Identification by Mutagenesis of Arginines in the Substrate Binding Site of the Porcine NADP-dependent Isocitrate Dehydrogenase*

(SReceived for publication, October 1, 1999, and in revised form, December 3, 1999)

Sambanthamurthy Soundar, Brenda L. Danek, and Roberta F. Colman‡
From the Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Pig heart mitochondrial NADP-dependent isocitrate dehydrogenase is the most extensively studied among the mammalian isocitrate dehydrogenases. The crystal structure of Escherichia coli isocitrate dehydrogenase and sequence alignment of porcine with E. coli isocitrate dehydrogenase suggests that the porcine Arg101, Arg110, Arg120, and Arg133 are candidates for roles in substrate binding. The four arginines were separately mutated to glutamine using a polymerase chain reaction method. Wild type and mutant enzymes were each expressed in E. coli, isolated as maltose binding fusion proteins, then cleaved with thrombin, and purified to yield homogeneous porcine isocitrate dehydrogenase. The R120Q mutant has a specific activity, as well as Km values for isocitrate, Mn2+, and NADP+ similar to wild type enzyme, indicating that Arg120 is not needed for function. The specific activities of R101Q, R110Q, and R133Q are 1.73, 1.30, and 19.7 μmol/min/mg, respectively, as compared with 39.6 units/mg for wild type enzyme. The Km values for Mn2+, but not for NADP+, are also elevated indicating that binding of the metal-isocitrate complex is impaired in these mutants. It is proposed that the positive charges of Arg110 and Arg133 normally strengthen the binding of the negatively charged isocitrate by electrostatic attraction. The R101Q mutant shows smaller, but significant increases in the Km values for isocitrate and Mn2+; however, the marked decrease in kcat suggests a role for Arg101 in catalysis. The Vmax of wild type enzyme depends on the ionized form of an enzymic group of pK 5.5, and this pK is similar for the R101Q and R120Q enzymes. In contrast, the pK is elevated for R110Q and R133Q enzymes increases to 6.4 and 7.4, respectively, indicating that the positive charges of Arg110 and Arg133 lower the pK of the nearby catalytic base to facilitate its ionization. These results may be understood in terms of the structure of the porcine NADP-specific isocitrate dehydrogenase generated by the Insight II Modeler Program, based on the x-ray coordinates of the E. coli enzyme.

The mitochondrial NADP-specific pig heart isocitrate dehydrogenase (EC 1.1.1.42) catalyzes the divergent metal ion-dependent oxidative decarboxylation of isocitrate to α-ketoglutarate, and it is considered that the metal-tribasic isocitrate complex is the preferred substrate (1). The enzyme is a homodimer (2, 3), with a subunit mass of 46,600 Da consisting of 413 amino acids of determined sequence (4). A 13C-NMR study using specifically enriched isocitrate demonstrated that all three carboxyls of the substrate remain fully ionized from pH 5.5 to 7.5 when bound to the enzyme, although the carboxylates of free isocitrate become protonated over this pH range (5). This result could be due to the presence of positively charged groups in the region of the substrate binding site. The first evidence of the importance of arginines in the function of NADP-dependent isocitrate dehydrogenase came from the inactivation of the pig heart enzyme by 2,3-butanedione (6). A maximum of four arginines were implicated in catalytic activity and, because isocitrate markedly decreased the inactivation rate, it was suggested that at least some of these residues were at or near the isocitrate binding site.

Whereas no crystal structure of a mammalian isocitrate dehydrogenase has yet been determined, the structure of the Escherichia coli NADP-dependent isocitrate dehydrogenase is known (7–9). In this bacterial enzyme, Arg119, Arg129, and Arg153 interact with the carboxylates of isocitrate, and Ser113 is also close to the substrate (8). Alignment of the amino acid sequence of E. coli isocitrate dehydrogenase with that of the porcine enzyme reveals only about 12% identity (plus about 34% similarity). However, there are conserved amino acids among those known to interact with the substrate in the E. coli enzyme. Fig. 1 shows the amino acid sequence alignment in this region between three eukaryotic enzymes (pig, rat, and yeast) and the E. coli enzyme. In the porcine enzyme, Arg110 and Arg133 line up, respectively, with the E. coli Arg119 and Arg153. Arg101 of the pig isocitrate dehydrogenase is aligned with E. coli’s Arg112, which is close to Ser113. There some uncertainty in the alignment in the middle of the region illustrated; however, Arg120 is the only such residue in this portion of the porcine enzyme and may be comparable to Arg132 or Arg129 of the E. coli enzyme.

