Malic enzyme (ME) is a family of enzymes that catalyze a reversible oxidative decarboxylation of L-malate to pyruvate with simultaneous reduction of NAD(P)⁺ to NAD(P)H. According to the cofactor specificity, the mammalian enzyme can be categorized into three isozymes. The cytosolic (c) and mitochondrial (m) NADP⁺-dependent MEs utilize NADP⁺ as the cofactor. The mitochondrial NAD(P)⁺-dependent ME can use either NAD⁺ or NADP⁺ as the cofactor. In addition, the m-NAD(P)-ME isoform can be inhibited by ATP and allosterically activated by fumarate. In this study, we delineated the determinants for cofactor specificity and isoform-specific inhibition among the ME isoforms. Our data strongly suggest that residue 362 is the decisive factor determining cofactor preference. All the mutants containing Q362K (Q362K, K346S/Q362K, Y347K/Q362K, and K346S/Y347K/Q362K) have a larger $k_{cat,NAD}$ value compared with the $k_{cat,NAD}$ value, indicating that the enzyme has changed to use NADP⁺ as the preferred cofactor. Furthermore, we suggest that Lys-346 in m-NAD(P)-ME is crucial for the isoform-specific ATP inhibition. The enzymes containing the K346S mutation (K346S, K346S/Y347K, K346S/Q362K, and K346S/Y347K/Q362K) are much less inhibited by ATP and have a larger $k_{cat,ATP}$ value. Kinetic analysis also suggests that residue 347 functions in cofactor specificity. Here we demonstrate that the human K346S/Y347K/Q362K m-NAD(P)-ME has completely shifted its cofactor preference to become an NADP⁺-specific ME. In the triple mutant, Lys-362, Lys-347, and Ser-346 work together and function synergistically to increase the binding affinity for NADP⁺.

Malic enzyme (ME) is a family of divalent metal ion (Mn²⁺ or Mg²⁺)-dependent oxidative decarboxylases. It catalyzes a reversible interconversion of L-malate to pyruvate and CO₂ concomitant with the reduction of NAD(P)⁺ to NAD(P)H (1–4). The ME family is broadly distributed throughout nature and plays an important role in the metabolic pathway of organisms. Since the sequences and structural information of this enzyme family became available, malic enzyme has been characterized as a new family, distinct from other oxidative decarboxylases (5–9). In mammals, the enzyme can be divided into three isozymes according to the cofactor specificity. Both the cytosolic (c) and mitochondrial (m) NADP⁺-dependent malic enzymes utilize NADP⁺ as the cofactor and play a part in lipid metabolism by generating NADPH as the source for reductive biosynthesis (3, 10–12). The mitochondrial NAD(P)⁺-dependent malic enzyme (m-NAD(P)-ME) is a distinct isoform from the other two because it can use either NAD⁺ or NADP⁺ as the cofactor; however, it prefers NAD⁺ under physiological conditions (3, 13, 14). The m-NAD(P)-ME isoform is involved in the metabolism of glutamine with the production of pyruvate and NADH as the source of reducing equivalent for energy production (3, 15–20). It was found to be overexpressed in tumors and rapidly growing tissues, and it participates in the glutaminolysis of cancer cells that use glutamine as the energy source for their fast development (3, 13–20).

Crystal structures of malic enzyme from various sources reveal that the enzyme is a homotetramer with a double dimer structure consisting of a tighter dimer interface and a looser tetramer interface (5, 6, 9) (Fig. 1A, PDB code 1PJ3). The difference in kinetic properties among the malic enzyme isoforms is that human m-NAD(P)-ME, but not the other two isoforms, is an allosteric enzyme and demonstrated positive cooperativity with its substrate L-malate. The activator fumarate is bound at the dimer interface and allosterically activates the human m-NAD(P)-ME isoform by decreasing the $K_w$ value of the substrates and increasing the $k_{cat}$ value (18, 21–28). Additionally, human m-NAD(P)-ME is more sensitive to ATP inhibition than the other two isoforms (18, 21, 29, 30). A crystal structure of human m-NAD(P)-ME complexed with ATP has suggested that ATP has two binding sites; one is at the active center, and the other is at the tetramer interface, called the exo site (21). Although ATP can bind at the exo site, site-directed mutagenesis and kinetic studies suggest that ATP is an active-site inhibitor rather than an allosteric inhibitor for the human m-NAD(P)-ME (29, 30). The binding of ATP at the exo site may be involved in the subunit association of the enzyme (29). The unique allosteric regulation for human m-NAD(P)-ME may have an important effect on cancer cells because of their unusual metabolic pathway (18–20).

Malic enzymes show a high degree of sequence homology, except in the cofactor binding region, which is not conserved among the malic enzyme isoforms. Multiple sequence alignments reveal that the three amino acid residues at positions 346, 347, and 362 display an isoform-specific distribution...
In this study, we focused on exploring the contribution of these residues to the cofactor specificity of human m-NAD(P)-ME. Site-directed mutagenesis was used in the combination of mutations for the three amino acid residues. In this study, the respective amino acid residue in human m-NAD(P)-ME was changed to that in human c-NADP-ME. According to the kinetic analysis, we have successfully demonstrated a mutant human m-NAD(P)-ME, which has completely changed to become an NADP$^+$-specific and ATP-resistant malic enzyme, just like a c-NADP-ME.

