Evaluation by Mutagenesis of the Roles of His^{309}, His^{315}, and His^{319} in the Coenzyme Site of Pig Heart NADP-Dependent Isocitrate Dehydrogenase†

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Received January 23, 2002; Revised Manuscript Received March 4, 2002

ABSTRACT: Sequence alignment predicts that His^{309} of pig heart NADP-dependent isocitrate dehydrogenase is equivalent to His^{339} of the *Escherichia coli* enzyme, which interacts with the coenzyme in the crystal structure [Hurley et al. (1991) *Biochemistry 30*, 8671−8688], and porcine His^{315} and His^{319} are close to that site. The mutant porcine enzymes H309Q, H309F, H315Q, and H319Q were prepared by site-directed mutagenesis, expressed in *E. coli*, and purified. The H319Q mutant has K_m values for NADP, isocitrate, and Mn^{2+} similar to those of wild-type enzyme, and $V_{max} = 20.1$, as compared to 37.8 μmol of NADPH min^{-1} (mg of protein)$^{-1}$ for wild type. Thus, His^{319} is not involved in coenzyme binding and has a minimal effect on catalysis. In contrast, H315Q exhibits a K_m for NADP 40 times that of wild type and $V_{max} =$ 16.2 units/mg of protein, with K_m values for isocitrate and Mn^{2+} similar to those of wild type. These results implicate His^{315} in the region of the NADP site. Replacement of His^{309} by Q or F yields enzyme with no detectable activity. The His^{309} mutants bind NADPH poorly, under conditions in which wild type and H319Q bind 1.0 mol of NADPH/mol of subunit, indicating that His^{309} is important for the binding of coenzyme. The His^{309} mutants bind isocitrate stoichiometrically, as do wild-type and the other mutant enzymes. However, as distinguished from the wild-type enzyme, the His^{309} mutants are not oxidatively cleaved by metal isocitrate, implying that the metal ion is not bound normally. Since circular dichroism spectra are similar for wild type, H315Q, and H319Q, these amino acid substitutions do not cause major conformational changes. In contrast, replacement of His^{309} results in detectable change in the enzyme's CD spectrum and therefore in its secondary structure. We propose that His^{309} plays a significant role in the binding of coenzyme, contributes to the proper coordination of divalent metal ion in the presence of isocitrate, and maintains the normal conformation of the enzyme.

The mitochondrial NADP-dependent pig heart isocitrate dehydrogenase (EC 1.1.1.42) catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate. The enzyme is a homodimer (*1*, *2*), with a subunit mass of 46600 Da consisting of 413 amino acids of determined sequence (*3*). Direct binding experiments indicate that NADP−isocitrate dehydrogenase binds 1 mol of NADPH or $NADP^+$/mol of enzyme subunit (*4*). Manganous isocitrate inhibits the binding of NADPH, and reciprocally, NADPH inhibits the binding of manganous isocitrate (*5*). These findings suggest that binding to the NADPH and substrate sites is mutually exclusive.

Chemical modification with inactivation of pig heart NADP-dependent isocitrate dehydrogenase by diethyl pyrocarbonate implicates histidine in the region of the coenzyme binding site (*6*). ^{1}H NMR studies of this enzyme, in complexes with NADP, NADPH, and coenzyme fragments, also indicate the presence of histidines in the nucleotide binding site close to the adenine moiety and to the nicotinamide (*7*).

Isocitrate dehydrogenase requires a divalent metal ion for enzyme activity (*8*−*10*). Direct binding experiments indicate that the porcine enzyme binds 1 mol of isocitrate, and of Mn^{2+}, per mole of enzyme subunit and that the metal isocitrate complex is the preferred substrate for this enzyme (*8*). Previous studies of kinetics, metal binding, and affinity cleavage of this enzyme by Fe^{2+}-isocitrate suggest that metal ion occupies different sites in the absence and presence of isocitrate (*8*, *11*). Affinity cleavage studies of pig heart NADP-dependent isocitrate dehydrogenase in the presence of oxygen suggested that His^{309} is the target for Fe^{2+}-isocitrate cleavage and may be a coordination site for Mn^{2+}-isocitrate (*11*).

Although no complete crystal structure of any mammalian NADP-dependent isocitrate dehydrogenase has yet been published, the structures of the *Escherichia coli* and *Bacillus subtilis* enzymes have been solved (*12*, *13*). In the crystalline NADP−enzyme complex of the *E. coli* enzyme, His^{339} has been located near the adenine of the coenzyme (*12*, *14*). Sequence alignment of several NADP-dependent isocitrate dehydrogenases (Figure 1) predicts that His^{309} of the pig heart mitochondrial isocitrate dehydrogenase is equivalent to His^{339} of the *E. coli* enzyme and His^{345} of the *B. subtilis* enzyme (*12*, *13*). Therefore, His^{309} in pig heart NADP-dependent isocitrate dehydrogenase is probably in the region of the coenzyme binding site. His^{315} and His^{319} of this enzyme are located close to His^{309} and may be the histidines associated by NMR experiments with the coenzyme site.

† This work was supported by NIH Grant R01-HL67774.