In this paper, to test the roles of the positively charged Arg101, Arg110, Arg120, and Arg133 as determinants of isocitrate affinity and catalysis by porcine dehydrogenase, each of these arginines was mutated to the neutral glutamine. Here, we report the procedures for mutagenesis, expression, and purification of the mutant enzymes, as well as the characterization of the physical and kinetic properties of these enzymes. A preliminary version of this work has been presented (10).

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were synthesized by the Operon Technologies, Inc. (Alameda, CA). Plasmid pMal-c2, E. coli strain TB1, T4 DNA ligase, and amylase resin were obtained from New England Biolabs (Beverly, MA). The restriction enzymes, BamHI and Bsp1102I, as well as polymerase K were purchased from Life Technologies, Inc. Pfu DNA polymerase was obtained from Stratagene (La Jolla, CA). Dye-conjugated DNA primers were synthesized by LI-COR, Inc. (Lincoln, NE). Calf intestinal alkaline phosphatase, deoxynucleotid triphosphates, and Wizard Plus Maxi and Miniprep DNA purification systems

* This work was supported by United States Public Health Service Grants DK 39075 and T32 GM 08550 (to B. L. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ To whom correspondence should be addressed: Tel.: (302)831-2973; Fax: (302)831-6335; E-mail: rfcolman@udel.edu.

© 2000 by The American Society for Biochemistry and Molecular Biology, Inc.
Arg in Isocitrate Site of Pig NADP-Isocitrate Dehydrogenase

The abbreviations used are: kbp, kilobase pair(s); PCR, polymerase chain reaction.

1 The abbreviations used are: kbp, kilobase pair(s); PCR, polymerase chain reaction.
zymes. A small number of colonies resulted from self-ligated DNA. However, the ligation was not successful, and the restriction enzyme DNA was gel purified, it did not initially remove the overhang and was gel purified, it did not initially work. The ligation was performed at 25°C by monitoring the time-dependent reduction of NADP⁺ from the UV absorbance of NADPH at 340 nm. The standard assay solution (1 ml) was 30 mM triethanolamine chloride buffer (pH 7.4), 0.1 mM NADP⁺, 4 mM DL-isocitrate, and 2 mM MnSO₄, and specific activity was defined as the \( \frac{\text{d} \text{mol of NADPH produced}}{\text{d} \text{mg of protein}} \). The concentration of either coenzyme, isocitrate or Mn²⁺, was determined from \( E_{280} \) nm absorbance. 2 Although it appears that the mobility of the R120Q mutant is slightly retarded relative to the other samples, this difference is not considered significant because it was observed only in this gel and is probably due to uneven loading of this sample.

RESULTS

Expression and Purification of Wild Type and Mutant Enzymes—The porcine NADP-dependent isocitrate dehydrogenase, with glutamine substituted for arginine at each of the positions 101, 110, 120, and 133, were generated using expression vector pMALcIDP1 by a megagal PCR method (3). The first and second PCR runs yielded approximately 0.86 and 1.68 kb DNA, respectively. Although the 1.68-kb DNA was incubated with the BamHI and Bpu1102I restriction enzymes to remove the overhang and was gel purified, it did not initially ligate with the 6.22-kb vector DNA. However, the ligation was successful if the 1.68-kb DNA had been treated with proteinase K and SDS prior to incubation with the restriction enzymes. A small number of colonies resulted from self-ligated vector DNA on transformation, even though the vector DNA had been treated with alkaline phosphatase. Such colonies were avoided, and colonies that yielded plasmid DNA, which produced a 1.66-kb insert DNA upon BamHI/Bpu1102I digestion, were selected for expression. Nucleotide sequence analysis showed that the mutations had been introduced in all of the mutant plasmid DNAs and that the sequences of the PCR-amplified DNA were correct.

The cells were grown in 8 liters of culture medium, and the isopropyl thio-β-D-galactopyranoside-induced expression time was increased from 4 h (12) to 20–24 h to improve the level of expression of the fusion protein. The sonication of cells in one pulse for 10 min yielded more soluble fusion protein than obtained previously (12). The time for incubation of the fusion protein with thrombin was also increased from 18 to 48 h. Under these conditions only trace amounts of uncleaved fusion protein remained. This procedure yielded approximately 15 mg of pure protein as compared with the 1.2 mg obtained by the procedure reported previously (12).