**EXPERIMENTAL PROCEDURES**

**Expression, Purification, and Characterization of Human m-NAD(P)-ME and c-NADP-ME**—The detailed expression and purification protocols were described previously (3, 11, 12). Human m-NAD(P)-ME was overexpressed in *Escherichia coli* BL21 cells using a pRH281 vector with a trp promoter system that is modulated by -indole-3-acetic acid. Human c-NADP-ME was overexpressed in *E. coli* BL21 (DE3) cells using a pET21b vector with a T7 promoter system that is induced by isopropyl-β-D-1-thiogalactopyranoside. The human m-NAD(P)-ME was purified using ATP-agarose affinity chromatography (Sigma), and the human c-NADP-ME was purified using the HIS-SelectTM nickel affinity gel column (Sigma). The purified enzyme was buffer-exchanged and concentrated with Amicon Ultra-15 centrifugal filters (Millipore Corp.) and then stored in 30 mM Tris-HCl (pH 7.4) with 2 mM β-mercaptoethanol. The enzyme purity was examined by SDS-PAGE, and the protein concentration was determined by the Bradford dye method using bovine serum albumin as a standard (36).

**Site-directed Mutagenesis**—Single (K346S, Y347K, and Q362K), double (K346S/Y347K, K346S/Q362K, and Y347K/Q362K), and triple (K346S/Y347K/Q362K) mutants were constructed with the QuikChange™ kit (Stratagene), using the human m-NAD(P)-ME as the template for the mutagenic reaction. The PCR primers were as follows: 5′-GAAAATCTGGATTTGACTCCATCTGTTTGAATGTTTTGTTATTAGTTAAGGACG-3′ for K346S; 5′-CTGGATGTTTTGACAGAAAAGGTTTATTAGTTAAGG-3′ for Y347K; 5′-GAAAATAGATAGTTTAAAGAACCATTTCACCTACAACGC-3′ for Q362K, and 5′-CTGGATGTTTTGACCTAAAAGGTTTATAGTTAAGGACG-3′ for Q346S. The PCR products were cloned into the pET21b vector (Stratagene) and transformed into *E. coli* strain BL21 (DE3). The expression of the recombinant plasmids was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside, and the induced cells were harvested by centrifugation.

**FIGURE 1.** Crystal structure and sequence alignment of the cofactor binding site for human m-NAD(P)-ME. A, homotetrameric structure of human mitochondrial NAD(P)$^+$-dependent malic enzyme with pyruvate, NAD$^+$, Mn$^{2+}$, and fumarate (PDB code 1PJ3). Fumarate is shown as a sphere and colored in pink, and NAD$^+$ is shown as a ball-and-stick model. This figure was generated using PyMOL (DeLano Scientific LLC, San Carlos, CA). B, sequence alignments of malic enzymes. Alignments of 14 malic enzymes with amino acid sequences around the nucleotide binding pockets in the active site are shown from residues 336–364. Amino acid sequences of malic enzymes were searched using BLAST (37), and alignments were created using ClustalW (38). This figure was generated using the BioEdit sequence alignment editor program (39).

(Fig. 1B), and they may be the determinants of cofactor specificity. Previous studies have suggested that Lys-362 in pigeon c-NADP-ME is important for NADP$^+$ specificity (31). This corresponding residue in human m-NAD(P)-ME is instead a Gln, and thus this human isoform has dual cofactor specificity (32). Substitution of Gln-362 by Lys in human m-NAD(P)-ME changed it into an NADP$^+$-dependent enzyme (32).

Human m-NAD(P)-ME was purified using ATP-agarose affinity chromatography (Sigma), and the human c-NADP-ME was purified using the HIS-Select™ nickel affinity gel column (Sigma). The purified enzyme was buffer-exchanged and concentrated with Amicon Ultra-15 centrifugal filters (Millipore Corp.) and then stored in 30 mM Tris-HCl (pH 7.4) with 2 mM β-mercaptoethanol. The enzyme purity was examined by SDS-PAGE, and the protein concentration was determined by the Bradford dye method using bovine serum albumin as a standard (36).

**Site-directed Mutagenesis**—Single (K346S, Y347K, and Q362K), double (K346S/Y347K, K346S/Q362K, and Y347K/Q362K), and triple (K346S/Y347K/Q362K) mutants were constructed with the QuikChange™ kit (Stratagene), using the human m-NAD(P)-ME as the template for the mutagenic reactions. The PCR primers were as follows: 5′-GAAAATCTGGATTTGACTCCATCTGTTTGAATGTTTTGTTATTAGTTAAGGACG-3′ for K346S; 5′-CTGGATGTTTTGACAGAAAAGGTTTATTAGTTAAGG-3′ for Y347K; 5′-GAAAATAGATAGTTTAAAGAACCATTTCACCTACAACGC-3′ for Q362K, and 5′-CTGGATGTTTTGACCTAAAAGGTTTATAGTTAAGGACG-3′ for Q346S. The PCR products were cloned into the pET21b vector (Stratagene) and transformed into *E. coli* strain BL21 (DE3). The expression of the recombinant plasmids was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside, and the induced cells were harvested by centrifugation.
Human Mitochondrial Malic Enzyme

GGG-3′ for K346S/Y347K. The PCR was performed at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 2 min/kb of plasmid length with Pfu DNA polymerase for 16 cycles. The vector templates were digested with DpnI restriction enzyme and transformed into E. coli XL-1 cells. All mutation sites were confirmed by autosequencing.

