Ligands of the Mn^{2+} Bound to Porcine Mitochondrial NADP-Dependent Isocitrate Dehydrogenase, as Assessed by Mutagenesis†

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ABSTRACT: Pig heart mitochondrial NADP-dependent isocitrate dehydrogenase requires a divalent metal ion for catalysis, and metal-isocitrate is its preferred substrate. On the basis of the crystal structure of the enzyme−Mn^{2+}−isocitrate complex, Asp^{252}, Asp^{275}, and Asp^{279} were selected as targets for site-directed mutagenesis to evaluate the roles of these residues as ligands of the metal ion. The circular dichroism spectra of the purified mutant enzymes are similar to that of wild-type enzyme indicating there are no appreciable conformational changes. The K_m values for isocitrate and for Mn^{2+} are increased in the asparagine and histidine mutants at positions 252 and 275; while for cysteine mutants at the same positions, the K_m's are not changed appreciably. Mutants at position 279 exhibit only a small change in K_m for isocitrate. These results indicate that Asp^{252} and Asp^{275} are ligands of enzyme-bound $Mn^{2+}$and influence the binding of Mn^{2+}−isocitrate. Cysteine is an acceptable substitute for aspartate as a ligand of Mn^{2+}. The pK_{aes}'s of D252C and D275C enzymes are similar to that of the wild-type enzyme (about 5.2), while the pK_{aes} of D279C is a little lower (about 4.7). These findings suggest that the V_{max}'s of the D252C, D275C, and D279C enzymes depend on the ionizable form of the same group as in wild-type enzyme and neither Asp^{252}, Asp^{275}, nor Asp^{279} acts as the general base in the enzymatic reaction. For wild-type enzyme, the pK_{aes} varies with the metal ion used with $Mg^{2+} > Cd^{2+} > Mn^{2+} > Co^{2+}$, similar to the order of the pK's for these four metal-bound waters. We therefore attribute the pH dependence of V_{max} to the deprotonation of the metal-coordinated hydroxyl group of isocitrate bound to isocitrate dehydrogenase.

A divalent metal ion is required for the dehydrogenation of isocitrate to form enzyme-bound oxalosuccinate, and for the decarboxylation of this β-keto acid to yield α-ketoglutarate, as catalyzed by porcine mitochondrial NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42). One Mn^{2+} is bound per enzyme subunit of this dimeric enzyme (*1*, *2*), but the enzyme can also use other divalent metal ions, including Cd^{2+}, Zn^{2+}, Co^{2+}, and Mg^{2+} (*3*). On the basis of proton relaxation rates, it was proposed that water is displaced in forming the ternary enzyme−Mn^{2+}−isocitrate complex from the enzyme−metal complex, allowing for chelation of the Mn^{2+} by the substrate (*4*). Cadmium NMR studies of the enzyme−metal−isocitrate complex showed that the cadmium is bound to six oxygen-containing ligands, some of which were thought to be supplied by the substrate and others by the enzyme (*5*).

The pH dependence of V_{max} has been described by the requirement for the basic form of an ionizable group of pK 5.4; this pK increases in a solvent of lower dielectric constant, such as 20% ethanol, which is characteristic of the deprotonation of an uncharged acid such as a carboxyl group in the enzyme−substrate complex (*6*, *7*). Since ^{13}C NMR studies indicate that the three carboxyls of enzyme-bound isocitrate remain ionized from pH 5.5 to 7.5 (*8*), the pH dependence of V_{max} was interpreted as reflecting the ionization of an enzymic carboxyl group (*9*). However, this pK could also indicate the deprotonation of a metal-bound water or hydroxyl.

To aid in the choice of target sites for mutagenesis, the amino acid sequence of the porcine NADP-specific isocitrate dehydrogenase (*10*) was initially aligned with that of the *Escherichia coli*[1] enzyme, since that was the first isocitrate dehydrogenase of which the crystal structure was determined (*11*). On that basis, Asp^{253}, Asp^{275}, and Asp^{279} were considered as candidates for a role in the reaction catalyzed by the porcine enzyme and were replaced by asparagine (*9*). However, the sequence homology between the bacterial and mammalian enzyme is very low: only about 16% sequence identity is observed, and alternate alignments are possible.

We have recently reported the crystal structure of porcine mitochondrial NADP-dependent isocitrate dehydrogenase complexed with Mn^{2+}−isocitrate (*12*, *13*). The bound Mn^{2+} is hexacoordinate, having interactions with Asp^{252}, Asp^{275}, two water molecules, and two oxygens of isocitrate (the α-hydroxyl and an oxygen of the α-carboxylate), while Asp^{279} is a little further from the metal ion and is linked to it through the two Mn^{2+}-coordinated water molecules (*13*). Figure 1 illustrates the region of the metal site. We now evaluate the importance to enzymatic activity of Asp^{252} by mutating it to asparagine. As well, we replaced Asp^{252},

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[1] Abbreviations: PCR, polymerase chain reaction; CD, circular dichroism; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; kbp, kilobase pair; *E. coli*, *Escherichia coli*; *B. subtilis*, *Bacillus subtilis*.

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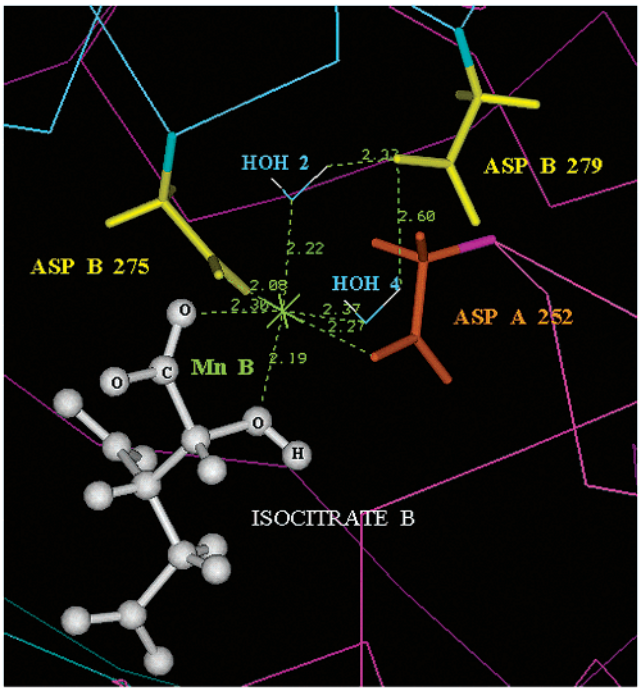

FIGURE 1: View of the active site of porcine mitochondrial NADP-dependent isocitrate dehydrogenase in subunit B, based on the crystal structure of Mn^{2+}−isocitrate complex (PDB 1LWD), as described in ref *13*. The peptide backbone of the B subunit is shown in cyan, and that of the A subunit is pink. The side chains of Asp^{275} and Asp^{279} of the B subunit are yellow, while that of Asp^{252} of the A subunit is orange. The structured waters (2 and 4), which are ligands of the Mn^{2+} (green), are shown. Isocitrate is white with the atoms of the α-carboxyl and α-hydroxyl group labeled. The distances between the Mn^{2+} and its six ligands are indicated, as are the distances between Asp^{279} and the two active site waters.

