The structure of crystalline porcine mitochondrial NADP-dependent isocitrate dehydrogenase (IDH) has been determined in complex with Mn\(^{2+}\)-isocitrate. Based on structural alignment between this porcine enzyme and seven determined crystal structures of complexes of NADP with bacterial IDHs, Arg\(^{331}\), Thr\(^{311}\), and Asn\(^{328}\) were chosen as targets for site-directed mutagenesis of porcine IDH. The circular dichroism spectra of purified wild-type and mutant enzymes are similar. The mutant enzymes exhibit little change in \(K_m\) for isocitrate or Mn\(^{2+}\), showing that these residues are not involved in substrate binding. In contrast, the Arg\(^{331}\) mutants, Asn\(^{328}\) mutants, and T311A exhibit 3-20-fold increase in the \(K_m^{NADP}\). We propose that Arg\(^{331}\) enhances NADP affinity by hydrogen bonding with the 3'-OH of the nicotinamide ribose, whereas Asn\(^{328}\) hydrogen bonds with N1 of adenine. The pH dependence of \(V_{\text{max}}\) for Arg\(^{331}\) and Asn\(^{328}\) mutants is similar to that of wild-type enzyme, but for all the Thr\(^{311}\) mutants, \(pK_a\) is increased from 5.2 in the wild type to ~6.0. We have previously attributed the pH dependence of \(V_{\text{max}}\) to the deprotonation of the metal-bound hydroxyl of isocitrate in the enzyme-substrate complex, prior to the transfer of a hydride from isocitrate to NADP's nicotinamide moiety. Thr\(^{311}\) interacts with the nicotinamide ribose and is the closest of the target amino acids to the nicotinamide ring. Distortion of the nicotinamide by Thr\(^{311}\) mutation will likely be transmitted to Mn\(^{2+}\)-isocitrate resulting in an altered \(pK_a\). Because porcine and human mitochondrial NADP-IDH have 95% sequence identity, these results should be applicable to the human enzyme.

We have described the crystal structure of porcine NADP-IDH complexed with Mn\(^{2+}\) and isocitrate (7). The enzyme-bound Mn\(^{2+}\) is hexacoordinate, with Asp\(^{252}\), Asp\(^{275}\), two water molecules, and two oxygens of isocitrate acting as ligands (7–9). Mutagenesis experiments have supported the importance of Asp\(^{252}\) and Asp\(^{275}\) in the Mn\(^{2+}\)-isocitrate site and have implicated Asp\(^{279}\) (8, 9), Arg\(^{101}\), Arg\(^{110}\), and Arg\(^{133}\) (10), Lys\(^{212}\), and Tyr\(^{140}\) (11), and Ser\(^{95}\), Asn\(^{97}\), and Thr\(^{78}\) (12) as participants in the substrate site. Our recent findings suggested that the pH dependence of the enzyme's \(V_{\text{max}}\) is caused by the deprotonation of the metal-bound hydroxyl of isocitrate in the enzyme-substrate complex prior to the transfer of a hydride to NADP (9).

Previous studies from our laboratory suggest that Arg\(^{314}\), His\(^{315}\), and Tyr\(^{316}\) interact with the 2'-phosphate of NADP and are determinants of the coenzyme specificity of isocitrate dehydrogenase (13, 14). His\(^{309}\) also contributes to the coenzyme binding site (13). Although the porcine mitochondrial NADP-specific isocitrate dehydrogenase has not been crystallized in complex with coenzyme, NADP has been modeled within the crystal structure of this mammalian enzyme using a structural alignment between the porcine enzyme-Mn\(^{2+}\)-isocitrate complex and seven determined crystal structures of bacterial IDH-NADP complexes (7). Fig. 1 shows a view of the model of the NADP-Mn\(^{2+}\)-isocitrate complex of pig mitochondrial NADP-IDH. To evaluate the location of NADP in this model, we have selected as targets for site-directed mutagenesis three amino acids distributed throughout the predicted coenzyme site: Arg\(^{331}\), Thr\(^{311}\), and Asn\(^{328}\). Arg\(^{331}\) and Thr\(^{311}\) are positioned close to the nicotinamide ribose and 5'-phosphate, whereas Asn\(^{328}\) is near the adenine ring (Fig. 1). A structure-based alignment of two regions of the amino acid sequences of NADP-isocitrate dehydrogenases from six species is shown in Fig. 2. Arg\(^{331}\) is conserved among mammals but not in lower organisms, while Thr\(^{311}\) and Asn\(^{328}\) are conserved in all species. The results of this report demonstrate that all three of these amino acids contribute to and delineate the extent of the coenzyme binding site of the mitochondrial porcine NADP-dependent isocitrate dehydrogenase.