**Enzyme Kinetic Analysis**—The malic enzyme reaction was assayed in a reaction buffer containing 50 mM Tris-HCl (pH 7.4) with saturated concentrations of l-malate, NAD+, or NADP+ and MgCl2 in the absence or presence of 3 mM fumarate in a total volume of 1 ml at 30 °C. The absorbance at 340 nm was continuously monitored in a Beckman DU 7500 spectrophotometer. In this process, 1 unit of enzyme was defined as the amount of enzyme producing 1 µmol of NAD(P)H per min under the assay conditions. An absorption coefficient of 6.22 mM−1 cm−1 for NAD(P)H was used in the calculations. Apparent Michaelis constants for the substrates were determined by varying one substrate concentration near its $K_m$ value, whereas the other components were kept constant at the saturation levels. All calculations were carried out using the Sigma Plot 8.0 program (Jandel, San Rafael, CA).

The ATP inhibition experiment was assayed with 50 mM Tris-HCl (pH 7.4), 40 mM malate (pH 7.4), 10 mM MgCl2, and 1.0 mM NAD+ or NADP+ (pH 7.4) at a series of ATP concentrations, ranging from 0 to 3 mM. The $K_v$ value of all the enzymes was assayed with the reaction buffer consisting of 50 mM Tris-HCl (pH 7.4), 40 mM malate (pH 7.4), and 10 mM MgCl2 at a series of ATP concentrations around its $K_v$ value and at a series of NAD+ or NADP+ (pH 7.4) concentrations around its $K_v$ value. Equation 1 was globally fitted to the total data set, which describes a competitive inhibition pattern,

$$v = V_{max}[S]/([S] + K_m(1 + [ATP]/K_{ATP}))$$

where $v$ is the observed initial velocity; $V_{max}$ is the maximum rate of the reaction; $K_m$ is the Michaelis constant for the substrate, and $K_{ATP}$ is the inhibition constant for ATP.

To evaluate the cooperativity of l-malate, the Hill Equation 2 was fitted to the sigmoidal curves of [malate] versus initial rates,

$$v = V_{max}[malate]^h/(K_{0.5,malate} + [malate]^h)$$

The $K_{0.5,malate}$ value is the malate concentration at half-maximal velocity, and the Hill coefficient (h) is employed to assess the degree of cooperativity. All the kinetic data were analyzed using the program Sigma Plot 8.0 program (Jandel, San Rafael, CA).

To evaluate the contribution of a side chain to the cofactor specificity in WT relative to its change in a mutant, the change in free energy change ($\Delta G$) of any mutation can be estimated by Equation 3,

$$\Delta G = -RT \cdot \ln((v_{cat,mut}/K_{cat,mut})/(v_{cat,WT}/K_{cat,WT}))$$

where $R$ is the gas constant, and $T$ is the absolute temperature.

**RESULTS**

**Kinetic Properties of Recombinant Human m-NAD(P)-ME and c-NADP-ME**—Kinetic parameters of human malic enzymes utilizing NAD+ or NADP+ as the cofactor were determined in the absence and presence of fumarate (Table 1). The $K_{m,NAD}$ and $K_{m,NADP}$ values of m-NAD(P)-ME were 1.23 and 1.69 mM, respectively, demonstrating the dual cofactor specificity of this isoform. c-NADP-ME displayed much higher affinity for NADP+ than for NAD+; the $K_{m,NAD}$ and $K_{m,NADP}$ values of this isoform were 13.1 mM and 2.9 µM, respectively. For m-NAD(P)-ME, the $k_{cat,NAD}$ value was significantly higher than the $k_{cat,NADP}$ value (where and $k_{cat,NAD}$ is the catalytic constant using NAD+ as the cofactor and $k_{cat,NADP}$ is the catalytic constant using NADP+ as the cofactor); the values were 203 and 18 s−1, respectively. In contrast, for c-NADP-ME, the $k_{cat,NADP}$ value was larger than the $k_{cat,NAD}$ value, which were 140 and 32 s−1, respectively. The $k_{cat,NADP}/k_{cat,NAD}$ and $k_{cat,NADP}/K_{cat,NAD}$ values of m-NAD(P)-ME were 165 and 10.6 mm−1 s−1, respectively, and for c-NADP-ME, they were 2.4 and 48386 mm−1 s−1, respectively (Table 2). The $k_{cat,NADP}/K_{cat,NAD}$ value of m-NAD(P)-ME was about 70-fold higher than that of c-NADP-ME, whereas the $k_{cat,NADP}/K_{cat,NAD}$ value of c-NADP-ME was about 4560-fold higher than that of m-NAD(P)-ME (Table 2). Furthermore, the values of NADP+/NAD+ specificity ($k_{cat,NADP}/K_{cat,NADP}/K_{cat,NAD}$) for m-NAD(P)-ME and c-NADP-ME were 0.06 and 20,160, respectively (Table 2), demonstrating the differential cofactor specificity of these two human enzyme isoforms.