Asp^{275}, or Asp^{279} by other potential metal ligands (cysteine and histidine) and characterized the purified enzymes to determine whether a sulfur or nitrogen ligand can substitute for an oxygen in binding the divalent metal ion. We also present evidence supporting the interpretation of the pH dependence of V_{max} as due to the deprotonation of the α-hydroxyl of isocitrate bound to the metal ion.

EXPERIMENTAL PROCEDURES

Materials. Buffer components, biochemicals, and chemicals were purchased from Sigma Chemical Co. The restriction enzymes *Bam*HI, *Bpu*11021, and proteinase K were obtained from Life Technologies Inc. (Rockville, MD). The QuikChange-XL Kit and *Pfu* DNA polymerase were purchased from Stratagene (La Jolla, CA). Oligonucleotides were synthesized by Bio-Synthesis, Inc. (Lewisville, TX). T4 DNA ligase, *E. coli* strain TB1, plasmid pMal-c2, and amylose resins were purchased from New England Biolabs (Beverly, MA). Human thrombin was obtained from Enzyme Research Lab, Inc. (South Bend, IN). The plasmid DNA purification kit was supplied by QIAGEN Inc. (Valencia, CA). DEAE-cellulose (DE-52) was purchased from Whatman Inc. (Clifton, NJ). DL-Isocitrate and NADP were obtained from Sigma (St. Louis, MO).

Site-Directed Mutagenesis. A 1.2 kbp cDNA encoding pig heart NADP-dependent isocitrate dehydrogenase (IDP1) was cloned into vector pMAL-c2 (pMALcIDP1), as previously described (*9*, *14*, *15*). Site-directed mutagenesis of pMALcIDP1 was performed either by using the PCR[1] megaprimer method (*9*, *15*−*17*) or the QuikChange-XL Kit (Stratagene Inc.). D275C, D275H, D279C, and D279H enzymes were generated by the PCR megaprimer method. The oligonucleotides used to generate the mutations by the PCR megaprimer method were D275C forward primer (5′-GAACTACGATGGA*TGC*GTGCAGTCGGAC-3′), D275H forward primer (5′-GAACTACGATGGA*CAT*GTGCAGTCGGAC-3′), D279C forward primer (5′-GAGACGTGCAGTCG*TGC*ATCCTGGCCC-3′), and D279H forward primer (5′-GAGACGTGCAGTCG*CAT*ATCCTGGCCC-3′). The italicized codons are those nucleotides coding for mutations to cysteine or histidine. The universal primers *Bam*H1 reverse primer (5′-CTAGAGGATCCTTACTACTGCCGGCCCAGAGCTCTGTC-3′) and *Bpu* forward primer (5′-GGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCG-3′) were used for DNA amplification in PCR reactions. D252C, D252N, and D252H mutant enzymes were generated by the QuikChange XL Kit. The oligonucleotides used to generate mutant enzymes by the QuikChange method were D252C forward primer (5′-CACCGGCTCATT*TGT*GACATGGTGGCTCAG-3′), D252C reverse primer (5′-GACCTGAGCCACCATGTC*ACA*AATGAGCCGG-3′), D252N forward primer (5′-CACCGGCTCATT*AAT*GACATGGTGGCTCAG-3′), D252N reverse primer (5′-GACCTGAGCCACCATGTC*ATT*AATGAGCCGG-3′), D252H forward primer (5′-CACCGGCTCATT*CAT*GACATGGTGGCTCAG-3′) and D252H reverse primer (5′-GACCTGAGCCACCATGTC*ATG*AATGAGCCGG-3). The italicized codons are those mutated to cysteine, asparagine, or histidine. All mutations were confirmed by the BigDye terminator cycle sequencing method performed at the Agricultural DNA sequencing Facility, University of Delaware.

Expression and Purification of Wild-Type and Mutant Enzymes. The plasmids were expressed in *E. coli* strain TB1, and the recombinant wild-type and mutant isocitrate dehydrogenases were isolated as maltose binding proteins using an amylose column (*13*). The fusion proteins were cleaved by thrombin (*15*) and purified on a DE-52 ion exchange column as previously described (*18*). The purity of the proteins was assessed by SDS−polyacrylamide gel electrophoresis (*15*) and by N-terminal amino acid sequencing on an Applied Biosystem gas-phase sequencer (model Procise) equipped with an on-line microgradient delivery system (model 140C) and a computer (model 610 Macintosh). The protein concentration for the purified enzyme was determined from $E^{1\%}_{280nm} = 10.8$ (*19*). A subunit molecular mass of 46.6 kDa (*10*) was used to calculate the concentration of enzyme subunits.

Circular Dichroism of the Wild-Type and Mutant Enzymes. CD was conducted using a Jasco model J-710 spectropolarimeter at room temperature (Jasco, Inc., Easton, MD). Measurements of ellipticity as a function of wavelength were made as described previously (*9*), using 413 as the number of amino acids per subunit of NADP-dependent isocitrate dehydrogenase. The concentrations of wild-type and mutant enzymes were determined using the Bio-Rad assay, which is based on the method of Bradford (*20*).

Kinetics of Wild-Type and Mutant NADP-Dependent Isocitrate Dehydrogenases. The activity of the enzyme was monitored continuously by the reduction of NADP to

NADPH ($\epsilon_{340nm} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$) at 25 °C. The standard assay solution (1 mL) contained 30 mM triethanolamine hydrochloride buffer (pH 7.4), 0.1 mM NADP, 4 mM DL-isocitrate, and 2 mM $MnSO_4$, unless otherwise noted.

For K_m determinations, the concentration of NADP, isocitrate, or Mn^{2+} was varied, while the other substrates were maintained at saturating concentrations. The K_m values for other metals were determined by varying the concentrations of $CdSO_4$, $MgSO_4$, $ZnSO_4$, or $CoSO_4$ while maintaining the isocitrate and NADP at the saturating concentrations of 4 mM and 1 mM, respectively. The assay solution for measuring K_m at pH 5.5 contained 30 mM sodium acetate buffer, and the assay solution for measuring K_m at pH 7.4 contained 30 mM triethanolamine hydrochloride. The K_m values were determined from direct plots of velocity versus substrate concentration using SigmaPlot software, and the standard errors are given.

