme—Gln362 seems to be involved in the catalysis of human m-NAD-ME because the k_{cat(NAD)} values for Q362A and Q362N in the absence or presence of fumarate were notably smaller than those for the WT enzyme. Fig. 4C shows the hydrogen-bonding networks of Gln362. The amide side chain of this residue is hydrogen-bonded with Ala312 and Asn359. Mutation of Gln362 to Ala causes the enzyme to retain less than half of the enzyme activity compared with the WT enzyme, and the enzyme activity of Q362N is only ~20% of that of the WT enzyme, suggesting that Gln362 plays an important role in catalysis. Because Gln362 is not in the catalytic triad (5), it may not directly participate in the catalytic reaction, but the hydrogen-bonding network involving Gln362 might be very important in maintaining the correct active-site geometry that is essential for catalysis. To our surprise, the catalytic constant (k_{cat}) for Q362N was smaller than that for Q362A. Because the amide side chain is retained in Q362N, there might be some unanticipated hydrogen-bonding interactions between Asn362 and other amino acid residues that are unfavorable for cofactor binding, causing incorrect active-site structure for catalysis.

In summary, we have provided kinetic evidence demonstrating the determinants of the dual cofactor specificity and substrate cooperativity of m-NAD-ME. Our results provide excellent explanations for elucidating the differences between m-NAD-ME and c-NADP-ME in cofactor specificity and malate cooperative binding.

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