Fig. 2 shows that the wild type and mutant enzymes eluted after the second amylose column were homogeneous, as evaluated by polyacrylamide gels containing sodium dodecyl sulfate. The subunit molecular size of the four mutant proteins is similar to that of wild type enzyme. 2 The sequences of the porcine and E. coli isocitrate dehydrogenases differ in nine of the first ten amino acids. The N-terminal sequencing of these purified wild type and mutant porcine enzymes indicated that they are not contaminated with the E. coli enzyme.

Gel Filtration of the Wild Type and Mutant Enzymes—To test the molecular size of the native wild type and mutant enzymes, they were subjected to gel filtration on a Superose-12 column, equilibrated with 0.1 M triethanolamine chloride, pH 7.7, containing 10% glycerol and 0.3 M Na₂SO₄. The wild type and mutant enzymes all elute between aldolase and bovine serum albumin with an average elution volume of 10.8 ml. The results indicate that wild type and mutant isocitrate dehydrogenases exist as dimers in solution. The molecular weight of the isocitrate dehydrogenases was determined using a plot of...
Mn$^{2+}$ that of wild type, and it has a 18-fold. The R110Q and R133Q mutants exhibit increased 19.7 240

Wild Type 39.6 0.21 ± 0.02 5 ± 0.19
R101Q 1.73 62 ± 5.54 5 ± 0.48
R120Q 1.30 20 ± 1.46 18 ± 0.96
R133Q 34.9 0.50 ± 0.02 6 ± 0.26

Table I

| Enzyme       | Specific activity | $K_m$-Mn$^{2+}$ | $K_m$-NADP$^+$ |
|--------------|------------------|----------------|----------------|
|   Wild Type  |       39.6       | 0.21 ± 0.02    | 5 ± 0.19      |
| R101Q       |       1.73       | 62 ± 5.54      | 5 ± 0.48      |
| R120Q       |       1.30       | 20 ± 1.46      | 18 ± 0.96     |
| R133Q       |       34.9       | 0.50 ± 0.02    | 6 ± 0.26      |

As described under “Experimental Procedures,” the assay for specific activity was conducted in 30 mM triethanolamine chloride, pH 7.4, with 4 mM DL-isocitrate, 2 mM Mn$^{2+}$, and 0.1 mM NADP$^+$. The $K_m$ values for Mn$^{2+}$ and NADP$^+$ were determined at pH 7.4 by varying the concentration of Mn$^{2+}$ or NADP$^+$ while maintaining the other substrates at the standard concentrations. The data were analyzed by Lineweaver-Burk plots.

**Circular Dichroism Spectra of the Wild Type and Mutant Enzymes**—Circular dichroism spectra of the expressed enzymes were measured to ascertain whether these mutations cause conformational change in the enzyme. The spectra of all mutants are similar to that of the wild type enzyme; all exhibit minima at 208 and 225 nm. These results indicate that the mutations do not cause appreciable change in the secondary structure of these isocitrate dehydrogenases.

**Kinetic Parameters of the Wild Type and Mutant Enzymes**—Table I shows the specific activities of wild type and mutant enzymes under standard conditions, as described under “Experimental Procedures.” The purified wild type enzyme expressed in E. coli cells exhibits a specific activity, which is similar to that of the enzyme isolated previously from pig heart mitochondria in our laboratory (15). The R101Q and R110Q mutants showed marked decreases in specific activity, whereas the R133Q mutant is 50% as active as wild type, indicating that the functions of these three mutant enzymes are impaired. The mutant R120Q has a specific activity similar to that of wild type.

All four mutants exhibit values of $K_m$ for the coenzyme, NADP$^+$, similar to that of wild type enzyme, as shown in Table I. These results indicate that these arginines are not involved in coenzyme binding. The R120Q mutant also has a $K_m$ for Mn$^{2+}$ similar to that of wild type indicating that Arg120 is not involved in Mn$^{2+}$ binding. In contrast, the R101Q, R110Q, and R133Q mutants show $K_m$ values for Mn$^{2+}$ that are increased 300-, 100-, and 1200-fold, respectively, consistent with a role for these arginines in isocitrate-Mn$^{2+}$ binding.