The pH−rate profiles for the reaction catalyzed by wild-type and mutant enzymes were determined over the range pH 5−8, using the buffers previously described (*9*). The reaction rates were measured using 4−16 mM isocitrate, 0.5−2.0 mM NADP, and 2−8 mM metal (Mn^{2+}, Cd^{2+}, Co^{2+}, or Mg^{2+}), as indicated for each enzyme.

RESULTS

Expression and Purification of Wild-Type and Mutant Enzymes. The pig heart NADP-dependent isocitrate dehydrogenase mutants with cysteine and histidine substituted for aspartate at positions 275 and 279 were generated using expression vector pMALcIDP1 and the megaprimer PCR method (*9*, *15*−*17*). The mutant enzymes with asparagine, cysteine, and histidine replacing aspartate at position 252 were generated using the QuikChange-XL Kit. The wild-type and mutant enzymes were expressed in *E. coli* and purified as described in Experimental Procedures. The purity of these enzymes was assessed by SDS−polyacrylamide gel electrophoresis (data not shown). The wild-type and mutant enzymes were homogeneous and expressed well and have the same subunit molecular mass (about 46.6 kDa). The N-terminal amino acid sequences of these porcine isocitrate dehydrogenase preparations confirmed their purity and, since the sequences of the *E. coli* and porcine enzymes differ in 9 of the first 10 amino acids, demonstrate that the recombinant porcine enzyme is not contaminated with the *E. coli* isocitrate dehydrogenase.

Circular Dichroism Spectra of Wild-Type and Mutant Enzymes. CD spectra of wild-type and mutant enzymes were measured to evaluate whether changes in secondary structure occur with these amino acid substitutions. The spectra of all the mutant enzymes are very similar to that of the wild-type enzyme (data not shown). All the enzymes exhibit CD spectra typical of α-helical proteins with double minima at 208 and 222 nm. These results suggest that the mutations do not cause appreciable conformational changes.

Kinetic Characteristics of Wild-Type and Mutant Enzymes. The NADP-dependent isocitrate dehydrogenase is isolated without any endogenous metal ion, as shown previously (*3*), but is activated by Mn^{2+}, Mg^{2+}, Cd^{2+}, Zn^{2+}, or Co^{2+}. The kinetic parameters of wild-type and mutant porcine NADP-dependent isocitrate dehydrogenases are summarized in Table 1. Under the standard assay conditions at pH 7.4, containing 4 mM isocitrate, 2 mM Mn^{2+}, and 0.1 mM NADP, the specific activities of all the Asp^{252}, Asp^{275}, and Asp^{279} mutants are greatly decreased. Except for D252C mutant, all the mutants exhibit activities less than 4% that of wild-type, and the specific activities of D252H and D275H mutants are, respectively, about 0.05% and 0.01% that of wild-type. The D252C mutant has a specific activity of 6.85 μmol min^{-1} mg^{-1} measured under "standard assay" conditions (i.e., 0.1 mM NADP), and upon extrapolation to saturating concentrations of NADP (∼1 mM), a V_{max} of 12.0 μmol min^{-1} mg^{-1} is obtained. Table 1 records the V_{max} values of wild-type and mutant isocitrate dehydrogenases extrapolated to saturating concentrations of NADP.

Table 1: Kinetic Parameters at pH 7.4 for Wild-Type and Mutant Porcine NADP-Dependent Isocitrate Dehydrogenases for Isocitrate, NADP, and Mn^{2+} [a]

enzyme	K_m(isocitrate)[b] (μM)	K_m(NADP)[c] (μM)	K_m(Mn)[d] (μM)	V_{max} [e] (μmol/min/mg)
wild-type	8.37 ± 0.96	5.59 ± 0.42	0.33 ± 0.06	42.9 ± 1.0
D252N	1422 ± 31	92.4 ± 19.3	1003. ± 202	0.08 ± 0.01
D252H	117 ± 12	78.1 ± 12.0	219. ± 59	0.02 ± 0.00
D252C	8.7 ± 0.9	76.6 ± 2.3	0.31 ± 0.05	12.0 ± 1.3
D275H	44.6 ± 6.4	4.25 ± 0.68	241. ± 85	0.0044 ± 0.001
D275C	10.2 ± 1.0	12.9 ± 1.0	0.13 ± 0.02	1.64 ± 0.23
D279H	6.38 ± 0.72	8.85 ± 0.79	0.12 ± 0.03	0.70 ± 0.07
D279C	3.71 ± 1.12	4.14 ± 0.76	0.29 ± 0.09	0.18 ± 0.01

[a] The kinetic parameters were measured in 30 mM triethanolamine hydrochloride (pH 7.4). Unless specified otherwise, the concentration of NADP, isocitrate, or Mn^{2+} was varied, while the other substrates were maintained at the concentrations in the standard assay described in Experimental Procedures. [b] The K_m values for isocitrate for all mutants were determined using 1 mM NADP to ensure saturation of the coenzyme site; for D252N, the Mn^{2+} concentration was 10 mM, while for all the other mutants 2 mM Mn^{2+} was used. The K_m values for isocitrate for D275N and D279N were previously reported to be 103 and 1.4 μM, respectively (*9*). [c] The K_m values for NADP for D275N and D279N were previously determined as 24 and 10 μM, respectively (*9*). [d] The K_m values for Mn were determined at pH 7.4 containing 1.0 mM NADP and 4 mM isocitrate. [e] Obtained by extrapolating the dependence of v_i on [NADP] to infinite concentrations of coenzyme.

The K_m values for isocitrate of the asparagine and histidine mutants at positions 252 and 275 are increased 5−170- fold compared with wild-type (8.37 μM), while the K_m values for the cysteine mutants at the same positions are not changed appreciably (Table 1). In contrast, for the mutants at position 279, there is no appreciable change in the K_m for isocitrate. Thus, replacement of Asp^{252} or Asp^{275} in general leads to an increase in K_m for isocitrate, while replacement of Asp^{279} has a minimal effect on the K_m for isocitrate. The exceptions are replacement of Asp^{252}/Asp^{275} by cysteine: since normal K_m values for isocitrate are exhibited by D252C and D275C, it appears that cysteine is an acceptable substitute for aspartate in determining the affinity between enzyme and isocitrate.