For all mutant m-NAD(P)-MEs, the $K_{m,NADP}$ values did not show significant changes when compared with that of WT m-NADP-ME, and they were reduced by fumarate. The $K_{m,NADP}$ values of these mutants, however, displayed significant differences. Previous studies indicated that the Q362K mutant becomes an NADP-specific, nonallosteric, and noncooperative enzyme (32). The K346S enzyme showed only a slight decrease in $K_{m,NADP}$ suggesting that this residue is not the decisive factor for the cofactor specificity of human m-NAD(P)-ME. The Y347K enzyme displayed an 8.5-fold decrease in $K_{m,NADP}$ indicating that Lys-347 may be one of the determinants of NADP+ specificity of c-NADP-ME (Table 1). All of the double mutant enzymes displayed much smaller $K_{m,NADP}$ values. The $K_{m,NADP}$ values for K346S/Y347K, K346S/Q362K, and Y347K/Q362K were 13.1 mM and 2.9 µM, respectively. For Y347K/Q362K, the $K_{m,NADP}$ value was decreased by about 100-fold compared with that of WT m-NAD-P-ME, but it was still about 6-fold higher than that of c-NADP-ME (Table 1). The $K_{m,NADP}$ value of K346S/Y347K/Q362K was only 1/600 of the WT m-NAD(P)-ME enzyme was about 1.5. The ratios for single mutants were decreased by 1 order of magnitude from 1.5 to 0.2–0.5. The ratios for double mutants were further decreased by 2 orders of magnitude with values ranging from 0.03 to 0.04. The ratio for the triple mutant was decreased by 1000-fold, with a value of 0.001 (Table 1).

The $k_{cat,NADP}$ values of most mutant m-NAD(P)-MEs were reduced, except for Y347K and K346S/Y347K, which were still as active as WT with NAD+ as the cofactor. The $k_{cat,NADP}$ value for the double mutants, K346S/Q362K and Y347K/Q362K, and the triple mutant K346S/Y347K/Q362K was 55, 60, and 26 s−1,
respectively, and they were reduced by 3–8-fold when compared with that of the WT m-NAD(P)-ME (Table 1). On the other hand, all the mutant enzymes have demonstrated a significantly elevated $k_{\text{cat}}$ when NADP$^+$ was used as the cofactor. The $k_{\text{cat,NADP}}$ values of these single mutants were around 100 s$^{-1}$. However, the $k_{\text{cat,NADP}}$ values of double and triple mutants were not further increased, and their $k_{\text{cat,NADP}}$ values were close to that of c-NADP-ME. The ratio of $k_{\text{cat,NADP}}/k_{\text{cat,NAD}}$ for the WT m-NAD(P)-ME enzyme was about 0.1. The K346S, Y347K, and K346S/Y347K mutant enzymes have a $k_{\text{cat,NADP}}/k_{\text{cat,NAD}}$ ratio similar to that of WT m-NAD(P)-ME. The mutants containing Q362K increased the ratio of $k_{\text{cat,NADP}}/k_{\text{cat,NAD}}$ by more than 20-fold. The value of $k_{\text{cat,NADP}}/k_{\text{cat,NAD}}$ for Q362K, K346S/Q362K, and Y347K/Q362K was around 2. The value for the triple mutant K346S/Y347K/Q362K increased to 4, which is 43-fold greater than that of WT m-NAD(P)-ME and comparable with that of c-NADP-ME (Table 1).

The $k_{\text{cat,NADP}}/K_{\text{m,NADP}}$ and $k_{\text{cat,NADP}}/K_{\text{m,NAD}}$ values of K346S/Y347K/Q362K were 13.3 and 35,985 mM$^{-1}$ s$^{-1}$, respectively, very similar to those of c-NADP-ME (2.4 and 48,386 mM$^{-1}$ s$^{-1}$, respectively) (Table 2), indicating that the catalytic efficiency of the triple mutant was almost the same as that of c-NADP-ME. The $k_{\text{cat,NADP}}/K_{\text{m,NADP}}$, $k_{\text{cat,NADP}}/K_{\text{m,NAD}}$ value of the triple mutant m-NAD(P)-ME was about 12-fold less than that of WT m-NAD(P)-ME, whereas at the same time the $k_{\text{cat,NADP}}/K_{\text{m,NADP}}$ value of the triple mutant was about 3400-fold larger than that of WT m-NAD(P)-ME and has reached a magnitude similar to that of c-NADP-ME, revealing only about a 1.3-fold difference (Table 2). Moreover, the value of NADP$^+$/NAD$^+$ specificity for the triple mutant m-NAD(P)-ME was about 45,100-fold higher than that of WT m-NAD(P)-ME (2706 and 0.06, respectively, see Table 2). According to these kinetic results, we suggested that the K346S/Y347K/Q362K mutant m-NAD(P)-ME had successfully changed to become an NADP$^+$–specific enzyme, just like a c-NADP-ME.

In the presence of fumarate, the $k_{\text{cat,NADP}}$ values of the WT and mutant m-NAD(P)-ME were elevated, whereas their $K_{\text{m,NADP}}$ values were reduced, indicating that these enzymes can still be allosterically activated by fumarate (Table 1). Nevertheless, these mutant enzymes were less activated by fumarate when using NADP$^+$ as the factor, indicating that these mutant enzymes, which are changed to favor NADP$^+$ as the cofactor, can achieve the maximal enzyme activities in the absence of fumarate.