**EXPERIMENTAL PROCEDURES**

**Materials—**DL-Isocitrate, NADP, other biochemicals, chemicals, and buffer components were purchased from Sigma. Oligonucleotides were synthesized by Bio-Synthesis, Corp. (Lewisville, TX). The QuickChange XL Kit and Pfu DNA polymerase were obtained from Stratagene (La Jolla, CA). Escherichia coli strain TB1, plasmid pMal-c2, and amylose resin were purchased from New England Biolabs (Beverly, MA). The DNA purification kit was supplied by Qiagen Inc. (Valencia, CA). Human thrombin was obtained from Enzyme Research Lab, Inc. (South Bend, IN). DEAE-cellulose (DE-52) was purchased from Whatman Inc. (Clifton, NJ).

**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 (15). To facilitate separation from the E. coli isocitrate dehydrogenase, the enzyme was

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The porcine mitochondrial NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42) (NADP-IDH) is a divalent metal-dependent enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate using NADP as a cofactor. The enzyme is a homodimer (1, 2), with a molecular mass of 46.6 kDa and 413 amino acids per subunit (3), which binds 1 mol of NADPH or NADP\(^+\) per mol of enzyme subunit (4). NADP-dependent isocitrate dehydrogenase is not only involved in the citric acid cycle, but also has an important role in preventing oxidative damage in mitochondria through NADPH regeneration (5, 6).

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1 The abbreviation used is: NADP-IDH, NADP-dependent isocitrate dehydrogenase.
enzy\textsubscript{m}es—CD spectra were measured at room temperature using a Jasco model J-710 spectropolarimeter (Jasco, Inc., Easton, MD). Measurements of ellipticity as a function of wavelength were made as described previously (8, 10), using 413 as the number of amino acids per subunit (11, 12). The oligonucleotides (forward and reverse primers) used to generate the mutant enzymes by the QuikChange method are listed in Table I. The underlined codons are those for the mutated amino acids.

Circular Dichroism Spectra of the Wild-type and Mutant Enzymes—The activity of NADP-dependent isocitrate dehydrogenase was determined using the 6.22 spectropolarimeter (Jasco, Inc., Easton, MD). Measurements of ellipticity as a function of wavelength were made as described previously (8, 10), using 413 as the number of amino acids per subunit (11, 12). The oligonucleotides (forward and reverse primers) used to generate the mutant enzymes by the QuikChange method are listed in Table I. The underlined codons are those for the mutated amino acids.

Circular Dichroism Spectra of the Wild-type and Mutant Enzymes—CD spectra were measured at room temperature using a Jasco model J-710 spectropolarimeter (Jasco, Inc., Easton, MD). Measurements of ellipticity as a function of wavelength were made as described previously (8, 10), using 413 as the number of amino acids per subunit (11, 12). The oligonucleotides (forward and reverse primers) used to generate the mutant enzymes by the QuikChange method are listed in Table I. The underlined codons are those for the mutated amino acids.

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The pH dependence of $V_{\text{max}}$ for the reaction catalyzed by wild-type and mutant isocitrate dehydrogenases were determined over the range pH 5–8, using the buffers previously described (9). The reaction rates were determined using 4 mM isocitrate, 0.1–10 mM NADP, and 2–6 mM Mn$^{2+}$, as indicated for each enzyme.