Table 1 also reports the K_m values for Mn^{2+} at pH 7.4. For the asparagine and histidine mutants at position 252, as well as the histidine mutant at position 275, these values are increased 700−3000-fold as compared to that of wild-type enzyme. In contrast, for the two mutants in which cysteine replaces aspartate (D252C, D275C), the K_m for Mn^{2+} is very similar to that of wild-type enzyme. Among the D279 mutants, there is little change in the K_m for Mn^{2+} as compared to wild-type enzyme. These results indicate that Asp^{252} and Asp^{275} are directly involved in the binding of Mn^{2+} by the

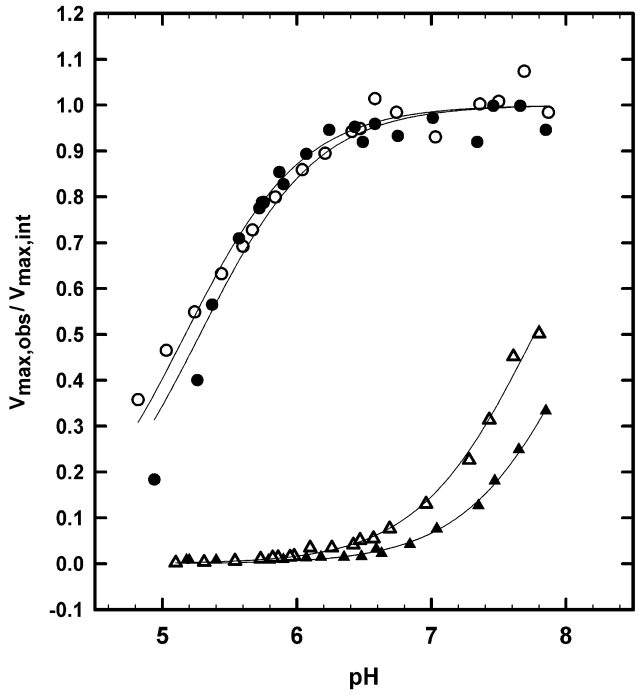

FIGURE 2: pH−V_{max} profiles for wild-type and mutant isocitrate dehydrogenases. For each enzyme, every observed maximum velocity was divided by its own intrinsic maximum velocity ($V_{max,obs}/V_{max,int}$) so that the shapes of the profiles can easily be compared. The profiles for the wild-type (○), D252C (●), D252N (△), and D252H (▲) are shown. For wild-type, the constant concentrations used over the entire pH range were 4 mM isocitrate, 2 mM Mn^{2+}, and 1 mM NADP; for D252C, 8 mM isocitrate, 4 mM Mn^{2+}, and 1 mM NADP were used; for D252N and D252H, 16 mM isocitrate, 8 mM Mn^{2+}, and 2 mM NADP were used. The curves were superimposable over the entire pH range tested when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5 or 1 mM NADP were used for wild-type, when 8 mM isocitrate, 4 mM Mn^{2+}, and 1 or 2 mM NADP were used for D252C, and when 8 or 16 mM isocitrate, 4 or 8 mM Mn^{2+}, and 1 or 2 mM NADP were used for D252N and D252H, indicating that these enzymes were saturated with all substrates.

enzyme and that cysteine is an acceptable substitute for aspartate in its interaction with Mn^{2+}.

All the D252 mutants exhibit significantly higher K_m values for NADP compared to wild-type enzyme (5.59 μM), whereas the D275 and D279 mutants display either no or small changes in K_m for NADP. The results of Table 1 suggest that Asp^{252} and Asp^{275} are involved in isocitrate binding, while Asp^{252} also affects the coenzyme binding.

pH Dependence of V_{max}. The pH−V_{max} profiles of wild-type and mutant isocitrate dehydrogenases were measured using saturating concentrations of substrate and coenzyme (4−16 mM isocitrate, 2−8 mM Mn^{2+}, and 1−2 mM NADP). The V_{max} of the recombinant wild-type enzyme depends on the basic form of an ionizable group of the enzyme−substrate complex. The dependence of V_{max} on pH was analyzed in terms of the equation

$$V_{max,obs} = \frac{V_{max,int}}{1 + \frac{[H^+]}{K_{aes}}} \quad (1)$$

where $V_{max,obs}$ is the maximum velocity measured at each pH, $V_{max,int}$ is the intrinsic maximum velocity, which is independent of pH, and K_{aes} is the dissociation constant of an ionizable group of the enzyme−substrate complex. In the cases of the D252N, D252H, D275N, and D275H mutants, $V_{max,obs}$ goes to a low constant rate ($V_{max,lim}$) at low pH and the $V_{max,lim}$ is about 3% of $V_{max,int}$. In these cases, ($V_{max,obs} - V_{max,lim}$) replaced $V_{max,obs}$ and ($V_{max,int} - V_{max,lim}$) replaced $V_{max,int}$ in eq 1. The pH−rate profiles were plotted as $V_{max,obs}$ or ($V_{max,obs} - V_{max,lim}$) against pH. Figure 2 illustrates the pH-dependence of V_{max} for wild-type and D252 mutant enzymes. To facilitate comparisons among these enzymes, for each enzyme $V_{max,obs}$ (or $V_{max,obs} - V_{max,lim}$) was divided by its own $V_{max,int}$ (or $V_{max,int} - V_{max,lim}$). Over the pH range 5−8, the pH−V_{max} profile for D252C is similar to that of wild-type, whereas those for D252H and D252N are shifted upward to yield higher apparent pK_{aes} values.

Table 2: Kinetic Parameters for the pH−V_{max} Profiles for Wild-Type and Mutant Enzymes[a]

enzyme	pK_{aes}	$V_{max,int}$ (μmol min^{-1} mg^{-1})
wild-type	5.24 ± 0.03	42.9 ± 1.0
D252C	5.24 ± 0.06	12.0 ± 1.3
D275C	5.00 ± 0.03	1.64 ± 0.23
D279C	4.69 ± 0.11	0.18 ± 0.01
D252H	8.18 ± 0.07	0.05 ± 0.00
D275H	8.27 ± 0.10	0.07 ± 0.01
D279H	5.53 ± 0.09	1.13 ± 0.07
D252N	7.76 ± 0.06	0.57 ± 0.04

[a] The pK_{aes} values were measured in different pH buffers containing various concentrations of isocitrate, Mn^{2+}, and NADP. The pH−V_{max} curves were superimposable when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5 or 1 mM NADP were used for wild-type, when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5, 1, or 2 mM NADP were used for D252C, when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5 or 1 mM NADP were used for D275C, when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5 or 1 mM NADP were used for D279C, when 4, 8, or 16 mM isocitrate, 2, 4, or 8 mM Mn^{2+}, and 0.5, 1, or 2 mM NADP were used for D252H, when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5 or 1 NADP were used for D275H, when 4 or 8 mM isocitrate, 2 or 4 mM Mn^{2+}, and 0.5 or 1 mM NADP were used for D279H, and when 16 mM isocitrate, 8 mM Mn^{2+}, and 1 or 2 mM NADP were used for D252N. These results indicate that these enzymes were saturated with substrates over the full pH range and that the measured values were actually V_{max} values as a function of pH. The substrate concentrations are all high relative to the K_m values. For example, for wild-type, D252C, D275C, and D279C, at pH 5.5 the K_m values for isocitrate were 5−20 μM, for NADP 13−87 μM, and for Mn^{2+} 2−9 μM. The pK_{aes} value of D275N was previously reported to be 7.73 (*9*).