Table II shows the kinetic constants for the substrate, isocitrate, wild type, and mutant enzymes. The R120Q mutant has $V_{max}$ and $K_m$ values similar to those of wild type enzyme, indicating that Arg120 is not involved in the function of the enzyme. The R101Q mutant exhibits a $k_{cat}$ value that is only 4% that of wild type, and it has a $K_m$ for isocitrate that is increased 19-fold. The R110Q and R133Q mutants exhibit $k_{cat}$ values that are, respectively, 10 and 53% that of wild type. However, they have $K_m$ values for isocitrate that are increased more than 400- and 165-fold, respectively, as compared with wild type. These data indicate that the affinity of the enzyme for isocitrate is greatly impaired by replacing Arg101 and Arg133. The last column in Table II shows the catalytic efficiency ($k_{cat}/K_m$) of mutant enzymes, compared with that of wild type enzyme. Whereas mutant R120Q is similar to that of wild type, R101Q and R133Q mutants are 1000-fold poorer in efficiency compared with that of wild type. The R110Q mutant exhibits a 10,000-fold decrease in efficiency compared with wild type, because it has both a high value for $K_m$ for isocitrate and a lower $V_{max}$.

**Dependence of Velocity on pH for Wild Type and Mutant Enzymes**—The pH dependence of $V_{max}$ was determined for the recombinant wild type and mutant enzymes from about pH 5.2 to 7.8, for comparison with the pH rate profile previously reported for the enzyme isolated from pig hearts (17-19). The enzyme has been shown to be stable over this pH range. Although the $K_m$ values for isocitrate are relatively high for some of the mutant enzymes, we have ascertained that each enzyme is saturated with respect to isocitrate over the entire pH range at the concentration of the substrate chosen for that enzyme (4 mM isocitrate for the wild type and R120Q enzymes, 8 mM isocitrate for R101Q enzyme, and 20 mM isocitrate for R110Q and R133Q enzymes).

The dependence of observed $V_{max}$ on pH was analyzed in accordance with the equation,

$$V_{max} = V_{max}/(1 + [H]^nK_{aes})$$  

where $V_{max}$ is the maximum velocity observed at a given pH, $V_{max}$ is the intrinsic pH independent maximum velocity, and $K_{aes}$ is the dissociation constant for the enzyme-substrate complex. The $pK_{aes}$ values for wild type and mutant enzymes are summarized in Table III. To allow comparisons of the shapes of the curves for the various enzymes, the activity of each mutant enzyme at each pH was expressed as a fraction of its own intrinsic maximum velocity ($V_{max}'$, $V_{max}'$). Fig. 3 shows the plots of ($V_{max}'$, $V_{max}'$) for both wild type and mutant enzymes. The points in the figure are experimental data, whereas the lines represent fits to Equation 1, for $V_{max}'$, $V_{max}'$ using the $pK_{aes}$ values in Table III. The pH-$V_{max}$ profile for the mutant R120Q is similar to that of wild type enzyme. For mutant R101Q, the p$K_{aes}$ is slightly lower than that of wild type enzyme. In contrast, both mutants R110Q and R133Q exhibit striking increases in p$K_{aes}$ to 6.43 and 7.40, respectively, as compared with 5.54 for wild type enzyme. These results suggest that the positive charges of Arg110 and Arg133 normally lower the pK of the nearby catalytic base to facilitate its ionization.

**DISCUSSION**

The dimeric bacterial and eukaryotic NADP-dependent isocitrate dehydrogenases carry out the same catalytic reaction, with similar kinetic parameters (12, 20). They also have about the same number of amino acids; yet the level of amino acid conservation is low (about 12% identity plus 34% similarity overall). Based on the sequence alignment of the mammalian and E. coli isocitrate dehydrogenases, consideration of the relatively few amino acids, which are conserved in all species, and analysis of the crystal structures of the E. coli enzyme, Arg101, Arg110, Arg132, and Arg133 were chosen for testing by mutagenesis as candidates for interaction with the carboxylates for isocitrate. Neutral glutamine was selected to replace arginine because it lacks the positive charge of the wild type amino acid but is polar and is similar in size to arginine. It is clearly desirable to change a single variable at a time in designing mutants to minimize generalized effects on the overall structure of the enzyme. In contrast, Jennings et al. (21), in constructing mutants of the rat cytoplasmic NADP-dependent isocitrate dehydrogenase, substituted the negatively charged glutamate for arginine and did not evaluate the effect of this substitution on the structure of the enzyme. If the arginine normally participates in electrostatic attraction of a carboxylate of isocitrate, then replacement of Arg by glutamate could
Arg in Isocitrate Site of Pig NADP-Isocitrate Dehydrogenase

The enzyme activity was measured under standard conditions at pH 7.4, as described under “Experimental Procedures,” except that the concentrations of isocitrate were varied. The kinetic parameters were calculated as described under “Experimental Procedures.”