**RESULTS**

**Expression and Purification of Wild-type and Mutant Enzymes**—The pig heart NADP-dependent isocitrate dehydrogenase mutant enzymes were generated using expression vector pMALcIDP1, and the QuikChange-XL kit method with oligonucleotides encoding substituted amino acids. At position 83, the positively charged arginine was substituted with glutamine (which is only slightly smaller, but lacks the hydrogen bonding potential) or lysine (to maintain the positive charge, but change the shape). At position 311, threonine was replaced by alanine (which is only slightly smaller, but retains the possibility of hydrogen bonding) or by asparagine (which is much larger, yet maintains the hydrogen bonding capability). At position 328, asparagine was changed to the negatively charged aspartate or to the neutral, but smaller, serine. The wild-type and all mutant enzymes expressed in *E. coli* as maltose fusion proteins, were cleaved by thrombin and purified, as described under “Experimental Procedures.” The wild-type and mutant enzymes each exhibit a single protein band on polyacrylamide gels containing SDS and each one has the same subunit molecular mass (about 46.6 kDa); representative samples are shown in Fig. 3. The N-terminal amino acid sequences of these purified porcine isocitrate dehydrogenases also demonstrate that these recombinant porcine enzymes are not contaminated with the *E. coli* isocitrate dehydrogenase, since the N-terminal sequences of the *E. coli* and porcine enzymes differ in 9 of the first 10 amino acids.

**Circular Dichroism Spectra of Wild-type and Mutant Enzymes**—CD spectra of wild-type and mutant isocitrate dehydrogenases were measured to evaluate whether there are changes in secondary structure in these mutant enzymes. The CD spectra of R83Q, R83K, T311A, T311S, T311N, N328S, and N328D mutant enzymes are very similar to that of the wild-type enzyme (data not shown). These results suggest that the mutations do not cause any appreciable conformational changes.

**Kinetic Characteristics of Wild-type and Mutant Enzymes**—The kinetic parameters of wild-type and mutant porcine NADP-dependent isocitrate dehydrogenases are summarized in Table II. The $K_m$ values for NADP were measured at pH 7.4, containing 4 mM isocitrate and 2 mM Mn$^{2+}$. Except for T311S and T311N mutant enzymes, all the mutant enzymes exhibit significantly higher $K_m$ values for NADP as compared with that of wild-type enzyme (5.59 $\mu M$). The $V_{\text{max}}$ values for NADP of glutamine and lysine mutant enzymes at position 83 are increased 3–15-fold compared with wild type. The $K_m$ value for NADP of the alanine mutant at position 311 is increased about 12 times that of wild type. In contrast, for the serine and asparagine mutant enzymes at the same position (311), the $K_m$ values for NADP are very similar to that of wild type. Replacement of asparagine by aspartate and serine at position 328 results, respectively, in about 4 and 20 times increase in $K_m$ for NADP. These results indicate that Arg$^{83}$, Thr$^{311}$, and Asn$^{328}$ are involved in coenzyme binding.

In contrast, the Arg$^{83}$, Thr$^{311}$, and Asn$^{328}$ mutant enzymes display either small or no changes in $K_m$ for isocitrate (6.4–15 $\mu M$) and Mn$^{2+}$ (0.1–0.4 $\mu M$) under saturating concentrations of NADP. These results indicate that Arg$^{83}$, Thr$^{311}$, and Asn$^{328}$ are not directly involved in the binding of isocitrate or Mn$^{2+}$ by the enzyme.