Table 2 summarizes the pK_{aes} values for wild-type and the D252, D275, and D279 mutant enzymes. For all the enzymes, substrate saturated the active site under the conditions used. Doubling the already high concentrations of isocitrate, Mn^{2+}, and NADP did not change the pH−V_{max} profiles. Table 2 shows that the pK_{aes} values of D252C and D275C are similar to that of the wild-type enzyme (about 5.2); the V_{max}'s of these two mutants probably depend on the ionizable form of the same group as in the wild-type enzyme. The pK_{aes} of the D279C enzyme is a little lower, 4.7. Since the pK of the −SH group of a typical cysteine residue is 9.1−10.8 (*21*), one might expect that, if a cysteine residue were located at the position of the amino acid that normally functions as the general base of the catalytic reaction, the pK_{aes} for the mutant enzyme would be 9−10. Since none of these cysteine mutants has a pK_{aes} of 9−10, these results suggest that neither Asp^{252}, Asp^{275}, nor Asp^{279}

Table 3: Effect of Metal Ions on pH−V_{max} Profile for Wild-Type NADP-Dependent Isocitrate Dehydrogenase[a]

metal	pK_{aes}	$V_{max,int}$ (μmol min^{-1} mg^{-1})
Mg^{2+}	5.73 ± 0.02	24.9 ± 2.2
Cd^{2+}	5.41 ± 0.06	42.6 ± 0.3
Mn^{2+}	5.24 ± 0.03	42.9 ± 1.0
Co^{2+}	5.07 ± 0.04	17.1 ± 0.7

[a] The pK_{aes} values for wild-type enzyme were measured in the presence of various concentrations of isocitrate, NADP, and divalent metal ion. With Mg^{2+}, the same values were obtained using 4 and 8 mM isocitrate, 2 and 4 mM Mg^{2+}, and 0.2 and 0.5 mM NADP; with Cd^{2+}, the same values were obtained using 4 and 8 mM isocitrate, 2 mM Cd^{2+}, and 0.25 and 0.5 mM NADP; with Co^{2+}, the same values were obtained using 4 and 8 mM isocitrate, 2 mM Co^{2+}, and 0.5 mM NADP; with Mn^{2+}, the same values were obtained when 4 and 8 mM isocitrate, 2 and 4 mM Mn^{2+}, and 0.2, 0.5, and 1 mM NADP were used. (These substrate concentrations are high relative to the K_m values. For example, for wild-type and mutant enzymes, the K_m values at pH 5.5 for these metal ions ranged from 2.4 to 25 μM. The K_m values at pH 7.4 for the metal ions, shown in Table 4, range from 0.5 to 25 μM.) These results indicate that the enzyme was saturated with substrate, coenzyme, and metal ion over the entire pH range and the curves obtained actually represent the dependence of V_{max} on pH.

acts as the general base in the enzymatic reaction.

The pK_{aes} values of D279H and D279C are both slightly altered as compared to wild-type (5.5 and 4.7, respectively); these results may reflect a relatively small perturbation of pK_{aes} of the same ionizable group seen in wild-type. In contrast, the pK_{aes} values of D252H, D252N, D275H, and D275N mutants are significantly higher than that of wild-type enzyme; furthermore, the values of $V_{max,int}$ are extremely low for all of these mutants.

Effect of Divalent Metal Ion on pH Dependence of V_{max} for Wild-Type Enzyme. Another candidate for the ionizable group reflected in the pH−V_{max} profile is the α-hydroxyl of enzyme-bound isocitrate, which is a ligand of the Mn^{2+}. The pK of a metal ion-bound water molecule depends on the particular metal ion to which it is bound (*22*, *23*). For example, the pK of isolated metal−hydroxyl is 11.5 for Mg^{2+}, 10.7 for Mn^{2+}, and 9.6 for Co^{2+} (*22*, *23*). Various values appear in the literature for Cd^{2+}, one of which is 11.7. Table 3 summarizes the results of the pH dependence of V_{max} for the wild-type isocitrate dehydrogenases in the presence of various divalent metal ions. Over the pH range 5−8, the shapes of the pH−V_{max} profiles of wild-type enzyme in the presence of Mg^{2+}, Cd^{2+}, Mn^{2+}, and Co^{2+} are similar with low values of $V_{max,obs}$ at low pH and increasing values of $V_{max,obs}$ as the pH is raised and the ionizable group becomes deprotonated. However, the pK value for the enzyme−substrate complex depends on the metal ion present. The order of the pK_{aes} obtained with the various metal ions, from highest to the lowest, is Mg^{2+} > Cd^{2+} > Mn^{2+} > Co^{2+}; this *order* is similar to that of the pK values of these four metal−hydroxyls (pK's from 11.5 to 9.6) (*22*, *23*), although the absolute values of the pK's differ. Similar data have been used to identify the ionizing catalytic group as the metal-bound water molecule in the case of carboxypeptidase A (*24*, *25*).

The $V_{max,int}$ of the wild-type enzyme in the presence of Cd^{2+} is similar to that in the presence of Mn^{2+}, while that of the enzyme in the presence of Mg^{2+} is lower (Table 3). $V_{max,int}$ of wild-type enzyme in the presence of Co^{2+} is about half of that in the presence of Mn^{2+}.

For wild-type enzyme, we have also measured, at pH 7.4, V_{max} and K_m for Mn^{2+}, Cd^{2+}, Zn^{2+}, Co^{2+}, and Mg^{2+}, and have compared these kinetic parameters with those obtained for the mutant enzymes in which one of the important aspartates was replaced by cysteine: D252C, D275C, D279C. Table 4 shows that the K_m values for Co^{2+}, Zn^{2+}, and Cd^{2+} are decreased in the D252C and D275C mutants, while the corresponding values for Mn^{2+} and Mg^{2+} are affected to a lesser extent. For the D279C mutant, the decreases in K_m for metal ions are *not* observed, supporting the conclusion that Asp^{279} is not a direct ligand of the enzyme-bound metal ion. Nevertheless, changing only one of the six metal ligands from aspartate to cysteine does not strikingly alter the metal specificity of isocitrate dehydrogenase. The V_{max} values decrease markedly in all the mutant enzymes when tested with any of the five metal ions.