### Table II

| Enzyme   | $K_m$-isocitrate ($\mu$M) | $V_{max}$ (umol/min/mg) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ $\mu$M$^{-1}$) | ($k_{cat}/K_m$)(Mutant)/($k_{cat}/K_m$)(WT) |
|----------|--------------------------|--------------------------|----------------------|----------------------------------------|------------------------------------------|
| Wild Type| 6 ± 0.43                 | 39.6 ± 0.82              | 30.7 ± 0.63          | 5.12                                   | 1.0                                      |
| R101Q    | 106 ± 2.19               | 1.73 ± 0.12              | 1.32 ± 0.09          | 1.25 × 10$^{-2}$                       | 2.4 × 10$^{-3}$                         |
| R110Q    | 2600 ± 101               | 3.98 ± 0.10              | 3.09 ± 0.08          | 1.19 × 10$^{-3}$                       | 2.3 × 10$^{-4}$                         |
| R120Q    | 9 ± 0.39                 | 34.9 ± 0.96              | 27.1 ± 0.74          | 3.01                                   | 0.59                                     |
| R133Q    | 990 ± 70.4               | 21.0 ± 0.35              | 16.3 ± 0.27          | 1.65 × 10$^{-2}$                       | 3.2 ± 10$^{-3}$                         |

$^a$ These values are higher than the specific activities given in Table I because the $V_{max}$ values given here were obtained by extrapolation to saturating concentrations of isocitrate for each mutant, whereas the “specific activity” given in Table 1 was measured under the “standard conditions,” which includes an isocitrate concentration of 4 mM. For wild type enzyme, 4 mM isocitrate is a saturating concentration, but that is not the case for the R110Q and R133Q mutants because of their high $K_m$ values for isocitrate.

### Table III

| Enzyme   | $pK_{max}$  |
|----------|-------------|
| Wild Type| 5.54 ± 0.02 |
| R101Q    | 5.20 ± 0.02 |
| R110Q    | 6.43 ± 0.04 |
| R120Q    | 5.60 ± 0.01 |
| R133Q    | 7.40 ± 0.05 |

### Fig. 3

$pH$-$V_{max}$ profiles for the wild type and mutant isocitrate dehydrogenases. For each enzyme, every $pH$ is divided by its own intrinsic $pH$-independent maximum velocity so that the shapes of the curves can readily be compared. The profiles for the wild type (○), R101Q (▲), R110Q (●), R120Q (●), and R133Q (●) are shown.

introduce a local, intense electrostatic repulsion between the enzyme and substrate, which would make it difficult to measure the altered kinetic parameters of the mutant enzymes. In fact, half of the Arg to Glu mutant rat cytoplasmic isocitrate dehydrogenases exhibited such low activity, that they were not characterized kinetically (21). In contrast, all of our arginine to glutamine mutants retained sufficient activity to allow measurement of a variety of kinetic parameters. These studies show that only the R120Q mutant is similar to the wild type enzyme in function, whereas the other mutants show impaired function either in isocitrate binding or in catalysis.

Wild type and mutant pig mitochondrial isocitrate dehydrogenases were found, in the present study, to elute at comparable positions on gel filtration fast protein liquid chromatography, indicating that they all have the normal dimeric structure. Furthermore, the circular dichroism spectra of the wild type and mutant enzymes are nearly superimposable, showing that the mutations do not cause appreciable change in the secondary structure of the enzymes.

The R120Q mutant enzyme has a specific activity, $K_m$ values for isocitrate, Mn$^{2+}$, and NADP$^+$ and $pH$-$V_{max}$ profile similar to those of wild type enzyme. These characteristics indicate that Arg$^{120}$ is not involved in either catalysis or substrate binding. The resemblance between the sequences of the mammalian and the E. coli isocitrate dehydrogenase is weak between Asp$^{125}$ and Met$^{149}$ of the E. coli enzyme (Fig. 1). Apparently, the porcine enzyme does not have an arginine equivalent to Arg$^{129}$ of the E. coli enzyme that forms hydrogen bonds and/or salt bridges to the β-carboxylate of isocitrate (8).

