Identification of Mn$^{2+}$-binding Aspartates from $\alpha$, $\beta$, and $\gamma$ Subunits of Human NAD-dependent Isocitrate Dehydrogenase*

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Sambanthamurthy Soundar, Molly O’Hagan, Kenneth S. Fomulu, and Roberta F. Colman

From the Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

The human NAD-dependent isocitrate dehydrogenase (IDH), with three types of subunits present in the ratio of 2$\alpha$:1$\beta$:1$\gamma$, requires a divalent metal ion to catalyze the oxidative decarboxylation of isocitrate. With the aim of identifying ligands of the enzyme-bound Mn$^{2+}$, we mutated aspartates on the $\alpha$, $\beta$, or $\gamma$ subunits. Mutagenesis target sites were based on crystal structures of metal-isocitrate complexes of *Escherichia coli* and pig mitochondrial NADP-IDH and sequence alignments. Aspartates replaced by asparagine or cysteine were 206, 230, and 234 of the $\alpha$ subunit and those corresponding to $\alpha$-Asp-206: 217 of the $\beta$ subunit and 215 of the $\gamma$ subunit. Each expressed, purified mutant enzyme has two wild-type subunits and one subunit with a single mutation. Specific activities of WT, $\alpha$-D206N, $\alpha$-D230C, $\alpha$-D234C, $\beta$-D217N, and $\gamma$-D215N enzymes are 22, 29, 1.4, 0.2, 7.3, and 3.7 $\mu$mol of NADH/min/mg, respectively, whereas $\alpha$-D230N and $\alpha$-D234N enzymes showed no activity. The $K_m$,$\mu$NAD+ for $\alpha$-D230C and $\gamma$-D215N are increased 32- and 100-fold, respectively, along with elevations in $K_m$,$\mu$isocitrate. The $K_m$,$\mu$NAD of $\alpha$-D230C is increased 16-fold, whereas that of $\beta$-D215N is elevated 10-fold. For all the mutants $K_m$,$\mu$isocitrate is decreased by ADP, indicating that these aspartates are not needed for normal ADP activation. This study demonstrates that $\alpha$-Asp-230 and $\alpha$-Asp-234 are critical for catalytic activity, but $\alpha$-Asp-206 is not needed; $\alpha$-Asp-230 and $\gamma$-Asp-215 may interact directly with the Mn$^{2+}$; and $\alpha$-Asp-230 and $\beta$-Asp-217 contribute to the affinity of the enzyme for NAD. These results suggest that the active sites of the human NAD-IDH are shared between $\alpha$ and $\gamma$ subunits and between $\alpha$ and $\beta$ subunits.

Our previous work on pig heart NAD-dependent isocitrate dehydrogenase showed that this enzyme has two binding sites per tetramer for each of its ligands: isocitrate, Mn$^{2+}$, NAD, ADP, NADH, and NADPH (4, 5). These binding studies either indicate that these distinctive subunits have specialized functions for particular ligand sites or that the binding site for each ligand is shared between two subunits (4, 5). The three types of subunits of the pig heart enzyme can be separated by chromatofocusing in the presence of urea (6). Isolated $\alpha$, $\beta$, and $\gamma$ subunits are either inactive or exhibit very low activity, but recombination of isolated $\alpha$ with either $\beta$ or $\gamma$ results in formation of either $\alpha\beta$ or $\alpha\gamma$, which have substantial catalytic activity (6). These observations suggest that dimers may be the minimal functional subunits. The active site may be either within the $\alpha$-subunits or shared between $\alpha$ and $\beta$ or between $\alpha$ and $\gamma$ subunits.

No structure of an NAD-dependent isocitrate dehydrogenase has yet been determined. However, the crystal structure of NADP-specific isocitrate dehydrogenase of *Escherichia coli* (7, 8), *Bacillus subtilis* (9), and pig mitochondria (10) are known. In the crystal structure of the manganese-isocitrate complex of pig mitochondrial NADP-specific IDH, $^2$Asp-252, -275, and -279 are either direct ligands of the Mn$^{2+}$ or interact through a water molecule (10), whereas in the *E. coli* enzyme, Asp-283, -307, and -311 are similarly located (7, 8). Amino acid sequence comparison of the three subunits of the human NAD-IDH enzyme with the subunits of the pig mitochondrial and *E. coli* NADP enzymes using ClustalW indicates relatively low sequence identity. However, certain amino acids in the *E. coli* and pig mitochondrial NADP-isocitrate dehydrogenases that are known to interact with Mn$^{2+}$ and isocitrate are conserved among the three subunits of the human NAD-IDH. We have previously reported on the roles of three conserved arginines of the NAD-dependent isocitrate dehydrogenase (11).