**Cooperativity Binding of L-Malate for Recombinant Human m-NAD(P)-ME and c-NADP-ME—**Human m-NAD(P)-ME is a cooperative enzyme for substrate malate binding, but human c-NADP-ME is not (Fig. 2, A and C, respectively). The initial velocities of WT and mutant m-NAD(P)-ME measured at various concentrations of malate, either with NAD$^+$ or NADP$^+$ as the cofactor, demonstrate a sigmoidal instead of a hyperbolic kinetic curve (Fig. 2, A and B, respectively, closed circles). The sigmoidal kinetics, however, can be abolished by fumarate (Fig. 2, A and B, respectively, open circles). Table 3 summarizes the results obtained by fitting the Hill equation to these sigmoidal curves. The $K_{0.5,\text{malate(NAD)}}$ and $K_{0.5,\text{malate(NADP)}}$ values of m-NAD(P)-ME were 9.9 and 19.2 mM without fumarate and 4.5 and 6.2 mM with fumarate, respectively (where $K_{0.5,\text{malate(NAD)}}$ is the half-saturation for l-malate, with NAD$^+$ as the cofactor,
and \( K_{0.5,\text{malate(NADP)}} \) is the half-saturation for L-malate, with NADP\(^+\) as the cofactor. For c-NADP-ME, the \( K_{0.5,\text{malate(NADP)}} \) and \( K_{0.5,\text{malate(NADP)}} \) values were 4.4 and 0.5 mM without fumarate and 4.3 and 0.6 mM with fumarate, respectively. There was about a 2-fold difference between \( K_{0.5,\text{malate(NADP)}} \) and \( K_{0.5,\text{malate(NADP)}} \) for m-NADP-ME. For c-NADP-ME, \( K_{0.5,\text{malate(NADP)}} \) was about 10-fold larger than \( K_{0.5,\text{malate(NADP)}} \). Similar to the \( K_{0.5,\text{malate(NADP)}} \) values, the \( K_{0.5,\text{malate(NADP)}} \) values of mutant m-NADP-ME did not show considerable changes compared with that of WT m-NADP-ME, and they were also reduced by fumarate. The \( K_{0.5,\text{malate(NADP)}} \) value of these mutants, except K346S, were at least 3.5-fold less than that of WT. For the K346S/ Y347K/Q362K mutant enzyme, the \( K_{0.5,\text{malate(NADP)}} \) value was about 14-fold less than that of WT enzyme and close to the value of human c-NADP-ME.

The Hill coefficient (\( h \)), with either NAD\(^+\) or NADP\(^+\) as the cofactor (\( h_{\text{NAD}} \) and \( h_{\text{NADP}} \)), demonstrates the degree of malate binding cooperativity for the enzyme (Table 3). For m-NADP-PME, both \( h_{\text{NAD}} \) and \( h_{\text{NADP}} \) values were around 2, and they were reduced to 1 with fumarate, indicating that the cooperative binding behavior of malate in the enzyme was abolished. The single mutants, K346S, Y347K, and Q362K, displayed partial cooperativity of malate binding, and their \( h_{\text{NAD}} \) and \( h_{\text{NADP}} \) values were around 1.3–1.5. The malate cooperativity for the double and triple mutant enzymes is limited; their \( h_{\text{NAD}} \) values were around 1.2–1.4. In contrast, the \( h_{\text{NADP}} \) values of double and triple mutant enzymes were totally reduced near to 1, indicating that the malate cooperative kinetics with NADP\(^+\) as the cofactor was no longer present in these mutant enzymes. For K346S/Y347K/Q362K, whose cofactor specificity is changed from NAD\(^+\) to NADP\(^+\), the kinetic behavior for malate binding, either with NAD\(^+\) or NADP\(^+\) as the cofactor, displayed a hyperbolic curve (Fig. 2, C and D, respectively), indicating that the mutant enzyme is no longer cooperative for malate binding, and it cannot be significantly activated by fumarate. c-NADP-ME, which is characterized as a noncooperative and nonallosteric enzyme, displayed hyperbolic kinetic behavior (Fig. 2, E and F). In view of the above results, we suggested that the triple mutant K346S/Y347K/Q362K was totally changed into an NADP\(^+\)-specific and noncooperative enzyme.

**Inhibitory Effect of ATP on m-NAD(P)-ME and c-NADP-ME—** Our previous studies have suggested that Lys-346 is important for ATP inhibition of human m-NAD(P)-ME (35). With NAD\(^+\) as the cofactor, the K346S enzyme became less inhibited by ATP (Fig. 3A, open circles). The double mutants, K346S/Y347K and K346S/Q362K, displayed similar inhibition by ATP to K346S (Fig. 3A, closed and open triangles, respectively). The other mutants without K346S, including Y347K, Q362K, and Y347K/Q362K (Fig. 3B, closed and open squares and closed diamonds, respectively), displayed more significant inhibition by ATP than WT m-NADP-ME. The effect of ATP inhibition on the triple mutant K346S/Y347K/Q362K (Fig. 3B, open diamonds), however, is less than that on Y347K, Q362K, and Y347K/Q362K, and its enzyme activity remains at a level similar to that of WT m-NADP-ME (Fig. 3B, closed circles). With NADP\(^+\) as the cofactor, the K346S enzyme is also less inhibited by ATP (Fig. 3C, open circles). In contrast to the enzyme with NAD\(^+\) as the cofactor, the K346S/Y347K and K346S/Q362K enzymes, with NADP\(^+\) as the cofactor, are much less inhibited by ATP (Fig. 3C, closed and open triangles, respectively). The residual enzyme activity of these two double mutant enzymes was higher than that of K346S (Fig. 3C, open circles). Indeed, the residual enzyme activity of K346S/Y347K was almost the same as that of c-NADP-ME (Fig. 3C), indicating that this mutant has become an ATP-resistant enzyme like c-NADP-ME. The Y347K and Q362K enzymes (Fig. 3D, closed and open squares, respectively) were also inhibited by ATP to a level similar to that of WT m-NADP-ME. However, with NADP\(^+\) as the cofactor, the Y347K/Q362K enzyme was much less inhibited by ATP (Fig. 3D, closed diamonds). The triple mutant K346S/ Y347K/Q362K, similarly to K346S/Y347K, which resists ATP inhibition, completely retained its enzyme activity (Fig. 3D, open diamonds).