DISCUSSION

On the basis of the crystal structure of porcine heart mitochondrial NADP-dependent isocitrate dehydrogenase complexed with Mn^{2+} and isocitrate (*13*) and the structure-corrected sequence alignment of porcine, *E. coli*, and *Bacillus subtilis* enzymes (*13*), Asp ^{252}, Asp^{275}, and Asp ^{279} were selected for site-directed mutagenesis to evaluate the roles of these three residues in the catalytic reaction. Asparagine was chosen to replace aspartate because it lacks the negative charge while retaining a similar structure and size. This replacement can be used to examine the importance of charge in the function of these residues. Cysteine and histidine were selected as replacements for aspartate because with an −SH group and an imidazole group, respectively, these amino acids often serve as protein ligands of metal ions. We sought to determine whether in isocitrate dehydrogenase a sulfur or nitrogen can substitute for an oxygen in binding divalent metal ion and to ascertain the functional consequences.

Replacement of aspartate at positions 252, 275, and 279 with asparagine, cysteine, or histidine results in large decreases in V_{max}. The K_m values for isocitrate and for Mn^{2+} of the asparagine and histidine mutants of Asp^{252} and Asp^{275} are increased drastically as compared to that of wild-type.

Table 4: Kinetic Parameters for Wild-Type and Mutant Porcine NADP-Dependent Isocitrate Dehydrogenases Using Various Ions at pH 7.4[a]

	K_m (μM)					V_{max} (μmol/min/mg)				
enzyme	Mg^{2+}	Cd^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Mg^{2+}	Cd^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}
WT	2.39 ± 0.58	5.21 ± 0.58	0.33 ± 0.06	3.94 ± 1.06	5.91 ± 1.09	24.9 ± 2.2	42.6 ± 0.3	42.9 ± 1.0	33.7 ± 2.9	17.1 ± 0.7
D252C	1.37 ± 0.00	0.50 ± 0.09	0.31 ± 0.05	0.40 ± 0.04	1.23 ± 0.25	4.56 ± 0.04	3.14 ± 0.06	12.0 ± 1.3	4.88 ± 0.08	5.06 ± 0.17
D275C	2.87 ± 0.63	1.34 ± 0.29	0.13 ± 0.03	<0.4	<0.4	1.36 ± 0.02	1.12 ± 0.03	1.64 ± 0.23	1.04 ± 0.03	1.04 ± 0.03
D279C	6.47 ± 0.68	24.7 ± 5.1	0.29 ± 0.09	9.51 ± 1.81	6.00 ± 2.03	0.11 ± 0.00	2.61 ± 0.13	0.18 ± 0.01	2.31 ± 0.11	0.23 ± 0.15

[a] The K_m and V_{max} values were measured in 30 mM triethanolamine hydrochloride (pH 7.4) containing 1 mM NADP and 4 mM isocitrate as described under Experimental Procedures.

Since the metal−isocitrate complex is considered to be the preferred substrate for the porcine NADP-dependent isocitrate dehydrogenase (*1*, *3*, *26*, *27*), these results can be explained by the crystal structure of this enzyme complexed with Mn^{2+} and isocitrate (*13*). The Mn^{2+} binding site is hexacoordinate and binds to six oxygens of neighboring groups. The Mn^{2+} interacts with two oxygens of isocitrate, the equatorial water w2, the axial water w4, Asp^{275} from one subunit and Asp^{252} from the other subunit of the dimer. The Asp^{279} residue is located farther away from Mn^{2+} and binds to metal through waters w2 and w4 and therefore has only an indirect interaction with the Mn^{2+}−isocitrate complex. Figure 1 shows the distances of Mn^{2+} to each of its ligands, as well as the distance of Asp^{279} to the two water molecules. Changing the negatively charged aspartate to neutral asparagine, cysteine, or histidine can affect the stability of the metal−isocitrate complex and likely cause the changes in enzymatic activity and the K_m values for Mn^{2+} and isocitrate. Mn^{2+} is close to both Asp^{252} and Asp^{275} (see Figure 1) and further from Asp^{279} (3.20 Å and 4.78 Å, respectively, to the two oxygens of its carboxylate). This difference in distance is consistent with the greater increase in K_m for isocitrate of D252 and D275 mutants than for that of the D279 mutant. However, even a "second shell" ligand, such as Asp^{279}, can influence the orientation of the substrate within the active site through its interaction with the direct ligands; this effect can reduce V_{max}. Such a role for the second shell ligands has been proposed in the case of carbonic anhydrase (*28*).

In general Mn^{2+} and Mg^{2+} being "hard" metal ions prefer "hard" ligands, such as carboxylates, while Co^{2+} and Zn^{2+} are "borderline" metals, which generally exhibit a preference for imidazole ligands, and Cd^{2+} is a "soft" metal ion with a preference for thiol ligands (*29*). By replacement of the ligands of the metal ion, in principle, it is possible to change the metal specificity of an enzyme. By substitution of a histidine for an aspartate, the activity of the Zn^{2+}-dependent alkaline phosphatase toward Co^{2+} was enhanced (*30*). For the porcine NADP-dependent isocitrate dehydrogenase, we have found that replacing either Asp^{252} or Asp^{275} by cysteine improves the enzyme's affinity for Co^{2+}, Zn^{2+}, and Cd^{2+} with little effect on the K_m for Mn^{2+} or Mg^{2+}. However, for isocitrate dehydrogenase, it appears that a change from six oxygen ligands to five oxygen ligands and one sulfur ligand produces only a small change in the metal specificity; the V_{max} values are decreased when the mutant enzymes are assayed with any of the metal ions. Since the cysteine mutants at positions 252 and 275 are more active than the histidine or asparagine mutants, the results for isocitrate dehydrogenase suggest that cysteine can partially substitute for aspartate.

Although the porcine isocitrate dehydrogenase was crystallized only as the Mn−isocitrate complex, a structural alignment was made of the porcine enzyme with seven NADP-bound *E. coli* isocitrate dehydrogenases to predict the location of NADP in the porcine enzyme (*13*). In the predicted structure of the NADP−porcine isocitrate dehydrogenase complex, Asp^{252} is closer to NADP than Asp^{275} and Asp^{279} residues. In fact, the negatively charged carboxylate of Asp^{252} is only 4.3 Å away from C5 of the positively charged nicotinamide of the coenzyme; whereas Asp^{275} and Asp^{279} are no closer than 6.05 and 5.61 Å, respectively, to the nicotinamide. The predicted proximity between Asp^{252} and enzyme-bound NADP is consistent with the marked increase in the K_m for NADP observed selectively for all the mutants in which the negatively charged Asp^{252} is replaced by a neutral amino acid.