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10.1021/bi0200716 CCC: $22.00
Published on Web 04/04/2002

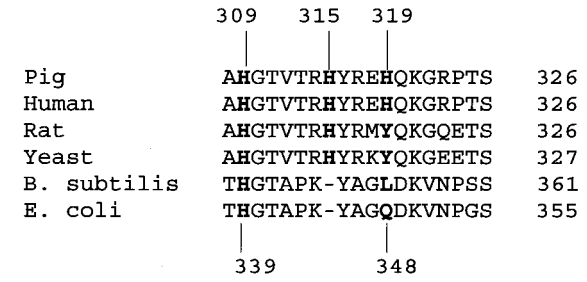

FIGURE 1: Amino acid sequence alignment of a selected region of NADP-dependent isocitrate dehydrogenase of porcine heart mitochondria (pig), human heart mitochondria (human), rat cytoplasm (rat), yeast mitochondria (yeast), *B. subtilis*, and *E. coli*. The alignment was done with CLUSTAL W. The boldface His residues were mutated to Gln or Phe for this study.

On the basis of the sequence alignment of three mammalian, yeast, *B. subtilis*, and *E. coli* enzymes (Figure 1) and comparison with the *E. coli* enzyme's crystal structure, as well as their proximity to the putative coenzyme site, His^{309}, His^{315}, and His^{319} were selected as targets for site-directed mutagenesis. A preliminary version of this study has been presented (*15*).

EXPERIMENTAL PROCEDURES

Materials. The restriction enzymes *Bam*HI and *Bpu* 11021 as well as proteinase K were purchased from Life Technologies Inc. Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). Oligonucleotides were synthesized by Operon Technologies, Inc. (Alameda, CA). Plasmid pMal-c2, *E. coli* strain TB1, T4 DNA ligase, and amylose resins were obtained from New England Biolabs (Beverly, MA). Calf intestinal alkaline phosphatase, deoxynucleotide triphosphates, and Wizard Plus Maxi and Miniprep DNA purification systems were purchased from Promega (Madison, WI). Bovine plasma thrombin and human thrombin were obtained from Sigma or Enzyme Research Lab, Inc. (South Bend, IN). The Ultraclean 15 DNA purification kit was obtained from MoBio Lab, Inc. (Solana Beach, CA), and the Superose-12 gel filtration column was purchased from Amersham Pharmacia Biotech. DEAE-cellulose (DE−52) was supplied by Whatman Inc. Sephadex G-150 and biochemicals were obtained from Sigma or Fisher, unless otherwise specified.

Site-Directed Mutagenesis. A 1.2 kbp[1] cDNA encoding pig heart NADP-dependent isocitrate dehydrogenase (IDP1) was cloned into vector pMAL-c2 (pMALcIDP1), as previously described (*16*−*18*). To facilitate separation from the *E. coli* isocitrate dehydrogenase, the enzyme was expressed as a maltose binding fusion protein with a thrombin cleavage site located between the maltose binding protein and the porcine isocitrate dehydrogenase, as described previously (*16*). Site-directed mutagenesis of pMALcIDP1 was performed using a PCR megaprimer method to produce a 1.6 kbp DNA, as we have previously described (*17*−*20*). The oligonucleotides used to generate mutant enzymes were H309Q reverse primer (5′-GTGACTGTCCCCTGAGCAGCCTCAGCTTC-3′), H309F reverse primer (5′-GTGACTGTCCCAAAAGCAGCCTCAGCTTC-3′), H315Q forward primer (5′-GTCACCCGCCAGTATAGGGAGCACCAG-3′), and H319Q forward primer (5′-GCCACTATAGGGAGCAGCAGAAGGGCCGG-3′). The underlined codons are those mutated to glutamine or phenylalanine. The Universal primers *Bam*HI reverse primer (5′-CTAGAGGATCCTTACTACTGCCGGCCCAGAGCTCTGTC-3′) and *Bpu* forward primer (5′-GGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCG-3′) were used for DNA amplification in PCR reactions. All mutations were confirmed by nucleotide sequence analysis via the dye primer cycle sequencing method using a LI-COR 4200 Long Readir Sequencer at the University of Delaware Cell Biology Core Facility.

Expression and Purification of Wild-Type and Mutant Enzymes. The recombinant wild-type and mutant enzymes expressed in *E. coli* as maltose binding fusion proteins were isolated as previously described (*16*−*18*). The pure fusion proteins were obtained by first eluting the fusion protein from an amylose column and then purifying it by gel filtration on Sephadex G-150. To obtain the pure wild-type or mutant enzymes, the fusion proteins were cleaved with thrombin for 24−48 h. The cleaved fusion proteins were further purified on a DE-52 ion-exchange column (*21*). The concentrations of fusion proteins and enzymes were determined by A_{280nm}.

SDS−*Polyacrylamide Gel Electrophoresis and Protein Sequencing*. The purity of the fusion proteins and free enzymes from wild-type and mutant enzymes was assessed by SDS−PAGE, as described (*11*). The purity of the wild-type and mutant enzymes was further analyzed by N-terminal amino acid sequencing on an Applied Biosystem gas-phase sequencer (Model 470) equipped with an on-line phenylthiohydantoin analyzer (Model 120) and a computer (Model 900A).

Enzyme Assays. The activity of NADP-dependent isocitrate dehydrogenase was monitored continuously at 25 °C by the reduction of NADP to NADPH ($\epsilon_{340nm} = 6.22 \times 10^3$ M^{-1} cm^{-1}). 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. The protein concentration for purified free isocitrate dehydrogenase was determined from $E^{1\%}_{280nm} = 10.8$ (*22*), and the protein concentration for maltose binding fusion protein was determined from $E^{1\%}_{280nm} = 12.8$ (*23*). Subunit molecular masses of 46.6 and 90 kDa (*3*, *23*) were used to calculate the concentration of free enzyme and fusion protein subunits.