Fig. 1 shows the amino acid sequence alignment in the region of the metal ligands of the pig mitochondrial and *E. coli* NADP-IDHs. Although there is relatively low homology among these enzymes, the $\alpha$-subunit Asp-206, -230, and -234 of the human NAD-IDH can be aligned with Asp-252, -275, and -279 of the pig mitochondrial NADP-IDH as well as with Asp-283, -307, and -311 of the *E. coli* NADP-IDH. In addition, the $\beta$ subunit Asp-217 and $\gamma$ subunit Asp-215 are comparable with Asp-252 of the pig NADP-IDH and Asp-283 of the *E. coli* enzyme.

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1 To whom correspondence should be addressed. Tel.: 302-831-2973; Fax: 302-831-6335; E-mail: rfcolman@udel.edu.

2 The abbreviations used are: IDH, isocitrate dehydrogenase; DTT, dithiothreitol; MES, 2-(N-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; HPLC, high performance liquid chromatography.
Asp in Mn$^{2+}$ Sites of Human NAD-isocitrate Dehydrogenase

IDH Pig IDC$^{39}$DMVAQLLKSSGGF-VWACNYNQVDG$^{40}$VQSP$^{41}$ILA
IDH α-Human LD$^{42}$YVCLAMNQPSQFDFVLMNQVG$^{43}$ILSP$^{44}$LCA
IDH β-Human LD$^{45}$NCNMQLVNQQFQDFVLMNQVG ILDN AA
IDH γ-Human VD$^{46}$NTTTQLVSQPQFQDFVLMNQVG IVNN VCA
IDH E. coli AD$^{47}$AFLQOILLRPARYIDVACMLNQGD$^{48}$YISP$^{49}$ALA

FIGURE 1. Amino acid sequence alignment of a selected segment of α, β, and γ subunits of human NAD-dependent isocitrate dehydrogenase, NADP-dependent isocitrate dehydrogenases from pig (heart) mitochondria, and E. coli. The star indicates that the amino acids at the position are identical, and the dot indicates that the amino acids are similar, using ClustalW. The Asp residues of the human enzyme subunits shown in boldface letters were mutated to Asn, and those indicated by boldface letters and underlined were replaced by Asn and Cys. The Asp residues of the porcine and E. coli enzymes numbered in superscript are aligned with the boldface Asp of the human enzyme subunits.

In the present study, in order to evaluate their roles in the human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation, we studied human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation, we studied human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation. In order to evaluate their roles in the human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation, we studied human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation. In order to evaluate their roles in the human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation, we studied human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation. In order to evaluate their roles in the human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation, we studied human NAD-dependent isocitrate dehydrogenase in the binding of metal ion, in catalytic activity, and in ADP activation.

EXPERIMENTAL PROCEDURES

Materials—Ammonium sulfate, citric acid, rotenone, adenine-5′-diphosphate, β-NAD, DL-isocitrate (trisodium salt), triethanolamine chloride, Coomassie Brilliant Blue-R, DL-dithiothreitol (DTT), ampicillin (monosodium salt), MES, and PIPES were obtained from Sigma. Cadmium sulfate, cobalt nitrate, magnesium sulfate, manganese sulfate, SDS, acrylamide/bisacrylamide, yeast extract, trypotone, isopropyl thio-β-D-galactopyranoside, Tris base, and other high grade chemicals were purchased from Qiagen Co. Low molecular weight standard proteins were from Amersham Biosciences and QIA Prep Spin miniprep kits were purchased from Qiagen Co.