It has been postulated that the enzymatic oxidative decarboxylation of isocitrate is initiated by the abstraction of a proton from the C2-hydroxyl of isocitrate prior to the transfer of a hydride to NADP (*26*). A general base was thought necessary to facilitate removal of the hydroxyl proton. In previous studies (*9*, *15*), it was speculated that Asp^{275} or Asp^{279} may function as the catalytic base and the pH-dependence of V_{max} described by pK 5.4−5.6 was attributed to ionization of Asp^{275} or Asp^{279} in the enzyme−substrate complex. However, the present study indicates that when cysteine replaced aspartate at position 252, 275, or 279, the pK_{aes} values were changed to 5.24, 5.00, and 4.69, respectively, far lower than the pK of 8−9 expected if a deprotonated −SH were functioning as a general base. (In the earlier studies of the pH dependence of V_{max}, the enzyme was not saturated with NADP in the lower pH range, which led to a little higher apparent value of the pK_{aes}. In the present study in which the enzyme is fully saturated with NADP over the entire pH range used, pK_{aes} for wild-type enzyme is 5.2.) These findings indicate that none of these aspartates functions as a general base. On the basis of the present evidence and the recent crystal structure, we postulate that the pH dependence of V_{max} is due to the deprotonation of the metal-coordinated hydroxyl group of isocitrate and the pK_{aes} of 5.2 in wild-type enzyme is due to the ionization of this Mn^{2+}-bound hydroxyl proton, even though the pK is far below that of the α-hydroxyl group of free isocitrate. An enzyme-bound water (w6) was suggested as the proton acceptor and as a participant in a proton relay to another enzyme-bound water (w8), which is solvent-accessible (*13*). It is significant that in NMR studies of ^{13}C-enriched isocitrate bound to porcine isocitrate dehydrogenase, no signal was observed for the α-hydroxyl carbon resonance, a result interpreted as indicating immobilization of this group (*8*). These earlier results are consistent with the new information that the α-hydroxyl of isocitrate is a ligand of Mn^{2+}. There are many cases of enzyme−metal-bound water exhibiting a pK orders of magnitude lower than that of free water. For example, Zn^{2+}-bound water of carboxypeptidase A has a pK_a of about 6.1 at 25 °C (*24*), and the Zn^{2+}-bound water of carbonic anhydrase II exhibits a pK_a of 6.8 (*31*). It was pointed out for carbonic anhydrase that the electrostatic environment around the zinc site modulates the pK_a of zinc-bound water (*31*), and this statement can certainly be applied to the present case of the pK of the α-hydroxyl of isocitrate liganded to Mn^{2+} while bound to isocitrate dehydrogenase. The positively charged Arg^{101}, Arg^{110}, and Arg^{133} are 4−5 Å from the −OH of enzyme-bound isocitrate, and Lys^{212} is about 3.7 Å from the −OH (*13*); the proximity of all of these positive charges greatly lowers the pK of the α-hydroxyl of isocitrate below its value in free isocitrate, as demonstrated by mutagenesis experiments in which the neutral glutamine replaces the normal positively charged amino acid (*15*, *32*).

The observation (Table 2) of elevated pK_{aes} values for isocitrate dehydrogenase mutants D252H, D252N, D275H, and D275N (7.7−8.3) can be explained in two ways. It may be that in these severely crippled enzymes, an amino acid

residue at another location acts as a surrogate general base, albeit poorer than the original ionizable group. Alternatively, since the replacement histidine or asparagine are not as effective ligands of the Mn^{2+} as the original aspartates, the p*K* of the metal-bound α-hydroxyl of isocitrate in these mutant enzymes may be decreased less as compared to free isocitrate.

Further support for the assignment of the p*K* of 5.2 to the ionization of the Mn^{2+}-bound hydroxyl of enzyme-bound isocitrate comes from the dependence of the p*K* of the enzyme−substrate complex on the metal ion used in the enzymatic reaction (Table 3). The order (from highest to lowest) of the p*K* values of wild-type enzyme in the presence of different metal ions is similar to the order of the p*K* values of different metal−water complexes ($Mg^{2+} \cong Cd^{2+} > Mn^{2+} > Co^{2+}$) (*22*, *23*). Auld and Vallee were the first to suggest, for carboxypeptidase A, that the decreasing p*K* from 6.36 to 5.33 in changing the required metal ion from Mn^{2+} to Co^{2+} reflected the ionization of the metal-coordinated water bound to the enzyme (*33*). Makinen et al. (*24*) confirmed the metal ion-dependent shift in p*K* for carboxypeptidase A, although the absolute values (obtained under different conditions) were not the same: 7.25 with Zn^{2+} and 6.08 with Co^{2+}. Conversely, the observation of the *same* pK_a value for the Co^{2+}-, Mn^{2+}-, Zn^{2+}-, and Cd^{2+}-substituted dihydroorotase was used to indicate that the pK_a value of 6.56 was *not* due to an enzyme−metal−water complex but rather to a critical amino acid at the active site (*34*).

The present study of site-directed mutagenesis of ligands of Mn^{2+} bound to the porcine mitochondrial NADP-dependent isocitrate dehydrogenase is consistent with the recently reported crystal structure of the enzyme−substrate complex. Asp^{252} and Asp^{275} provide direct ligands to the Mn^{2+}, while Asp^{279} is in the second coordination shell (Figure 1). The pH dependence of V_{max} is best explained as the ionization of the metal-liganded hydroxyl of isocitrate bound to the enzyme. This NADP-dependent isocitrate dehydrogenase is a dimer of identical subunits. However, each of the two active sites has contributions from the other subunit. For example, Asp^{252} from one subunit is a ligand of the Mn^{2+} bound to the other subunit of the enzyme. Thus, the homodimeric form of porcine NADP-dependent isocitrate dehydrogenase is likely to be required for a catalytically active enzyme.