Kinetics of Wild-Type and Mutant Enzymes. For K_m determinations, the concentration of NADP, isocitrate, or Mn^{2+} was varied, and the other substrates were maintained at saturating concentrations. The K_m values were determined from Lineweaver−Burk plots, and error measurements were determined from analysis of Excel plots. The pH dependence of V_{max} for the reaction catalyzed by the wild-type and mutant enzymes was determined over the range of pH 5−8, using the buffers previously described (*17*). The reaction rates were measured using 4 mM isocitrate, 2 mM $MnSO_4$, and 0.1 mM NADP for all the enzymes except H315Q, for which 1 mM NADP was used for the rate determination.

Binding Experiments. The enzymes were dialyzed against 50 mM triethanolamine hydrochloride at pH 7.7 containing

[1] Abbreviations: PCR, polymerase chain reaction; CD, circular dichroism; FPLC, fast-performance liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; EDTA, disodium ethylenediaminetetraacetate; IPTG, isopropyl thiogalactoside; kbp, kilobase pair; *E. coli*, *Escherichia coli*; *B. subtilis*, *Bacillus subtilis*.

10% glycerol and 150 mM Na_2SO_4 for 4 h before the binding experiments were conducted. To measure the stoichiometry of binding of NADPH, 0.5 mL of wild-type or mutant enzyme (11−16 μM) was mixed with 0.5 mL of NADPH solution (46−54 μM) and placed in a Centricon tube (Amicon) (3 mL). The mixed solution was centrifuged at 5000 rpm for 5 min, and the initial filtrate (about 200 μL) was removed. The residual mixture was centrifuged for another 30 min, and about 500 μL of filtrate was collected for NADPH measurement. The total concentration and the concentration of free NADPH in the filtrate were measured spectrophotometrically at 259 and 340 nm by using extinction coefficients of 14900 and 6200 M^{-1} cm^{-1}, respectively. The concentration of bound nucleotide was calculated from the difference between the total and free ligand concentrations.

To measure the stoichiometry of binding of isocitrate, 0.5 mL of wild-type or mutant enzyme (13−17 μM) was mixed with 0.5 mL of D-isocitrate (80−90 μM) containing 4 mM $MnSO_4$ and placed in a Centricon tube (3 mL). The mixture was then centrifuged at 5000 rpm for 5 min. The initial filtrate was removed, the residual mixture was further centrifuged for 30 min, and about 500 μL of filtrate was collected. The final filtrate (0.5 mL) was mixed with 0.5 mL of buffer containing 30 mM triethanolamine hydrochloride, 2 mM $MnSO_4$, and 200 μM NADP (pH 7.4) as the assay buffer. Five microliters of 0.1 mg/mL wild-type isocitrate dehydrogenase was used to determine enzymatically the concentration of isocitrate. The measurements were performed spectrophotometrically at 25 °C and 340 nm. The initial concentration and the concentration of free isocitrate in the final filtrate were determined from the maximum absorbance at 340 nm divided by the extinction coefficient of 6200 M^{-1} cm^{-1}. The concentration of bound isocitrate was calculated from the difference between the total and free isocitrate concentration.

Circular Dichroism of the Wild-Type and Mutant Enzymes. CD spectra were measured at room temperature on a Jasco Model J-710 recording spectropolarimeter (Jasco, Inc., Easton, MD). Measurements of the ellipticity as a function of wavelength were made as described previously (*17*), using 413 as the number of amino acids per subunit of free NADP-dependent isocitrate dehydrogenase and 803 per subunit for the fusion protein (*3*, *23*).

RESULTS

Expression and Purification of Wild-Type and Mutant Enzymes. The pig heart mitochondrial NADP-dependent isocitrate dehydrogenase mutants, with glutamine substituted for histidine at positions 309, 315, and 319 and phenylalanine substituted for His[309], were generated using the expression vector pMALcIDP1 and the megaprimer PCR method (*17*−*19*, *24*). The wild-type and mutant enzymes were expressed in *E. coli* as the maltose binding fusion proteins and purified by chromatography on an amylose column and gel filtration on Sephadex G-150. The gel patterns observed upon SDS−polyacrylamide gel electrophoresis indicate that the maltose binding fusion proteins of wild type, H319Q, and H315Q were expressed well, were more than 95% pure, and have similar subunit molecular masses (90 kDa). The wild-type, H319Q, and H315Q enzymes, after cleavage by thrombin and further purification by chromatography on DEAE-cellulose, were homogeneous and have the same subunit molecular masses (46.6 kDa) (Figure 2). The H309F enzyme is shown in Figure 5, lane 1. The sequences of the pig heart and *E. coli* isocitrate dehydrogenases differ in nine of the first ten amino acids. The N-terminal amino acid sequencing of these purified wild-type and mutant enzymes showed that they are pure and not contaminated with the *E. coli* enzyme.