Site-directed Mutagenesis—The complete recombinant human NAD-dependent IDH was expressed in E. coli XL Gold ultracompentent cells (Stratagene). The colonies with positive inserts were subcultured by growing overnight at 37 °C in LB medium (250 ml) supplemented with ampicillin (0.1 mg/ml). These E. coli XL Gold cell transformants harboring pHDHβγ2γ plasmid were used to express human NAD-dependent isocitrate dehydrogenase instead of the IDH-deficient E. coli EB 106 (DE 3) cells used in our previous work (11), because the yield of the pure recombinant IDH was greater. (In our earlier study, each point mutation was introduced into the cDNA encoding a single subunit, and the plasmid encoding the complete enzyme was subsequently laboriously assembled from the separate DNA plasmids encoding one mutant subunit and two wild-type subunits (11)).

To express the enzyme, five 6-liter flasks, each containing 2 liters of LB medium with 0.1 mg/ml ampicillin, were inoculated with freshly grown E. coli XL cells (2% v/v) and were grown at 37 °C while shaking at 200–220 rpm for 4 h or until the growth reached a midlog phase or cell density of $A_{600 \, \text{nm}} = 0.4–0.6$. The flasks were then placed temporarily in chilled water to lower the culture temperature to room temperature. Protein expression was induced in cells by the addition of isopropyl-thio-β-D-galactopyranoside to a final concentration of 0.4 mM, and the flasks were shaken at the lower speed of 100–140 rpm with minimal aeration at 23–25 °C for 20–22 h. (Incubation at the lower temperatures of 15 and 20 °C did not improve the yield of expressed proteins.) The 10 liters of cell culture was then centrifuged at 5000 × g for 10 min to separate the cells from media and suspended in a total volume of 250 ml of cold 11 mM citrate-Tris buffer, pH 7.2, containing 10% glycerol, 0.2 mM MnSO$_4$, and 0.1 mM DTT. These suspensions were stored at −80 °C. Frozen cells, ∼80–100 ml at a time, were

Transformation and Expression of Recombinant Human IDH Proteins in E. coli—Wild type or mutant plasmid DNA from the PCR was used to transform E. coli XL 10 Gold ultracompentent cells (Stratagene). The colonies with positive inserts were subcultured by growing overnight at 37 °C in LB medium (250 ml) supplemented with ampicillin (0.1 mg/ml). These E. coli XL Gold cell transformants harboring pHDHβγ2γ plasmid were used to express human NAD-dependent isocitrate dehydrogenase instead of the IDH-deficient E. coli EB 106 (DE 3) cells used in our previous work (11), because the yield of the pure recombinant IDH was greater. (In our earlier study, each point mutation was introduced into the cDNA encoding a single subunit, and the plasmid encoding the complete enzyme was subsequently laboriously assembled from the separate DNA plasmids encoding one mutant subunit and two wild-type subunits (11)).
partially thawed and lysed, using sonication conditions described previously, while keeping the preparation in ice (11). The lysate was centrifuged at 16,000 × g for 30 min, and the clear supernatant (crude extract) was separated. The protein concentration and isocitrate dehydrogenase activity were determined in this crude extract.

**Purification of Recombinant IDHs from the Crude Extract**—The isolation and purification of enzymes from the crude extract was modified from the previous procedure (11). Unlike IDH-deficient *E. coli* EB 106 cells, the *E. coli* XL cells used in this study possess constitutive NADP-dependent IDH enzyme in addition to the recombinant human NAD-dependent IDH enzyme. Since the human NAD-IDH and the *E. coli* NADP-IDH exhibit very different isoelectric points and have different molecular sizes under native conditions, they can be differentially precipitated from the crude extract by ammonium sulfate fractionation; in addition, they are bound and eluted from ion exchange resins under different conditions. The human NAD-IDH was further purified to homogeneity by gel filtration.