Both the R110Q and R133Q mutant enzymes feature striking increases in $K_m$ values for isocitrate (400- and 165-fold, respectively) and elevated $K_m$ values for Mn$^{2+}$ (100- and 1200-fold, respectively). These results indicate that positive charges of Arg$^{110}$ and Arg$^{133}$ normally stabilize the binding of Mn-isocitrate by electrostatic interaction with the negatively charged isocitrate. Conversely, elimination of the positive charge by substitution of the neutral glutamine for arginine weakens the affinity of isocitrate for the enzyme. In addition, the R110Q and R133Q mutant enzymes exhibit upward shifts of their $pH$-$V_{max}$ profiles to yield $pK_{max}$ values of 6.4 and 7.4, respectively, as compared with 5.5 for the wild type enzyme. Previous studies (17–19) have reported on the dependence of $V_{max}$ on $pH$ for the pig heart NADP-dependent isocitrate dehydrogenase. This titration curve could best be described by a requirement for catalysis of the unprotonated form of an enzymatic functional group of $pK$ about 5.6. Because the $pK$ increased when the catalytic activity was measured in 20% ethanol, a solvent of lower dielectric constant (17), but did not change when the temperature was varied from 10 to 30 °C (19), the $pK$ was attributed to ionization of a carboxyl group in the enzyme-substrate complex. Because $^{13}$C-NMR evidence indicated that enzyme-bound isocitrate remained fully ionized over this $pH$ range (5), the $pK$ of 5.6 can be attributed to an enzymatic carboxyl group. The isocitrate dehydrogenase reaction is thought to be initiated by removal of a proton from the -OH of isocitrate prior to the transfer of a hydride to NADP$^+$, a general base on the enzyme has been postulated to catalyze this proton transfer reaction. A recent mutagenesis study in which aspartic acids of the pig heart enzyme were replaced by asparagines suggests that either Asp$^{275}$ or Asp$^{279}$ functions as the catalytic base because the D275N and D279N mutants exhibited large changes in $pK_{max}$ (22). Our present results on the arginine to glutamine substitutions at positions 110 and 133 thus imply that the positive charges of Arg$^{110}$ and Arg$^{133}$ normally act to lower the $pK$ of Asp$^{275}$/Asp$^{279}$, when the positively charged arginines are substituted by neutral glutamines, the nearby Asp$^{275}$/Asp$^{279}$ becomes a weaker acid, as indicated by the increase in $pK_{max}$ of the enzymatic carboxyl group in the...
The increase in $pK_{\text{cat}}$ is greater in the R133Q than in the R110Q mutant enzyme, leading to the prediction that Arg$^{133}$ is closer than Arg$^{110}$ to the catalytic base of the enzyme.

The R101Q mutant enzyme exhibits a modest decrease in $k_{\text{cat}}$ with a modest increase in $K_m$ for isocitrate and an elevated $K_m$ for Mn$^{2+}$. The pH-$V_{\text{max}}$ profile is only slightly changed as compared with wild type enzyme. The results indicate that Arg$^{101}$ may have a role in catalysis. As shown in Fig. 1, porcine Arg$^{101}$ is aligned with E. coli Arg$^{112}$, which is next to Ser$^{113}$. The activity of E. coli isocitrate dehydrogenase is regulated by phosphorylation, with almost complete inactivation resulting from phosphorylation of Ser$^{113}$ (20, 23–25). Because replacement of Ser$^{113}$ by the negatively charged glutamate or aspartate also causes inactivation of the E. coli enzyme due to loss of the ability to bind isocitrate, it has been proposed that electrostatic repulsion and steric hindrance between the phosphoryl group and the γ-carboxylate group of isocitrate are the major causes of inactivation in the phosphorylated E. coli enzyme (24).

The pig heart enzyme is not regulated by phosphorylation; indeed in the eukaryotic enzymes, asparagine is located at the position equivalent to Ser$^{113}$ in the E. coli enzyme (Fig. 1), so these enzymes cannot be phosphorylated at that site. Nevertheless, it is not surprising that mutation of the nearby Arg$^{100}$ of rat cytosolic isocitrate dehydrogenase to the negatively charged glutamate causes inactivation (21), presumably from electrostatic repulsion of isocitrate. In our study of the porcine enzyme, we here show that even changing Arg$^{101}$ to the neutral glutamine results in a marked decrease in $k_{\text{cat}}$ (Table II), indicating that the positively charged arginine itself must be important in the overall catalytic reaction of isocitrate dehydrogenase.