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REFERENCES

1. Villafranca, J. J., and Colman, R. F. (1972) Role of metal ions in reactions catalyzed by pig heart triphosphopyridine nucleotide-dependent isocitrate dehydrogenase. I. Magnetic resonance and binding studies of the complexes of enzyme, manganous ion, and substrates, *J. Biol. Chem. 247*, 209−214.
2. Bailey, J. M., and Colman, R. F. (1985) Affinity Labeling of $NADP^+$-Specific Isocitrate Dehydrogenase by a New Fluorescent Nucleotide Analogue, 2-[(4-Bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-Bisphosphate, *Biochemistry 24*, 5367−5377.
3. Colman, R. F. (1972) Role of metal ions in reactions catalyzed by pig heart triphosphopyridine nucleotide-dependent isocitrate dehydrogenase. II. Effect on catalytic properties and reactivity of amino acid residues, *J. Biol. Chem. 247*, 215−223.
4. Villafranca, J. J. and Colman, R. F. (1974) Frequency and temperature dependence of the proton relaxation rates of solvent and substrate interaction with isocitrate dehydrogenase bound Mn(II), *Biochemistry 13*, 1152−1160.
5. Ehrlich, R. S., and Colman, R. F. (1989) Multinuclear NMR studies of the divalent metal binding site of NADP-dependent isocitrate dehydrogenase from pig heart, *Biochemistry 28*, 2058−2065.
6. Colman, R. F. (1968) A glutamyl residue in the active site of triphosphopyridine nucleotide-dependent isocitrate dehydrogenase of pig heart, *J. Biol. Chem. 243*, 2454−2464.
7. Seelig, G. F., and Colman, R. F. (1978) Characterization of the physicochemical and catalytic properties of human heart NADP-dependent isocitrate dehydrogenase, *Arch. Biochem. Biophys. 188*, 394−409.
8. Ehrlich, R. S., and Colman, R. F. (1987) Ionization of isocitrate bound to pig heart $NADP^+$-dependent isocitrate dehydrogenase: ^{13}C NMR study of substrate binding, *Biochemistry 26*, 3461−3466.
9. Grodsky, N. B., Soundar, S., and Colman, R. F. (2000) Evaluation by site-directed mutagenesis of aspartic acid residues in the metal site of pig heart NADP-dependent isocitrate dehydrogenase, *Biochemistry 39*, 2193−2200.
10. Haselbeck, R. J., Colman, R. F., and McAlister-Henn, L. (1992) Isolation and sequence of a cDNA encoding porcine mitochondrial NADP-specific isocitrate dehydrogenase, *Biochemistry 31*, 6219−6223.
11. Hurley, J. H., Dean, A. M., Koshland, D. E., Jr., and Stroud, R. M. (1991) Catalytic mechanism of $NADP^+$-dependent isocitrate dehydrogenase: Implications from the structures of magnesium-isocitrate and $NADP^+$ complexes, *Biochemistry 30*, 8671−8678.
12. Endrizzi, J., Zhang, G., Chen Xiong, W., Hurley, J. H., Remington, S. J., and Colman, R. F. (1996) Crystallization and preliminary diffraction analysis of porcine heart mitochondrial $NADP^+$-dependent isocitrate dehydrogenase, *Acta Crystallogr. D52*, 1024−1026.
13. Ceccarelli, C., Grodsky, N. B., Ariyaratne, N., Colman, R. F., and Bahnson, B. (2002) Crystal structure of porcine mitochondrial $NADP^+$-dependent isocitrate dehydrogenase complexed with Mn^{2+} and isocitrate, *J. Biol. Chem. 277*, 43454−43462.
14. Soundar, S., Jenning, G. T., McAlister-Henn, L., and Colman, R. F. (1996) Expression of pig heart mitochondrial NADP-dependent isocitrate dehydrogenase in *Escherichia coli*, *Protein Expression Purif. 8*, 305−312.
15. Soundar, S., Danek, B. L., and Colman, R. F. (2000) Identification by mutagenesis of arginines in the substrate binding site of the porcine NADP-dependent isocitrate dehydrogenase, *J. Biol. Chem. 275*, 5606−5612.
16. Tao, B. Y., and Lee, K. C. P. (1994) in *PCR Technology: Current Innovations* (Griffrin, H. G., and Griffrin, A. M., Eds.), p 74, CRC Press, Cleveland, OH.
17. Sarkar, G., and Sommer, S. S. (1990) The "megaprimer" method of site-directed mutagenesis, *BioTechniques 8*, 404−407.
18. Lee, P., and Colman, R. F. (2002) Implication by site−directed mutagenesis of Arg314 and Tyr316 in the coenzyme site of pig mitochondrial NADP-dependent isocitrate dehydrogenase, *Arch. Biochem. Biophys. 401*, 81−90.
19. Johanson, R. A., and Colman, R. F. (1981) Cysteine in the manganous-isocitrate binding site of pig heart TPN-specific isocitrate dehydrogenase. I. Kinetics of chemical modification and properties of thiocyano enzyme, *Arch. Biochem. Biophys. 207*, 9−20.
20. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
21. Cohn, E. J., and Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, p 445, Reinhold Publishing Corp, New York.
22. Lippard, S. J., and Berg, J. M. (1994) *Principles of Bioinorganic Chemistry*, p 25, University Science Books, Mill Valley, CA.
23. Huheey, J. E., Keiter, E. A. and Keiter, R. L. (1993) *Inorganic Chemistry*, 4th ed., p 328, Harper Collins College Publisher, New York.

24. Makinen, M. W., Kuo, L. C., Dymowski, J. J., and Jaffer, S. (1979) Catalytic role of the metal ion of carboxypeptidase A in ester hydrolysis, *J. Biol. Chem. 254*, 356−366.
25. Christianson, D W., and Lipscomb, W.N. (1989) Carboxypeptidase A, *Acc. Chem. Res. 22*, 62−69.
26. Colman, R. F. (1983) Aspects of the structure and function of isocitrate dehydrogenases, *Pept. Protein Rev. 1*, 41−69.
27. Ehrlich, R. S., and Colman, R. F. (1976) Influence of substrates and coenzymes on the role of manganous ion in reactions catalyzed by pig heart triphosphopyridine nucleotide-dependent isocitrate dehydrogenase, *Biochemistry 15*, 4034−4041.
28. DiTusa, C.A., McCall, K. A., Christensen, T., Mahapatro, M., Fierke, C.A., and Toone, E. J. (2001) Thermodynamics of metal ion binding. 2. Metal ion binding by carbonic anhydrase variants, *Biochemistry 40*, 5345−5351.
29. Mildvan, A. (1970) Metals in Enzyme Catalysis, in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. II, Chapter 9, pp 445−536, Academic Press, New York.
30. Wojciechowski, C. L., and Kantrowitz, E. R. (2002) Altering of the metal specificity of *Escherichia coli* alkaline phosphatase, *J. Biol. Chem. 277*, 50476−50481.
31. Lesburg, C. A., Huang, C. C., Christianson, D. W., and Fierke, C. A. (1997) Histidine → carboxamide ligand substitutions in the zinc binding site of carbonic anhydrase II alter metal coordination geometry but retain catalytic activity, *Biochemistry 36*, 15780−15791.
32. Kim, T. K., Lee, P., and Colman, R. F. (2003) Critical role of Lys212 and Tyr140 in porcine NADP-dependent isocitrate dehydrogenase, *J. Biol. Chem. 278*, 49323−49331.
33. Auld, D. S., and Vallee, B. L. (1970) Kinetics of carboxypeptidase A. The pH dependence of tripeptide hydrolysis catalyzed by zinc, cobalt, and manganese enzymes, *Biochemistry 9*, 4352−4359.
34. Huang, D. T. C., Thomas, M. A. W., and Christopherson, R. I. (1999) Divalent metal derivatives of the hamster dihydroorotase domain, *Biochemistry 38*, 9964−9970.

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