Ammonium sulfate precipitation was followed as described previously (11) to isolate NAD-IDH from the crude extract. The NAD-IDH precipitates predominantly between 30 and 50% saturated ammonium sulfate; this fraction was pooled and further purified. The protein precipitate was dissolved in 12 mM sodium citrate buffer, pH 5.7, containing 20% glycerol, 0.2 mM MnSO₄, and 0.1 mM DTT (Buffer A) and dialyzed against 6 liters of Buffer A with three changes, for 6 h each. The enzyme was applied to a DE-52 column (3 × 13 cm), which had previously been equilibrated with Buffer A. The column was eluted with the same buffer until the A₄₅₀ nm reached the baseline. A linear gradient was then started from 200 ml of buffer A to 200 ml of 50 mM citrate-Tris buffer, pH 7.7, containing 20% glycerol, 0.2 mM MnSO₄, and 0.1 mM DTT. The NAD-dependent isocitrate dehydrogenase typically elutes between 150 and 230 ml of the gradient. In contrast, the *E. coli* NADP-dependent IDH elutes later, from about 230 ml to the end of the gradient. Fractions (5 ml each) exhibiting specific activities more than 5–6.0 units/mg (wild type) were pooled and concentrated to ~10 ml by ultrafiltration.

The pool was dialyzed with three changes, against 6 liters of 12 mM sodium citrate buffer, pH 5.7, containing 20% glycerol and 0.1 mM DTT (Buffer B). To facilitate NAD-IDH binding to the cation exchange resin, MnSO₄ was not added to the dialysis or to the elution buffers. Some loss of IDH activity occurred at this step. The dialyzed enzyme was applied to a cellulose phosphate cation exchange column (2.5 × 11.0 cm). The column was washed with ~125 ml of Buffer B until the A₄₅₀ nm reached the baseline. The traces of NADP-IDH carried over from the DE-52 column eluted in the wash, since *E. coli* IDH does not bind to cellulose phosphate under these conditions. A linear gradient, consisting of 100 ml of Buffer B and 100 ml of 0.15 M sodium citrate buffer, pH 5.7, containing 20% glycerol and 0.1 mM DTT, was used to elute the NAD-IDH. The enzyme elutes between 75 and 150 ml of the gradient. Fractions of 5.0 ml were collected in tubes containing MnSO₄ (final concentration, 2 mM) to maintain enzyme stability. Aliquots of fractions showing high specific activity were screened by SDS-PAGE. Generally, the fractions containing the NAD-IDH exhibit the presence of two minor impurities with subunit molecular sizes of 90 and 45 kDa. These were pooled and concentrated to a 1–1.5-ml volume.

After dialyzing this pool against 4 liters of 50 mM citrate-Tris, pH 7.2, buffer containing 20% glycerol, 0.2 mM MnSO₄, and 0.1 mM DTT (Buffer C) for 6 h, the enzyme was separated from the other proteins by elution through an Ultragel ACA 34 (molecular mass cut-off 350 kDa) gel filtration column (1.0 × 124 cm). The proteins were eluted using Buffer C at the rate of 7.0–7.5 ml/h, and ~1.0-ml volume fractions were collected. Three distinct proteins were observed, with the NAD-dependent isocitrate dehydrogenase being eluted as the middle peak. Protein fractions that were homogenous on SDS-PAGE and exhibited high specific activity for NAD-IDH were pooled and used for characterization and kinetic studies. Pure mammalian NADP-dependent isocitrate dehydrogenase was recognized by the appearance of two close bands in approximately equal intensity, with an upper band (β and γ subunits) of 39,000 Da and a lower band of 37,000 Da (α subunits) (3, 14).

**Protein Determination and Assay for NAD and NADP-dependent IDH Activity**—In the early stages of protein purification from the crude extract, the protein concentration was determined from the A₄₅₀ nm after correcting for the ratio of A₃₄₀/A₄₅₀ (15). In the pure preparations, the protein concentrations in mg/ml were calculated using E₃₄₀ = 6.45 (4). Enzyme activity was determined by monitoring at 340 nm the time-dependent increase in UV absorbance of NADH produced by reduction of NAD at 25 °C in a 1-ml standard assay mixture containing 33 mM Tris acetate buffer, pH 7.2, 20 mM dl-isocitrate, 1 mM MnSO₄, and 1 mM NAD as the final concentrations. The specific activity was expressed as μmol/min/mg of protein. To minimize interference from NADH oxidase while assaying for IDH activity in crude preparations or ammonium sulfate fractions containing lower amounts of expressed mutant enzymes, the NADH oxidase inhibitor, 10 μM of rotenone (dissolved in 100% ethanol), was added to the 1 ml of standard assay solution to give a final concentration of 2.5 μM rotenone. Since the normal *E. coli* cells have NADP-dependent IDH at the early steps of purification (ammonium sulfate fractionation and DEAE-cellulose chromatography), enzyme samples were also assayed for NADP-dependent IDH. The NADP-IDH activity was determined at 25 °C by monitoring the increase in UV absorption of NADPH at A₃₄₀ nm in a 1-ml assay mixture containing 30 mM triethanolamine chloride, pH 7.4, buffer, 0.1 mM NADP⁺, 4 mM dl-isocitrate, and 2 mM MnSO₄.