The inhibition constants of these enzymes either with NAD\(^+\) or NADP\(^+\) as the cofactor, \( K_{\text{ATP(NAD)}} \) and \( K_{\text{ATP(NADP)}} \), have been determined (where \( K_{\text{ATP(NAD)}} \) is the inhibition constant for ATP inhibition of human m-NAD(P)-ME and c-NADP-ME—Our previous studies have suggested that Lys-346 is important for ATP inhibition of human m-NAD(P)-ME (35). With NAD\(^+\) as the cofactor, the K346S enzyme became less inhibited by ATP (Fig. 3A, open circles). The double mutants, K346S/Y347K and K346S/Q362K, displayed similar inhibition by ATP to K346S (Fig. 3A, closed and open triangles, respectively). The other mutants without K346S, including Y347K, Q362K, and Y347K/Q362K (Fig. 3B, closed and open squares and closed diamonds, respectively), displayed more significant inhibition by ATP than WT m-NADP-ME. The effect of ATP inhibition on the triple mutant K346S/Y347K/Q362K (Fig. 3B, open diamonds), however, is less than that on Y347K, Q362K, and Y347K/Q362K, and its enzyme activity remains at a level similar to that of WT m-NADP-ME (Fig. 3B, closed circles). With NADP\(^+\) as the cofactor, the K346S enzyme is also less inhibited by ATP (Fig. 3C, open circles). In contrast to the enzyme with NAD\(^+\) as the cofactor, the K346S/Y347K and K346S/Q362K enzymes, with NADP\(^+\) as the cofactor, are much less inhibited by ATP (Fig. 3C, closed and open triangles, respectively). The residual enzyme activity of these two double mutant enzymes was higher than that of K346S (Fig. 3C, open circles). Indeed, the residual enzyme activity of K346S/Y347K was almost the same as that of c-NADP-ME (Fig. 3C), indicating that this mutant has become an ATP-resistant enzyme like c-NADP-ME. The Y347K and Q362K enzymes (Fig. 3D, closed and open squares, respectively) were also inhibited by ATP to a level similar to that of WT m-NADP-ME. However, with NADP\(^+\) as the cofactor, the Y347K/Q362K enzyme was much less inhibited by ATP (Fig. 3D, closed diamonds). The triple mutant K346S/ Y347K/Q362K, similarly to K346S/Y347K, which resists ATP inhibition, completely retained its enzyme activity (Fig. 3D, open diamonds).

The inhibition constants of these enzymes either with NAD\(^+\) or NADP\(^+\) as the cofactor, \( K_{\text{ATP(NAD)}} \) and \( K_{\text{ATP(NADP)}} \), have been determined (where \( K_{\text{ATP(NAD)}} \) is the inhibition constant...
Human Mitochondrial Malic Enzyme

of ATP for NAD\textsuperscript{+}, and $K_{i,ATP(NADP)}$ is the inhibition constant of ATP for $\text{NADP}^+$). All malic enzymes displayed competitive inhibition patterns with similar inhibition constants either with $\text{NAD}^+$ or $\text{NADP}^+$ as the cofactor (data not shown). The $K_{i,ATP(NAD)}$ value was 0.67 mM for m-NAD(P)-ME, and 17.3 mM for c-NADP-ME (Table 4), indicating that m-NAD(P)-ME is more sensitive to ATP inhibition. As suggested previously, Lys-346 is critical for the isoform-specific inhibition, and mutations involving Lys-346 (K346S, K346S/Y347K, K346S/ Q362K) displayed an elevated $K_{i,ATP(NAD)}$ value (1.27–2.29 mM) either with $\text{NAD}^+$ or $\text{NADP}^+$ as the cofactor. In contrast, the $K_i$ values of the Y347K, Q362K, and Y347K/Q362K enzymes were smaller than that of WT m-NAD(P)-ME (0.15–0.27 mM), suggesting that the binding affinity of ATP for these mutants may be associated with the additional positive charge introduced into the active site. The triple mutant K346S/Y347K/Q362K, however, displayed a $K_{i,ATP(NADP)}$ value (0.72 mM) smaller than that of c-NADP-ME (17.3 mM). These data suggested that there are some additional factors responsible for the isoform-specific inhibition by ATP.

**DISCUSSION**

Human m-NAD(P)-ME is distinct from the other two isoforms

**TABLE 3**

Kinetic parameters derived from the Hill equation for human m-NAD(P)-ME and c-NADP-ME

|          | $K_{i,\text{malate}(\text{NAD})}$ | $h_{\text{NAD}}$ | $K_{i,\text{malate}(\text{NADP})}$ | $h_{\text{NADP}}$ |
|----------|----------------------------------|------------------|----------------------------------|------------------|
| WT m-NAD(P)-ME | (−) | 0.73 ± 0.03 | 1.00 ± 0.00 | 0.73 ± 0.03 | 1.00 ± 0.00 |
| K346S m-NAD(P)-ME | (+) | 1.13 ± 0.06 | 1.00 ± 0.00 | 1.13 ± 0.06 | 1.00 ± 0.00 |
| Y347K m-NAD(P)-ME | (−) | 0.56 ± 0.02 | 1.00 ± 0.00 | 0.56 ± 0.02 | 1.00 ± 0.00 |
| Q362K m-NAD(P)-ME | (+) | 0.72 ± 0.04 | 1.00 ± 0.00 | 0.72 ± 0.04 | 1.00 ± 0.00 |
| K346S/Y347K m-NAD(P)-ME | (−) | 0.56 ± 0.02 | 1.00 ± 0.00 | 0.56 ± 0.02 | 1.00 ± 0.00 |
| K346S/Q362K m-NAD(P)-ME | (+) | 0.72 ± 0.04 | 1.00 ± 0.00 | 0.72 ± 0.04 | 1.00 ± 0.00 |
| Y347K/Q362K m-NAD(P)-ME | (−) | 0.56 ± 0.02 | 1.00 ± 0.00 | 0.56 ± 0.02 | 1.00 ± 0.00 |
| K346S/Y347K/Q362K m-NAD(P)-ME | (+) | 0.72 ± 0.04 | 1.00 ± 0.00 | 0.72 ± 0.04 | 1.00 ± 0.00 |
| c-NADP-ME | (−) | 0.73 ± 0.03 | 1.00 ± 0.00 | 0.73 ± 0.03 | 1.00 ± 0.00 |
| (+) | 0.73 ± 0.03 | 1.00 ± 0.00 | 0.73 ± 0.03 | 1.00 ± 0.00 |