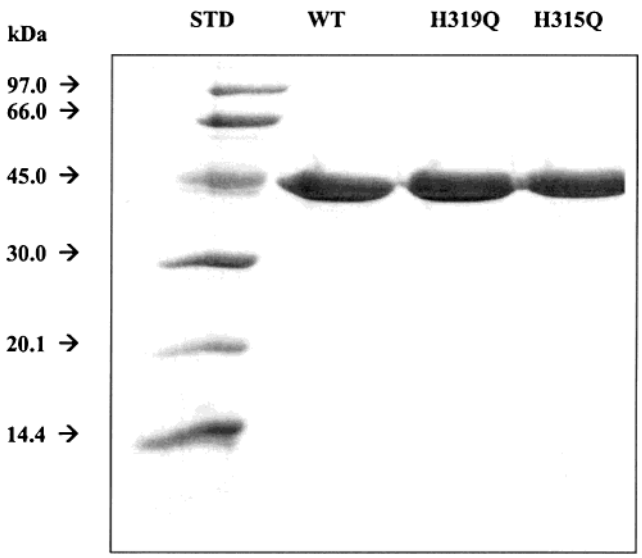

FIGURE 2: SDS−PAGE of purified wild-type and mutant enzymes: wild type (WT), His[319] mutant (H319Q), His[315] mutant (H315Q), and protein standards (STD) phosphorylase *b* (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Kinetic Characteristics of Wild-Type and Mutant Enzymes. The kinetic parameters of wild-type and mutant enzymes are summarized in Table 1. The specific activity of the H315Q mutant (measured at the "standard" assay concentration of 0.1 mM NADP) is greatly decreased to 4.1 μmol min^{-1} mg^{-1}, as compared to that of the wild-type enzyme (37.8 μmol min^{-1} mg^{-1}), while the specific activity of the H319Q mutant is 20.1 μmol min^{-1} mg^{-1}. Neither the H309Q nor H309F mutant shows any detectable activity.

The H319Q mutant has a K_m value for NADP similar to that of the wild type (5.59 μM), while the H315Q mutant displays a 40-fold increase in K_m for NADP as compared to the wild type. These results indicate that His[315] of pig heart NADP-dependent isocitrate dehydrogenase is in the region of the NADP binding site. When V_{max} is measured at saturating concentrations of coenzyme as well as substrates, the V_{max} values of the H315Q and H319Q mutants are about half that of the wild-type enzyme (Table 1, last column).

For the H315Q mutant, the K_m values for Mn^{2+} and isocitrate were determined using the saturating NADP concentration of 1 mM instead of the normal concentration of 0.1 mM NADP (standard conditions). Table 1 illustrates that the H315Q and H319Q mutants exhibit K_m values for Mn^{2+} and isocitrate similar to those of wild type measured under the same conditions. These results suggest that the H315Q and H319Q mutants are not involved in metal or isocitrate binding.

NAD is known to be a poor coenzyme for the pig mitochondrial NADP-dependent isocitrate dehydrogenase, with V_{max} only 2% of that observed with NADP (*6*). However, NAD acts as a competitive inhibitor with respect

Table 1: Kinetic Parameters for Wild-Type and Mutant NADP-Dependent Isocitrate Dehydrogenases

enzyme	specific activity[a] (μmol min^{-1} mg^{-1})	K_m(NADP)[b] (μM)	K_m(Mn^{2+})[b] (μM)	K_m(Mn^{2+})[c] (μM)	K_m(DL-isocitrate)[b] (μM)	K_m(DL-isocitrate)[c] (μM)	V_{max}[e] (μmol min^{-1} mg^{-1})
wild type	37.8 ± 4.65	5.59 ± 0.42	0.33 ± 0.02	2.6 ± 0.31	8.37 ± 0.96	8.83 ± 0.62	37.8 ± 4.65
H315Q	4.1 ± 0.89	218 ± 5.66		2.7 ± 0.42		8.67 ± 0.62	16.2 ± 0.89
H319Q	20.1 ± 1.50	5.13 ± 0.42	0.39 ± 0.01		8.25 ± 0.75		20.1 ± 1.50
H309Q	<0.0003	—[d]	—[d]	—[d]	—[d]	—[d]	<0.0003
H309F	<0.0003	—[d]	—[d]	—[d]	—[d]	—[d]	<0.0003

[a] The specific activities were measured in 30 mM triethanolamine hydrochloride, pH 7.4, containing 4 mM DL-isocitrate, 2 mM Mn^{2+}, and 0.1 mM NADP under the standard assay conditions described under Experimental Procedures. [b] The K_m values for Mn^{2+}, isocitrate, and NADP were determined at pH 7.4 by varying the concentrations of Mn^{2+}, isocitrate, or NADP while maintaining the other substrates or coenzyme at the standard concentrations. The data were analyzed by Lineweaver−Burk plots. [c] NADP = 1.0 mM. [d] The K_m values for H309 mutants could not be determined because the enzyme activities were too low. [e] V_{max} values were measured by maintaining the substrates at saturating concentrations for each enzyme (wild type and mutant).

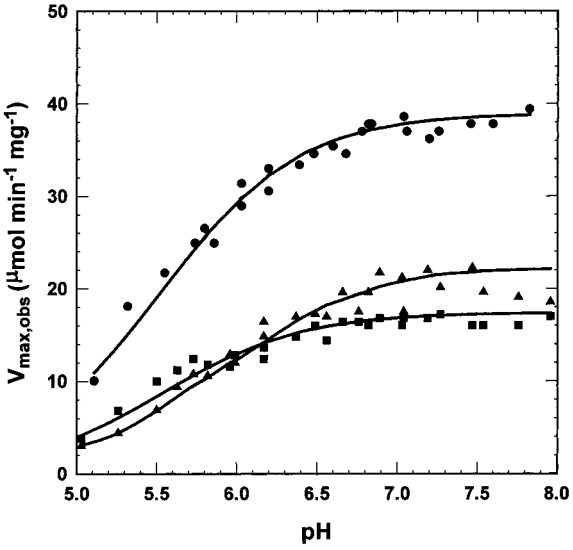

FIGURE 3: pH−V_{max} profiles for the wild-type and mutant isocitrate dehydrogenases. The activites in different pH buffers were measured for wild-type (●), H319Q (▲), and H315Q (■) enzymes, as described in Experimental Procedures.