Because there is not yet a three-dimensional structure for any mammalian NADP-dependent isocitrate dehydrogenase, we have generated an energy-minimized structure for the porcine NADP-specific isocitrate dehydrogenase, using the Modeller Program of Insight II, based on the sequence alignment and the X-ray coordinates of the E. coli enzyme complexed with metal isocitrate. Fig. 4 shows the portion of the model, which includes the substrate and the four arginine residues selected for mutation in this study. Arg$^{120}$ is far away from the substrate site in this model: the closest distance between this arginine’s guanido group and isocitrate is 19.5 Å to the α-carboxylate. This long distance is consistent with our experimental results that the R120Q enzyme is similar to wild type, with little change in the specific activity, $K_m$ values or $pK_{\text{cat}}$. For the rat cytosolic isocitrate dehydrogenase, even replacement of the corresponding Arg$^{119}$ by the negatively charged Glu caused no decrease in $V_{\text{max}}$ although there is about a 15-fold increase in the $K_m$ for isocitrate, probably due to electrostatic repulsion (21). We conclude that Arg$^{120}$ in the porcine mitochondrial isocitrate dehydrogenase is not needed for function.

Fig. 4 shows both Arg$^{110}$ and Arg$^{133}$ close to the substrate isocitrate. Arg$^{110}$ is only about 2.9 Å to the β-carboxylate of isocitrate, suggesting possible involvement in either hydrogen bonding or electrostatic interaction, whereas it is about 4.8 Å to the α-carboxylate of isocitrate. Arg$^{133}$ is located about equidistant to the α- and β-carboxylates (−4.6 Å), suggesting that it likely participates in electrostatic attraction of isocitrate. This model is consistent with the appreciable increases in the $K_m$ for isocitrate in both the R110Q and R133Q enzymes, with the $K_m$ effect for R110Q being greater, in agreement with the more striking proximity of Arg$^{110}$ to isocitrate. In the crystal structure of the E. coli enzyme, similar roles have been shown for the corresponding Arg$^{119}$ and Arg$^{153}$ (8).

The model of the pig isocitrate dehydrogenase is also consistent with an effect of the positively charged Arg$^{110}$ and Arg$^{133}$ in lowering the $pK_a$ of the catalytic base, postulated to be Asp$^{275}$ or Asp$^{279}$ (22). Both of these arginines are closer to Asp$^{275}$ (which may strengthen the case for this residue as the catalytic base), with Arg$^{133}$ estimated as 4.9 Å from the carboxylate of Asp$^{275}$, whereas Arg$^{110}$ is 6.8 Å from the same carboxylate. This difference in distances is consistent with the greater increase in $pK_a$ values upon eliminating the positive charge in the R133Q as compared with the R110Q mutant.

Arg$^{101}$ is located no closer than 12.9 Å from isocitrate in the model (Fig. 4) and therefore cannot be directly involved in binding substrate in the active site. This conclusion is consistent with the observation that the R101Q mutant enzyme exhibits smaller increases in the $K_m$ for isocitrate than do the R110Q or R133Q mutants. Similarly, Arg$^{101}$ is not close to the proposed enzymic general base, Asp$^{275}$ or Asp$^{279}$ (14.7 and 21.0 Å, respectively), consistent with the result that the $pK_{\text{cat}}$ is not increased in the R101Q mutant. The role of Arg$^{101}$ cannot be established conclusively from the model. It is possible that even at this distance the positive charge of Arg contributes to orientation of the isocitrate that is favorable for the catalytic reaction. An alternative explanation can also be envisioned: examination of the model for pig isocitrate dehydrogenase indicates that Arg$^{101}$ is located along a channel from the exterior solvent to the active site at which isocitrate is bound in the enzyme-substrate complex. Isocitrate may traverse this channel in the “on” reaction, whereas α-ketoglutarate may leave the active site via this channel in the “off” rate. Thus, Arg$^{101}$ may play a role in the overall reaction distinct from that involving removal of a proton from isocitrate by the catalytic base. How-
ever, a definitive assignment of a role for Arg<sup>101</sup> must await additional experiments and the experimental determination of the enzyme’s structure.