**FIGURE 2.** Cooperativity of malate for human WT and K346S/Y347K/Q362K m-NAD(P)-ME. The assay mixture contains 10 mM MgCl\textsubscript{2}, 1 mM NAD\textsuperscript{+} or NADP\textsuperscript{+}, and various concentrations of malate in a 50 mM Tris-HCl (pH 7.4) buffer system without (closed circles) or with 3 mM fumarate. Left panels (A, C, and E) show these curves using NAD\textsuperscript{+} as the cofactor, and right panels (B, D, and F) show these curves using NADP\textsuperscript{+} as the cofactor. A and B, WT m-NAD(P)-ME; C and D, K346S/Y347K/Q362K m-NAD(P)-ME; E and F, c-NADP-ME.
for its dual cofactor specificity (3). Previous studies have pointed out that Gln-362 is one of the determinants controlling the dual cofactor preference of human m-NAD(P)-ME (32). A single mutation of glutamine to lysine in human NAD(P)-ME has altered it to become an NADP-ME, which is characterized as a nonallosteric, noncooperative, and NADP-specific enzyme (32). However, the K_{m,NADP} value of the human Q362K enzyme is still higher than that of human c-NADP-ME by about 100-fold (Table 1). Additional factors may be involved in the cofactor specificity. The focus of this study was to demonstrate that the human K346S/Y347K/Q362K m-NAD(P)-ME mutant has completely shifted its cofactor specificity from NAD\(^+\) to NADP\(^+\) and abolished malate cooperativity. In addition, the human NADP\(^+\)-specific mutant enzyme is less activated by fumarate and less inhibited by ATP.

Kinetic Properties of the Human Triple Mutant K346S/Y347K/Q362K m-NAD(P)-ME—The human K346S/Y347K/Q362K m-NAD(P)-ME mutant has completely transformed into an NADP\(^+\)-specific malic enzyme. The specificity constant (k_{cat}/K_m) of this triple mutant is very close to that of c-NADP-ME (Table 2). Our data suggest that the switch in cofactor preference of the human m-NAD(P)-ME is attributed mainly to the substitution of Gln-362 by Lys, because the Q362K enzyme really shifted its factor specificity from NAD\(^+\) to NADP\(^+\) by decreasing K_{m,NADP} and increasing k_{cat,NADP}, and most importantly, because the k_{cat,NADP} value is now larger than that of k_{cat,NADP}. Mutation of Tyr-347 to Lys in the Q362K mutant enzyme has a further effect of decreasing the K_{m,NADP} by over 10-fold and slightly increasing k_{cat,NADP}. Substitution of Lys-346 by Ser in the Y347K/Q362K mutant enzyme further decreases K_{m,NADP} to a level that is similar to human c-NADP-ME. The changes in free energy change of these human mutants relative to that of WT m-NAD(P)-ME could also account for these facts (Table 2). The changes in free energy change with NAD\(^+\) as the cofactor (ΔΔG_{NADP}) for the human m-NAD(P)-ME and c-NADP-ME (32). However, with NADP\(^+\) as the cofactor, the change in free energy change (ΔΔG_{NADP}) for these Q362K-substituted enzymes significantly becomes negative (−2.2 to −4.9 kcal/mol), clearly indicating that the cofactor preference is shifted from NAD\(^+\) to NADP\(^+\) by replacing Gln-362 with Lys. Both the ΔΔG_{NAD} and ΔΔG_{NADP} values for Y347K are negative (−0.2 and −2.4 kcal/mol, respectively), whereas those for K346S are positive for ΔΔG_{NAD} (0.46 kcal/mol) and negative for ΔΔG_{NADP} (−1.27 kcal/mol). The enzyme has changed to favor the use of NADP\(^+\) over NAD\(^+\) as the cofactor because of the substitutions of residues 346 and 347. The ΔΔG_{NAD} and ΔΔG_{NADP} values for K346S/Y347K are −0.07 and −3.49 kcal/mol, respectively. Although the human K346S/Y347K enzyme favors NADP\(^+\) over NAD\(^+\), both values for the double mutant are negative, suggesting that the dual cofactor specificity is still contained in the enzyme, even though Lys-346 and Tyr-347 have been replaced with Ser and Lys, respectively. The change in free energy change of the human K346S/Y347K/Q362K m-NAD(P)-ME mutant with NADP\(^+\) as the cofactor is −4.89 kcal/mol, very close to that for human c-NADP-ME (−5.07
m-NAD(P)-ME containing Q362K (Q362K, K346S/Q362K, human m-NAD(P)-ME. All of the human mutants most important factor governing the cofactor specificity of human m-NAD(P)-ME (PDB code 1GQ2). In this study, we suggest that residue 362 is the most crucial residue in the human triple mutant enzyme, Lys-362, Lys-347, and Ser-346 work together and function synergistically to increase the affinity for NADP⁺ in the active site.