to NADP when the two coenzymes are added together (*6*). We have now compared NAD as a competitive inhibitor of the H315Q mutant and wild-type enzymes. The K_i values for NAD are similar for the two enzymes: 18.2 mM for H315Q and 17.8 mM for the wild type. Since the K_m for NADP increases 40-fold in the H315Q enzyme, while the K_i value for NAD does not change, it is likely that His^{315} is located in the vicinity of the 2′-phosphate that is unique for NADP.

pH Dependence of V_{max}. It has previously been shown for the NADP-dependent enzyme isolated from pig hearts (*25*) and for the recombinant enzyme (*17*, *18*) that V_{max} depends on the basic form of an ionizable group of the enzyme−substrate complex which has a p*K* of about 5.5. We have now compared the pH dependence of V_{max} for the wild-type, H315Q and H319Q enzymes to evaluate whether there were any effects on the important ionizable group of the enzyme caused by these mutations. Figure 3 shows the plots of $V_{max,obs}$ (μmol min^{-1} mg^{-1}) against pH for the wild-type and mutant enzymes. Measurements were made using 2 mM Mn^{2+}, 4 mM isocitrate, and 0.1 mM NADP for the wild-type and H319Q mutant. For H315Q, the higher concentration of NADP (1 mM) was used for measuring the V_{max} in order to ensure saturation of the enzyme with coenzyme. Over the pH range 5−8, the pH−rate profiles for H315Q and H319Q are similar to that of wild type. The data of these graphs were analyzed by the equation $V_{max,obs} = V_{max,int}/(1 + [H^+]/K_a)$, where $V_{max,obs}$ is the maximum velocity observed at a given pH, $V_{max,int}$ is the intrinsic pH-independent maximum velocity, and K_a is the dissociation constant for the ionizable group of the enzyme−substrate complex which was analyzed by a double-reciprocal plot of $1/V_{max,obs}$ versus $[H^+]$. The pK_{aes} values were 5.52, 5.49, and 5.54 for the wild-type, H315Q, and H319Q mutant enzymes, respectively. These results indicate that the V_{max} of the wild-type and mutant enzymes depends on the ionized form of an enzymic group of p*K* 5.5. This ionizable enzymic amino acid is not His^{315} or His^{319}, and replacing either of these two histidines does not perturb the p*K* of the ionizable amino acid.

Ability of Wild-Type and Mutant Enzymes To Bind NADPH and Isocitrate. Since the His^{309} mutants had no detectable activity, kinetics could not be used to evaluate whether their substrate and/or coenzyme sites were disrupted. The ability of mutant and wild-type enzymes to *bind* NADPH or isocitrate was tested by using ultrafiltration to separate the free NADPH or free isocitrate from the enzyme-bound ligands with which they are in equilibrium. Since the total concentrations of NADPH or isocitrate were high relative to the total enzyme concentrations and to the dissociation constants for wild-type enzyme−NADPH and enzyme−isocitrate, the stoichiometry of ligand binding could be assessed, as we have described previously (*4*, *26*). The total and free ligand concentrations were measured, and the bound ligand was calculated from the difference between the total and free ligand concentration, as described in Experimental Procedures. Table 2A shows that a stoichiometry of about 1 mol of isocitrate bound/mol of enzyme subunit was obtained for the wild-type, H315Q, and H319Q enzymes. Mutant enzymes with replacement of His^{309} gave good yields of the fusion of maltose binding protein and isocitrate dehydrogenase. However, after cleavage by thrombin, the His^{309} mutants were obtained in low yield. Therefore, the His^{309} mutant enzymes were studied as the fusion proteins and compared with wild-type fusion proteins. Table 2B exhibits similar results for isocitrate binding by the fusion protein of wild-type and H309 mutant isocitrate dehydrogenases: 1 mol of substrate is bound per mole of protein subunit; 1 mol of NADPH is bound per mole of enzyme subunit by wild-type and H319Q enzymes. In contrast, less than 0.25 mol of NADPH/mol of enzyme subunit was bound by the H315Q mutant under the same conditions (Table 2A), reflecting the poor affinity of this mutant enzyme for coenzyme (K_m for

Table 2: Stoichiometry of Binding of Isocitrate and NADPH by Wild-Type and Mutant NADP-Dependent Isocitrate Dehydrogenases[a]

enzyme	[bound D_s-isocitrate]/[enzyme subunit]	[bound NADPH]/[enzyme subunit]
(A) Isocitrate Dehydrogenase Alone[b]		
wild type	0.98	1.00
H319Q	0.98	1.00
H315Q	0.99	0.24
(B) Maltose Binding Protein−Isocitrate Dehydrogenase Fusion[c]		
wild type	0.91	0.93
H309F	0.90	0.13
H309Q	1.00	0.22

[a] The ultrafiltration experiments were performed at 25 °C in 50 mM triethanolamine hydrochloride buffer, pH 7.7, containing 10% glycerol and 150 mM Na_2SO_4, as described in Experimental Procedures. The concentrations used were as follows: enzyme, 11−16 μM; NADPH, 46−54 μM; and isocitrate, 80−90 μM. For the experiments in which the isocitrate stoichiometry was measured, 2 mM $MnSO_4$ was also present. [b] Homogeneous isocitrate dehydrogenase, obtained after cleavage of the fusion protein by thrombin followed by purification. [c] Purified fusion protein of maltose binding protein and isocitrate dehydrogenase.