The specific activity of wild-type enzyme, measured under the standard assay conditions at pH 7.4, containing 4 mM isocitrate, 2 mM Mn$^{2+}$, and 0.1 mM NADP, is 37.8 $\mu M$ min$^{-1}$ mg$^{-1}$. Table II also shows the $V_{\text{max}}$ values of wild-type and the mutant isocitrate dehydrogenases extrapolated to saturating concentrations of NADP. The $V_{\text{max}}$ value of wild-type enzyme is 42.9 $\mu M$ min$^{-1}$ mg$^{-1}$ and the corresponding values of R83Q, R83K, T311A, and N328S mutant enzymes are only a little lower, whereas the $V_{\text{max}}$ of N328D enzyme is reduced to 36% of that of wild-type enzyme. Notably, the T311N mutant enzyme has a markedly decreased $V_{\text{max}}$ value (less than 1% of the wild-type value); the relatively large size of the asparagine which replaces the threonine may so distort the binding of the coenzyme that the maximum catalytic rate is affected. In contrast, substitution of threonine at position 311 by serine not only does not decrease activity but actually gives a large increase in $V_{\text{max}}$ (82.4 $\mu M$ min$^{-1}$ mg$^{-1}$). However, it is notable that $k_{\text{cat}}K_m$ for the T311S mutant is similar to that of wild type (Table II).
The $pK_a$ values were measured in different pH buffers containing various concentrations of NADP, Mn$^{2+}$, and isocitrate. The pH-$V_{max}$ curves were superimposable when 0.5 or 1 mM NADP, 2 or 4 mM Mn$^{2+}$ and 4 or 8 mM isocitrate were used for wild type; when 1, 7, or 10 mM NADP, 2, 4, 5, or 6 mM Mn$^{2+}$ and 4 mM isocitrate were used for R83Q; when 1, 5, 7, or 10 mM NADP, 2 or 6 mM Mn$^{2+}$ and 4 mM isocitrate were used for R83K; when 1, 2, 5, 7, or 10 mM NADP, 2, 4, 5, or 6 mM Mn$^{2+}$ and 4 mM isocitrate were used for T311A; and when 1 or 2 mM NADP, 2 mM Mn$^{2+}$ and 4 mM isocitrate were used for N328S. For T311N and T311S, 0.1 mM NADP, 2 mM Mn$^{2+}$ and 4 mM isocitrate were used; and for N328D, 1 mM NADP, 2 mM Mn$^{2+}$ and 4 mM isocitrate were used. These results indicate that these enzymes were saturated with substrates over the full pH range and that the measured velocities 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, R83Q, R83K, and T311A, at pH 5.5 the $K_m$ values for NADP were 17–261 μM, and for Mn$^{2+}$ 1–6 μM.

### Table III

| Enzyme     | $pK_{a}$ | $V_{max, int}$ |
|------------|----------|----------------|
| Wild type  | 5.24 ± 0.03 | 42.9 ± 1.0 |
| R83Q       | 5.38 ± 0.04 | 32.4 ± 0.5 |
| R83K       | 5.25 ± 0.06 | 35.5 ± 0.7 |
| T311A      | 6.01 ± 0.02 | 31.1 ± 0.6 |
| T311S      | 6.19 ± 0.02 | 82.2 ± 0.9 |
| T311N      | 5.97 ± 0.03 | 0.33 ± 0.01 |
| N328S      | 5.63 ± 0.04 | 29.7 ± 0.5 |
| N328D      | 5.19 ± 0.02 | 15.1 ± 0.1 |

### 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 mM isocitrate, 2–6 mM Mn$^{2+}$, and 1–10 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 (9). The dependence of $V_{max}$ on pH was analyzed by Equation 1,

$$V_{max, obs} = \frac{V_{max, int}}{1 + \frac{[H^+]}{K_{a}}}$$

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 $K_{a}$ is the dissociation constant of an ionizable group of the enzyme-substrate complex. Over the pH range 5–8, the pH-$V_{max}$ profiles for all Arg$^{83}$ and Asn$^{328}$ mutant enzymes are similar to that of wild type, whereas those for Thr$^{311}$ mutant enzymes are shifted toward higher pH, yielding higher $pK_{a}$ values.

Table III summarizes the $pK_{a}$ values for wild type, as well as the Arg$^{83}$, Thr$^{311}$, and Asn$^{328}$ mutant enzymes. For all the enzymes, substrates saturated the active site under the conditions used. Increasing the already high concentrations of NADP to 10 mM and Mn$^{2+}$ to 6 mM did not change the pH-$V_{max}$ profiles. The $pK_{a}$ values of the Arg$^{83}$ mutant enzymes and N328D are similar to that of the wild-type enzyme (about 5.2 to 5.4). The $V_{max}$ values of these mutant enzymes probably depend on the ionizable form of the same group as in the wild-type enzyme. The $pK_{a}$ of the N328S mutant enzyme is slightly higher, 5.63, which may reflect a relatively small perturbation of the $pK_{a}$ of the same ionizable group seen in wild type. In contrast, the $pK_{a}$ values of all Thr$^{311}$ mutant enzymes are significantly higher than that of the wild-type enzyme. Although the $V_{max, int}$ of the Thr$^{311}$ mutant enzymes ranges from 0.33 to 82 μmol min$^{-1}$ mg$^{-1}$, all three Thr$^{311}$ mutant enzymes have similar $pK$ values, 6.0–6.2.