Furthermore, the human K346S/Y347K/Q362K m-NAD(P)-ME mutant has become a noncooperative and less allosteric enzyme with NADP⁺ as the cofactor, just like c-NADP-ME (Fig. 2). It is likely that this mutant has much smaller $K_{m,NADP}$ and $K_{0.5,\text{malate}}$ values than those of WT m-NAD(P)-ME and instead are similar to those of human c-NADP-ME. Furthermore, the triple mutant cannot be inhibited by ATP, also like the c-NADP-ME. However, the $K_i$ value of the triple mutant is smaller than that of c-NADP-ME (Table 4). It is also likely that the triple mutant has a much higher affinity for NADP⁺, which is indicated by a very small $K_{m,NADP}$ value. The mutant enzyme is protected by NADP⁺ binding in the nucleotide-binding site and thus is resistant to ATP inhibition.

**Contribution of Gln-362 in Human m-NAD(P)-ME—**The crystal structure of pigeon c-NADP-ME reveals that Lys-362 and Ser-346 interact with the 2'-phosphate of the NADP⁺ molecule and stabilize the binding of NADP⁺ (7, 9) (Fig. 4A, PDB code 1GQ2). In this study, we suggest that residue 362 is the most important factor governing the cofactor specificity of human m-NAD(P)-ME. All of the human mutants m-NAD(P)-ME containing Q362K (Q362K, K346S/Q362K, Y347K/Q362K, and K346S/Y347K/Q362K) have a $k_{cat,NADP}$ value higher than their $k_{cat,NAD}$ values. In the structure of pigeon c-NADP-ME, Lys-362 is ion-paired with the 2'-phosphate of NADP⁺ and participates in the electrostatic network of Asp-345 and Arg-345, making the carboxylic side chain of Asp-345 deviate from the 2'-phosphate of NADP⁺ (7, 9). Thus, Lys-362 may reduce the repulsive effect between Asp-345 and NADP⁺ and stabilize the binding of the NADP⁺ molecule in the active site. In human m-NAD(P)-ME, this residue is substituted by Gln, which is not involved in the binding of cofactor.

(FIG. 4B). Although there is no steric hindrance for the additional 2'-phosphate of NADP⁺, the neutral side chain of Gln-362 cannot neutralize the negative charge from Asp-345 and that may reduce the tolerance for the binding of NADP⁺ in human m-NAD(P)-ME. Thus, the human m-NAD(P)-ME isoform has a dual cofactor specificity.

**Contribution of Lys-346 in Human m-NAD(P)-ME—**Residue 346 among the ME isoforms demonstrates isoform-specific distribution (Fig. 1B). In c-NADP-ME and m-NADP-ME, this residue is a serine, in m-NAD(P)-ME, this residue is replaced with lysine. Mutation of Lys-346 to Ser in human m-NAD(P)-ME did not cause dramatic changes in the cofactor preference from NAD⁺ to NADP⁺. However, the double mutant K346S/Q362K shows slight improvement in decreasing $K_{m,NADP}$ and increasing $k_{cat,NADP}$, suggesting that Ser-346 plays a supplementary role in cofactor preference of the human m-NAD(P)-ME isoform. We have also suggested that the role of Lys-346 in human NAD(P)-ME is associated with the isoform-specific ATP inhibition (35). In this paper, we further demonstrate that human enzyme with the K346S mutation (K346S, K346S/Y347K, K346S/Q362K, and K346S/Y347K/Q362K) is much less inhibited by ATP and has a larger $K_{i,\text{ATP}}$ value than those enzymes without K346S (Fig. 3 and Table 4). In addition, the enzyme that introduces extra arginyl residue(s) (Y347K, Q362K, and Y347K/Q362K) becomes more sensitive to ATP. Thus, the isoform-specific ATP inhibition of human m-NAD(P)-ME seems to be related to the positive charge(s) in the active site.

**Contribution of Tyr-347 in Human m-NAD(P)-ME—**Kinetic analysis reveals that Tyr-347 is also a determinant of the dual cofactor specificity of human m-NAD(P)-ME. Mutation of Tyr-347 to Lys in human m-NAD(P)-ME revealed a decrease in $K_{m,NADP}$ similar to Q362K and a significant elevation in $k_{cat,NADP}$. However, the Y347K enzyme did not completely shift the cofactor specificity from NAD⁺ to NADP⁺, because the $k_{cat,NADP}$ value is still higher than $k_{cat,NAD}$ value of the mutant enzyme (Table 1). The Y347K/Q362K double mutant has a smaller $k_{m,NADP}$ value (0.018 mM) than that for K346S/Q362K (0.082 mM), implying that Lys-347 seems to be more important than Ser-346 in the preference of the mutant enzyme for NADP⁺. The positive charge of Lys on residue 347 might be helpful in support of the binding of NADP⁺ by keeping the electrostatic balance in the nucleotide-binding site of c-NADP-ME.

In summary, we elucidated the molecular basis for determining the dual cofactor specificity of human m-NAD(P)-ME. However, the determinants for the isoform-specific ATP inhibition are not completely identical to determinants of cofactor specificity for the enzyme. The additional factors involved in the isoform-specific inhibition need to be further investigated.
Human Mitochondrial Malic Enzyme

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