NADP = 218 μM). Similar results were obtained for both His[309] mutant fusion proteins: less than 0.25 mol of NADPH/mol of mutant fusion protein subunit was observed, in contrast to about 1 mol of NADPH/mol of wild-type fusion protein (Table 2B). These results indicate that His[315] and His[309] are involved in the binding of coenzyme, since replacement of either residue yields enzyme which binds NADP poorly. In contrast, His[319], His[315], and His[309] do not contribute to the binding of isocitrate, since mutant enzyme with substitution for any one of these histidines binds isocitrate stoichiometrically.

Affinity Cleavage of Pig Heart NADP-Dependent Isocitrate Dehydrogenase by Fe^{2+}-Isocitrate. Previous affinity cleavage studies of pig heart NADP-dependent isocitrate dehydrogenase showed that, in the presence of O_2, Fe^{2+} -isocitrate yields two major pairs of peptides on inactivation: 35 + 11 and 30 + 17 kDa (*11*). These peptides were shown to result from mutually exclusive cleavage between His[309]-Gly[310] or Asp[253]-Met[254] as coordination sites for Fe^{2+}-isocitrate (*11*). Since the affinity cleavage by Fe^{2+}-isocitrate occurs normally in the D253N mutant enzyme (*17*), it was concluded that Asp[253] is not the important target for Fe^{2+}-isocitrate. To locate the primary metal isocitrate site, we tested the H315Q, H319Q, and H309F mutant enzymes for affinity cleavage by Fe^{2+}-isocitrate. Figure 4 shows the affinity cleavage of wild-type, H319Q, and H315Q mutants in the presence of Fe^{2+}-isocitrate, as visualized by electrophoresis on polyacrylamide gels containing SDS. The affinity cleavage patterns are the same for all three enzymes: the two major bands seen are ~35 and 30−31 kDa with smaller bands consistent with the 11 and 17 kDa reported previously (*11*). Therefore, His[315] and His[319] of NADP-dependent isocitrate dehydrogenase are *not* responsible for the Fe^{2+}-isocitrate cleavage.

Figure 5 shows the results of experiments in which the H309F mutant enzyme was incubated in the presence of Fe^{2+} alone or Fe^{2+}-isocitrate. For the wild-type enzyme after incubation of enzyme for 3 h in the presence of Fe^{2+} alone, one pair of predominant fragments is observed (32 and 15 kDa), in addition to the intact enzyme (*11*). After incubation of enzyme in the presence of Fe^{2+}-isocitrate, 35 and 30 kDa

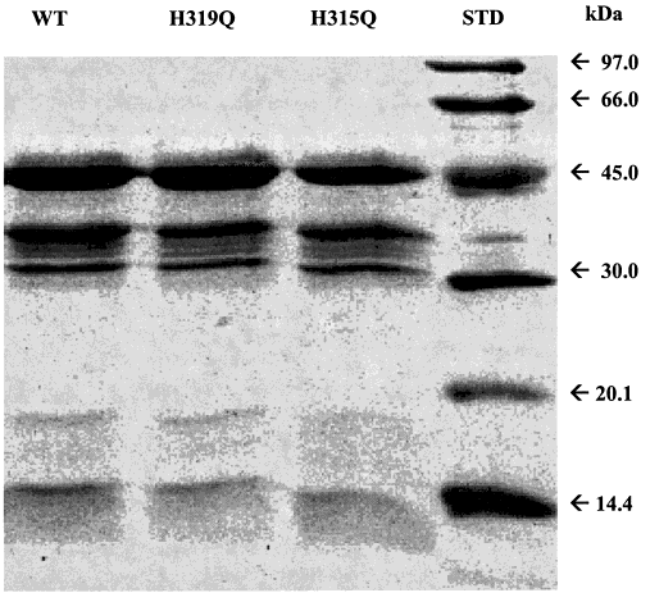

FIGURE 4: SDS−PAGE of wild-type, H319Q, and H315Q isocitrate dehydrogenases after inactivation by Fe^{2+}-isocitrate. Enzyme was incubated with ascorbate (20 mM) at 25 °C for 3 h in the presence of 20 μM $FeSO_4$ plus 4 mM isocitrate. Wild type (WT), His[319] mutant (H319Q), His[315] mutant (H315Q), and protein standards (STD) are shown. Electrophoresis was conducted as described in Experimental Procedures.

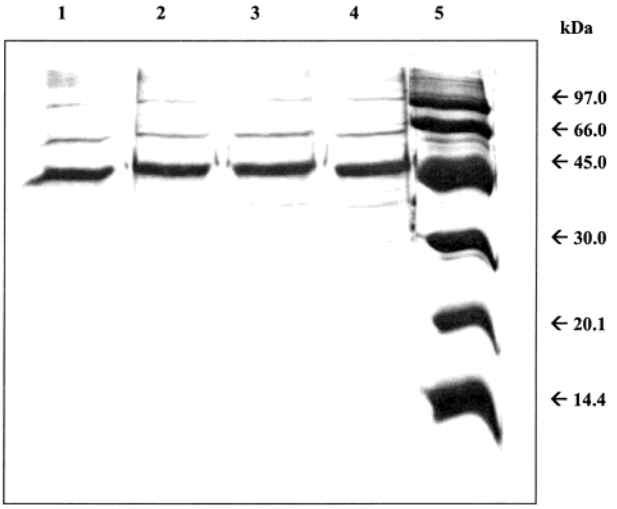

FIGURE 5: SDS−PAGE of the H309F mutant enzyme after inactivation by Fe^{2+} or Fe^{2+}-isocitrate. Enzyme (~10 μg) was incubated at 25 °C for 3 h with the following additions: lane 1, none; lane 2, 20 mM ascorbate; lane 3, 20 μM $FeSO_4$; lane 4, 20 μM $FeSO_4$ plus 4 mM isocitrate. Protein standards are shown in lane 5.