### DISCUSSION

In this study, the characterization of mutant enzymes with replacements at Arg$^{83}$, Thr$^{311}$, and Asn$^{328}$ have implicated these three amino acids in the coenzyme site of the porcine mitochondrial NADP-dependent isocitrate dehydrogenase. The Arg$^{83}$ and Asn$^{328}$ mutant enzymes, as well as the T311A enzyme, have strikingly elevated $K_m$ values for NADP, while the $K_m$ values for the other substrates are similar to those of wild-type enzyme. In the structure shown in Fig. 1, the guanidinium group of Arg$^{83}$ is close to the 3'-OH of the nicotinamide ribose, and to the 5'-O of that ribose, which is linked to a phosphorus of the pyrophosphate moiety. The distance between the 3'-OH and the closest nitrogen of Arg$^{83}$ is 4.6Å; however, there is an enzyme-bound water only 2.83Å from that N of Arg$^{83}$, which may mediate a hydrogen bond between the 3'-OH of the ribose and Arg$^{83}$. The closest distance is 5.7 Å between Arg$^{83}$ and the 5'-O of the pyrophosphate. It is apparent that the positive charge of the amino acid at position 83 contributes to the affinity between the enzyme and NADP because the $K_m$ for R83K is one-fourth that of R83Q. The structure of the NADP complex of the human cytosolic NADP-dependent isocitrate dehydrogenase has recently been reported as the second mammalian IDH to be crystallized (18); however, the functions of the amino acids near the coenzyme have not been evaluated by mutagenesis. The porcine mitochondrial NADP-IDH is 77% identical plus similar in amino acid sequence to human cytosolic IDH and an overlay of the structures of the porcine mitochondrial IDH (PDB 1LWD) and the human cytosolic IDH (PDB 1T09) shows the strong structural resemblance of the two enzymes. In the structure of the human cytosolic enzyme, the corresponding Arg$^{82}$ is also considered to form a hydrogen bond with the 3'-OH of the nicotinamide ribose of NADP (18).

In the porcine mitochondrial IDH (Fig. 1), Thr$^{311}$ is close to the oxygen in the ring of the furanose form of the nicotinamide ribose of NADP, as well as to the 5'-O of the ribose. The ability to form a hydrogen bond and the size of the amino acid at position 311 are important in NADP binding and in the catalytic reaction. Replacement of Thr$^{311}$ by alanine, which cannot form hydrogen bonds, results in more than a 10-fold increase in $K_m$. In contrast, when Thr$^{311}$ is replaced by serine, which can form hydrogen bonds, the $K_m$ is only 1.7× that of wild-type enzyme. Substitution of the larger asparagine for Thr$^{311}$ allows hydrogen bonding, but the enzyme-NADP complex must be distorted in accommodating the larger side chain. Thus, for the T311N enzyme, the $K_m$ is similar to that of the wild-type enzyme, but $V_{max}$ is less than 1% that of wild type. Thr$^{311}$ is the closest, of the three amino acids we have here mutated, to the enzyme-bound Mn$^{2+}$-isocitrate. In the catalytic dehydrogenation of isocitrate, a hydride is transferred from isocitrate to the N-4 position of the nicotinamide of NADP. It is reasonable that any perturbation of the enzyme-bound nicotinamide of NADP, which likely occurs when a larger amino acid is substituted at position 311, results in a decreased $V_{max}$. Indeed substitution of any amino acid at position 311 causes a change in the pH dependence of $V_{max}$, as indicated by the elevated $pK_{a}$ for all the Thr$^{311}$ mutant enzymes (from 5.2 in wild type to about 6.0 in all the Thr$^{311}$ mutant enzymes). We have previously attributed the pH dependence of $V_{max}$ to the deprotonation of the metal-bound hydroxyl of isocitrate in the enzyme-substrate complex (9). Although Thr$^{311}$ is about 12Å from the enzyme-bound Mn$^{2+}$ (Fig. 1), any distortion of the nicotinamide could readily be transmitted to the nearby Mn$^{2+}$-isocitrate with a concomitant change in the $pK_{a}$. It is notable that the corresponding Thr$^{311}$ in the human cytosolic IDH (18) occupies a similar location to that of Thr$^{311}$ in the mitochondrial enzyme, and it may have the same role in interacting with NADP.