The homology model of pig mitochondrial NADP-dependent isocitrate dehydrogenase has allowed us to visualize the substrate binding site of the enzyme and to summarize the results of mutagenesis experiments. The model is consistent with most of the experimental results. Whereas it cannot substitute for an actual crystal structure of the porcine enzyme, the homology model can be useful for suggesting additional sites for mutagenesis and for conceptualizing results until the protein structure is determined experimentally.

**Acknowledgments**—We thank Dr. Yu-Chu Huang for N-terminal sequencing of the proteins and Christine McDaniel for sequencing the nucleotides.

**REFERENCES**

1. Colman, R. F. (1983) *Pep. Protein Rev.* 1, 41–69
2. Kelly, J. H., and Plaut, G. W. E. (1981) *J. Biol. Chem.* 256, 330–334
3. Bailey, J. M., and Colman, R. F. (1985) *Biochemistry* 24, 5367–5377
4. Haselbach, R. J., Colman, R. F., and McAlister-Henn, L. (1992) *Biochemistry* 31, 6219–6223
5. Ehrlich, R. S., and Colman, R. F. (1987) *Biochemistry* 26, 3461–3466
6. Ehrlich, R. S., and Colman, R. F. (1977) *Biochemistry* 16, 3378–3383
7. Hurley, J. H., Thornness, P. E., Ramalingam, V., Helmers, N. H., Koshland, D. E., and Stroud, R. M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 8635–8639
8. Hurley, J. H., Dean, A. M., Koshland, D. E., and Stroud, R. M. (1991) *Biochemistry* 30, 8671–8678
9. Stoddard, B. L., Dean, A., and Koshland, D. E., Jr. (1993) *Biochemistry* 32, 9310–9316
10. Soundar, S., Danek, B., and Colman, R. F. (1999) *Biochemistry* 38, Suppl. 1, 138
11. Nagai, K., and Thogersen, H. C. (1987) *Methods Enzymol.* 153, 461–481
12. Soundar, S., Jennings, G. T., McAlister-Henn, L., and Colman, R. F. (1996) *Protein Expression Purif.* 8, 305–312
13. Tao, B. Y., and Lee, K. C. P. (1994) in PCR Technology: Current Innovations (Griffin, H. G., and Griffin, A. M., eds.) p. 74, CRC Press, Cleveland, OH
14. Blackshear, P. J. (1984) *Methods Enzymol.* 104, 237–255
15. Soundar, S., and Colman, R. F. (1993) *J. Biol. Chem.* 268, 5264–5271
16. Johanson, R. A., and Colman, R. F. (1981) *Arch. Biochem. Biophys.* 207, 9–20
17. Colman, R. F. (1968) *J. Biol. Chem.* 243, 2454–2464
18. Colman, R. F., and Chu, R. (1969) *Biochem. Biophys. Res. Commun.* 34, 528–535
19. Colman, R. F. (1973) *J. Biol. Chem.* 248, 8137–8143
20. Thornness, P. E., and Koshland, D. E., Jr. (1987) *J. Biol. Chem.* 262, 10422–10425
21. Jennings, G. T., Minard, K. I., and McAlister-Henn, L. (1997) *Biochemistry* 36, 13743–13747
22. Grodsky, N. B., Soundar, S., and Colman, R. F. (1999) *FASEB J.* 13, A1444
23. Hurley, J. H., Dean, A. M., Sohl, J. L., Koshland, D. E., and Stroud, R. M. (1999) *Science* 249, 1012–1016
24. Dean, A. M., and Koshland, D. E. (1990) *Science* 249, 1044–1046
25. Dean, A. M., Lee, M. H. I., and Koshland, D. E., Jr. (1989) *J. Biol. Chem.* 264, 20452–20456
Identification by Mutagenesis of Arginines in the Substrate Binding Site of the Porcine NADP-dependent Isocitrate Dehydrogenase
Sambanthamurthy Soundar, Brenda L. Danek and Roberta F. Colman

J. Biol. Chem. 2000, 275:5606-5612.
doi: 10.1074/jbc.275.8.5606

Access the most updated version of this article at http://www.jbc.org/content/275/8/5606

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 24 references, 9 of which can be accessed free at http://www.jbc.org/content/275/8/5606.full.html#ref-list-1