bands are the major distinguishable fragments (Figure 4). The affinity cleavage patterns obtained in the present study are very similar to the results for wild-type enzyme found in previous studies (*11*, *17*). In contrast, for the H309F mutant enzyme (Figure 5), no affinity cleavage is observed either in the presence of Fe^{2+} alone (lane 3) or in the presence of Fe^{2+}-isocitrate (lane 4), as compared to the controls (lanes 1 and 2). Thus, His[309] is probably responsible for Fe^{2+}-isocitrate cleavage of the wild-type enzyme in the presence of O_2, and this histidine may be a coordination site for Mn^{2+}-isocitrate.

Evaluation of Secondary Structure by Circular Dichroism. CD spectra were measured for the wild-type and mutant

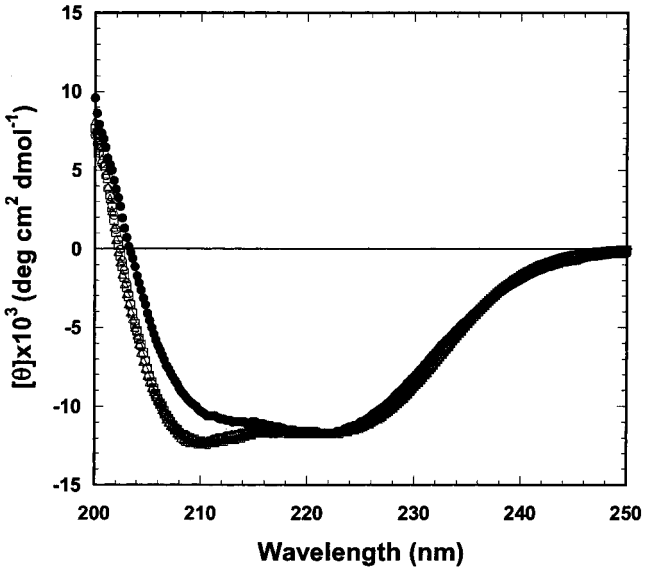

FIGURE 6: Circular dichroism (CD) spectra of the wild-type and mutant isocitrate dehydrogenases. The mean molar ellipticity [θ] $\times$ 10^3 (deg cm^2 dmol^{-1}) was calculated as described in Experimental Procedures. CD spectra of wild type (○), H319Q (□), H315Q (△), and H309F (●).

enzymes to evaluate whether changes in the secondary structure of the enzyme resulted from the mutations. Figure 6 shows the CD spectra of wild-type, H315Q, H319Q, and H309F enzymes. The spectra of the H315Q and H319Q mutants are identical to that of the wild type, showing that their secondary structures are not altered by the mutation. In contrast, the spectrum of the H309F mutant is distinguishable from that of the wild type: there is a pronounced decrease in the magnitude of ellipticity at 208 nm. The CD spectra of fusions of maltose binding protein with wild-type, H309Q, and H309F mutant isocitrate dehydrogenases were also measured. Wild type exhibits a CD spectrum typical of an α-helical protein with double minima at 208 and 222 nm. The spectra of H309F and H309Q fusion proteins are similar to each other but are significantly different from that of the wild-type fusion protein. The ellipticities of both H309 fusion proteins at 208 nm are less negative than that of the wild type, suggesting a somewhat lower α-helical content. The H309 mutants thus exhibit some change in secondary structure.

DISCUSSION

In this paper, site-directed mutagenesis has been used to evaluate the role of three histidine residues (His^{309}, His^{315}, and His^{319}) in the function of porcine NADP-dependent isocitrate dehydrogenase. Glutamine was chosen to replace histidine because it is similar in size and shape but lacks the general acid/base characteristics of His. Phenylalanine was selected as a replacement for histidine because it has a planar aromatic ring, which approximates one of the properties of the imidazole of histidine and might be expected to mimic some of the structural contributions of the histidine. Replacement of His^{319} does not affect the K_m for NADP and causes relatively little change in V_{max}; these results indicate that His^{319} is not involved in coenzyme binding and has a minimal effect on catalysis.

His^{309} is conserved in all species of NADP-dependent isocitrate dehydrogenase (Figure 1). H309Q and H309F mutants exhibit no detectable activity. Both of these mutants bind isocitrate normally (1 mol/mol of enzyme subunit) but bind coenzyme poorly. These results suggest that His^{309} contributes to the binding of coenzyme. His^{309} of the pig heart enzyme is equivalent in sequence to His^{339} of the *E. coli* enzyme and to His^{345}of *B. subtilis*. In the crystalline *E. coli* enzyme−NADP−Ca-isocitrate complex (PDB structure 1AI2), His^{339} is located near the adenine of the coenzyme where it is 2−3 Å from N9 and N6. In *B. subtilis* NADP-dependent isocitrate dehydrogenase, although the enzyme is a homodimer, the two crystallographic subunits are not structurally identical. In monomer A, the His^{345} residue adopts two alternate conformations: "flipping" toward and away from the bound citrate (a stand-in for isocitrate). In monomer B, His^{345} matches the orientation of the homologous His^{339} in the *E. coli* enzyme (*13*).