**SDS-PAGE**—Aliquots of fractions containing 10 μg of protein were analyzed in 15% polyacrylamide gels containing 0.1% SDS in a discontinuous pH electrophoresis system (16) to evaluate the purity of the protein samples during purification. The solutions for preparing the stacking and resolving gel, protein staining and destaining, and other electrophoresis conditions were described previously (17).

**CD of Recombinant Wild Type and Mutant Enzymes**—To evaluate the secondary structure of wild-type and mutant enzymes, the ellipticity was measured as a function of wavelength between 250 and 200 nm in a 0.1-cm path length quartz cell using a Jasco model J-710 spectropolarimeter. The wild-type and mutant proteins were dialyzed against 2 liters of 25 mM
triethanolamine chloride buffer, pH 7.4, containing 10% glycerol and 0.2 mM MnSO₄, and the same buffer was used to dilute the enzymes to 0.1 mg/ml. The mean residue molar ellipticity [θ] (degrees cm² dmol⁻¹), and molar concentrations of proteins were determined using average subunit molecular weights and number of residues per subunit, as described before (11).

**Determination of Amino Acid Sequence and Subunit Composition of Enzymes**—The α, β, and γ subunits of recombinant NAD-IDH enzymes were identified by comparing their N-terminal sequences with those of the known sequences of the enzyme subunits (13, 18), as described previously (11). The N-terminal amino acid sequences of wild type and mutant enzymes were determined using an Applied Biosystems protein/peptide sequencer (model Procise) equipped with an on-line Microgradient Delivery System (model 140 C) and a Macintosh computer (model 610). Since the N-terminal amino acid sequence of the α, β, and γ subunits are different, the subunit composition of wild-type and mutant enzymes was determined from the molar ratios of amino acids at positions 1, 6, and 7 upon sequencing the whole protein. Alternatively, to confirm the mutant subunit ratios of the enzymes, they were also determined from the SDS denatured subunits separated by a reverse phase column in HPLC and compared with those of wild-type enzyme, as described before (11).

**Michaelis-Menten Constants for Isocitrate, Metal Ions, and Coenzyme of Wild Type and Mutant Enzymes**—To determine the Kₘ, the concentration of coenzyme, DL-isocitrate, or metal ion was varied while the other substrates were maintained at the standard assay concentration. To determine the Kₘ,Mn⁺, 1.0–20.0 mM MnSO₄ for α-D230C, up to 25 mM Mn²⁺ for α-D215N, and 0.1–7.0 mM MnSO₄ for all other enzymes were used, whereas to measure the Kₘ,Cd²⁺, 0.05–20 mM CdSO₄ was used. The Kₘ,NAD was determined for α-D230C by using 1.0–10.0 mM NAD with 60 mM isocitrate and 20 mM MnSO₄, whereas for other enzymes, 0.01–1.0 mM NAD was used under the standard conditions. For the Kₘ determination for isocitrate, under the standard conditions, the ΔL-isocitrate concentration varied between 0.05 and 20 mM. For the Kₘ,isocitrate determination of α-D230C and γ-D215N, concentrations up to 50 or 60 mM isocitrate and 20 or 25 mM Mn²⁺, respectively, were included in the standard assay mixture. In general for ADP activation studies, 1 mM ADP was included while varying the isocitrate concentration under the standard conditions. For α-D230C, 5.0 mM ADP and 20.0 mM MnSO₄, whereas for γ-D215N, 5 mM ADP and 25 mM Mn²⁺, were included in the standard assay mixture with various isocitrate concentrations up to 20 and 60 mM, respectively.