Asn$^{328}$ of the porcine mitochondrial IDH is located at the other end of the coenzyme binding site near the adenine ring.
(Fig. 1). The nitrogen of the carbamido side chain of the asparagine is within hydrogen bonding distance (2.70 Å) of the N1 of the adenine, and thus can facilitate the binding of NADP. Replacement of Asn328 by the much smaller Ser results in a 20-fold increase in the $K_m^{NADP}$, likely reflecting the increased distance between Ser328 and the N1 of the purine ring. Substitution of the similarly sized but negatively charged Asp for Asn328 is also detrimental to the affinity between enzyme and NADP ($K_m^{NADP}$ is 4.4× that of wild-type enzyme), but less so than in the case of N328S. In the human cytosolic NADP-specific isocitrate dehydrogenase, Asn328 is also positioned to hydrogen bond to the N1 of the NADP adenine (18). Indeed, most of the amino acid side chains close to the NADP bound to the porcine mitochondrial IDH occupy similar locations in the crystal structure of the human cytosolic IDH (18). In previous studies from our laboratory, mutagenesis experiments indicated that Arg314 and Tyr316 (14), as well as His315 (13) of the porcine mitochondrial NADP-specific IDH dehydrogenase interact with the 2′-phosphate of NADP and contribute to the coenzyme specificity of the enzyme. His309 not only can affect coenzyme binding, but also influences the enzyme interaction with metal ion in the presence of isocitrate (13). Although sequence alignment indicates there is only 16% identity between the porcine and E. coli IDHs (7), amino acids corresponding to His309, Arg314, and Tyr316 have been observed in the NADP site of the crystalline isocitrate dehydrogenase of E. coli (19, 20). In the hyperthermophile Aeropyrum pernix, the adenine ring of NADP lies between the main chain of Asn356 and the side chain of His343 (21). The Asn356 and His343 of A. pernix IDH are equivalent to Asn328 and His309, respectively, of porcine mitochondrial NADP-IDH (Fig. 2). It has been pointed out that the use of NADP by prokaryotic IDHs is an ancient adaptation to growth on acetate (22), so it is not surprising that most of the amino acids which interact with the coenzyme are conserved. However, only in the other crystalline mammalian enzyme, human cytosolic NADP-IDH (18), are all of the amino acids identified in the coenzyme site of the porcine mitochondrial NADP-IDH found in approximately the same location.

The present study of site-directed mutagenesis in the coenzyme site of porcine mitochondrial NADP-dependent isocitrate dehydrogenase is consistent with the crystal structure of the Mn$^{2+}$-isocitrate complex of the same enzyme with NADP positioned in silico (7), and with the recently reported crystal structure of the human cytosolic IDH complexed with NADP (18). Arg314, Thr311, and Asn328 are all located close to enzyme-bound NADP where they are determinants of the enzyme affinity for coenzyme. In addition, Thr311 is in the vicinity of the nicotinamide ring of NADP, which must be close to the enzyme-bound metal-isocitrate, and therefore the amino acid at position 311 can indirectly influence the ionization of the metal-ligated hydroxyl of isocitrate in the active site. The porcine mitochondrial NADP-IDH has 95% amino acid sequence identity with human mitochondrial NADP-dependent isocitrate dehydrogenase. Therefore it is likely that the results of this study will be applicable to the human enzyme.

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