Previous affinity cleavage studies of the wild-type pig heart enzyme showed that Fe^{2+}-isocitrate in the presence of O_2 causes inactivation and mutually exclusive cleavages between Asp^{253}-Met^{254} or His^{309}-Gly^{310}, suggesting that Asp^{253} or His^{309} is a coordination site for Fe^{2+}-isocitrate (*11*). We later showed that Fe^{2+}-isocitrate produces inactivation and affinity cleavage in the D253N mutant (*17*), implying that Asp^{253} is not the important target of Fe^{2+}-isocitrate responsible for affinity inactivation and cleavage. In the present study, we have shown that the H309F mutant enzyme does *not* exhibit affinity cleavage in the presence of either Fe^{2+} alone or Fe^{2+}-isocitrate. Thus, His^{309} appears to be the critical target for Fe^{2+}-isocitrate cleavage and may be a coordination site for Mn^{2+}-isocitrate. Our present results on replacement of His^{309} of the pig heart NADP-dependent isocitrate dehydrogenase indicate that His^{309} normally plays a role both in binding coenzyme and in coordinating metal ion in the metal isocitrate site. Thus, the His^{309} of the pig NADP−isocitrate dehydrogenase may behave more like the *B. subtilis* His^{345} than the *E. coli* His^{339}: it may exist in alternate conformations to fulfill its two functions.

CD spectra were determined for wild-type and mutant enzymes to evaluate whether any mutations cause disruption in the secondary structural change in the enzyme. The results indicate that replacement of His^{315} or His ^{319} does not result in conformational change in the enzyme. In contrast, mutation at His^{309} causes detectable change in the secondary structure: the ellipticity at 208 nm is less negative than that of wild type, suggesting a lower α-helical content. On the basis of these findings, we postulate that His^{309} has multiple roles: it contributes to the binding of coenzyme, it interacts with metal in the presence of isocitrate, and it maintains the normal conformation of the enzyme. Although one might postulate that the loss of catalytic activity is an indirect result of the altered conformation, we consider that the complete conservation of His^{309} among isocitrate dehydrogenases of all species, as well as the proximity to the coenzyme of the equivalent histidine in the crystalline bacterial enzymes, argues for a direct role in the porcine enzyme of this residue in NADP binding.

In contrast to the case of the H309 mutants, the H315Q mutant exhibits a single major aberration: it has a greatly increased K_m value for NADP as compared to the wild-type enzyme, with a V_{max} that is only about half that of the wild-

type enzyme. In addition, the H315Q mutant exhibits decreased binding of NADPH (0.24 mol/mol of enzyme subunit) compared to wild type under the same conditions (1 mol/mol of subunit) but no change in K_m values for Mn^{2+} and isocitrate or in binding of isocitrate (1 mol/mol of subunit). His[315] is conserved in all eukaryotic NADP-specific isocitrate dehydrogenases (Figure 1). These results suggest that His[315] contributes to the binding of coenzyme in eukaryotes. The H315Q mutant exhibits no change in the pH dependence of V_{max} as compared to wild type and has the same pK_a (~5.5) for an ionizable group of the enzyme−substrate complex. This ionizable enzymic amino acid is not His[315]. ^{31}P NMR studies suggested that the 2′-phosphate groups of $NADP^+$, NADPH, and 2′-phosphoadenosine 5′-diphosphoribose are bound to isocitrate dehydrogenase in a similar environment, presumably to the same site on the enzyme, and exist in the dianionic form (*27*). The constant value of the ^{31}P chemical shift within the pH range 5.5−8.0 can be explained by a decrease in p*K* from that characteristic of a 2′-phosphate in free NADP/NADPH. Such a decrease in p*K* was attributed to proximity to positively charged amino acid residue(s) within the nucleotide binding site (*27*); however, it could also be due to hydrogen bonding (to His[315]), stabilizing the dianionic form of the phosphate.

NAD competes (albeit with weak affinity) with NADP in binding to the coenzyme site. NAD lacks the 2′-phosphate characteristic of NADP and responsible for its strong affinity for isocitrate dehydrogenase. In the H315Q mutant, the K_i for NAD does not change, while the K_m for NADP increases 40-fold as compared to the wild-type enzyme. The difference in the effect of replacing His[315] on the affinity of the enzyme for NAD and NADP suggests that His[315] interacts with the 2′-phosphate of NADP. Mutagenesis of Arg[314] and Tyr[316] of pig heart NADP−isocitrate dehydrogenase also produces increases in the K_m for NADP without corresponding changes in K_i for NAD (*28*). Thus, the three residues, Arg[314]-His[315]-Tyr[316], may all be located near or interact with the 2′-phosphate of NADP and contribute to the coenzyme specificity of this isocitrate dehydrogenase.

The crystal structure of the Mn-isocitrate complex of the pig mitochondrial isocitrate dehydrogenase has recently been determined using the MAD phasing method with selenium in the form of selenomethionine as the anomalous scatterer, and the structure is now being refined.[2] However, suitable crystals for structure determination of the coenzyme complex of the enzyme have not yet been obtained. The results of our mutagenesis experiments indicate that His[315] contributes to the enzyme's affinity for NADP and His[309] is a determinant of the affinity for coenzyme, influences the enzyme's interaction with divalent metal ion, and contributes to the normal conformation of pig mitochondrial NADP-dependent isocitrate dehydrogenase.

[2] C. Ceccarelli, N. B. Grodsky, N. Ariyaratne, R. F. Colman, and B. Bahnson, manuscript in preparation.

ACKNOWLEDGMENT

The authors thank Dr. Sambanthamurthy Soundar for supplying the H309Q mutant and Dr. Robert S. Ehrlich for helpful discussions.

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BI0200716