**Effect of Various Metal Ions on the pKₐ of the Wild-type Human NAD-IDH**—For determination of the pH–Vₘₐₓ profile, the enzyme activity of the wild type was determined at various pH values using the following buffers: sodium acetate, pH 4.4–5.8; MES, pH 5.4–6.6; PIPES, pH 6.2–7.4; and triethanolamine hydrochloride, pH 6.8–7.6. The final buffer concentration was 30 mM in 1 ml of standard assay solution. The metal ions were present at the following concentrations: MnSO₄ (1 or 2 mM), Co(NO₃)₂ (5 or 10 mM), and MgSO₄ (5 and 10 mM) in the 1-ml assay solutions in order to saturate the enzyme with metal ion over the entire pH range. At the lower metal ion concentrations, 20 mM isocitrate and 1 mM NAD were used in the assay, whereas 40 mM isocitrate and 2 mM NAD were used with the higher metal ion concentration. The results were the same with the two sets of substrate concentrations, indicating that the enzyme was saturated with respect to substrates. The dependence of the observed Vₘₐₓ on pH was analyzed in accordance with the equation,

\[
V_{\text{max,obs}} = \frac{V_{\text{max,int}}}{1 + H^+/K_{\text{aes}}} 
\]

where \(V_{\text{max,obs}}\) is the maximum velocity measured at each pH, \(V_{\text{max,int}}\) is the intrinsic maximum velocity, which is independent of pH, and \(K_{\text{aes}}\) is the dissociation constant of an ionizable group of the enzyme-substrate complex.

### RESULTS

**Site-directed Mutagenesis, Expression, and Purification of Recombinant Human NAD-IDHs**—Here, for the first time, site-directed mutagenesis was carried out on the complete plasmid harboring human NAD-dependent isocitrate dehydrogenase using the PCR-based QuickChange mutagenesis method. We have successfully replaced aspartates with asparagine or cysteine at positions 206, 230, and 234 in the α subunit, 217 in β subunit, or 215 in γ subunit. Each point mutation was accomplished in the target subunit without affecting the other subunits, as demonstrated by DNA sequencing of all subunits. The wild type or mutant plasmid DNA resulting from PCR was transformed into E. coli XL Gold competent cells (Stratagen), and cells with positive inserts were used to express the recombinant enzymes. The recombinant human NAD-IDHs were purified to homogeneity as described under “Experimental Procedures,” giving a yield of enzyme higher than that obtained from the IDH-deficient E. coli EB 106 cells that we used previously (11). Furthermore, as shown in Table 1, the mutagenesis and expression system described in this paper resulted in wild-type human NAD-dependent isocitrate dehydrogenase with the same specific activity as reported previously (4, 11).

The purity of the enzyme preparations was assessed by SDS-PAGE, as shown in Fig. 2. The wild type and all seven purified human mutant enzymes exhibit the two close bands with equal

### TABLE 1

Specific activities of recombinant wild-type and mutant human NAD-dependent isocitrate dehydrogenases

As described under “Experimental Procedures,” the specific activities for wild-type and mutant purified human NAD-IDHs were generally determined using standard assay solutions.

| Enzyme         | Specific activity (μmol/min/mg) |
|----------------|---------------------------------|
| Wild type      | 21.7                            |
| α-D206N        | 28.5                            |
| α-D230N        | 0.000                           |
| α-D230N        | 0.004                           |
| α-D230C        | 0.00                            |
| α-D230C        | 1.43                            |
| α-D234N        | 0.000                           |
| α-D234N        | 0.002                           |
| α-D234C        | 0.19                            |
| β-D217N        | 7.27                            |
| γ-D215N        | 0.00                            |
| γ-D215N        | 3.68                            |

* These mutant enzymes were assayed at standard assay conditions except for the use of 70 mM isocitrate plus 60 mM MnSO₄.

* The specific activity of this mutant enzyme was determined in assay solution containing 20 mM MnSO₄ under otherwise standard conditions.
human NAD-IDH, the purified enzymes were subjected to wild-type enzyme, reflecting appreciable amounts of all of the mutant enzymes are superimposable on that of the enzyme.

Enzymes

three subunits of human NAD-dependent isocitrate dehydrogenases, as shown in Table 2, indicate that the subunit composition of recombinant human NAD-IDH, we replaced Asp in Mn\(^{2+}\) and is also similar to that previously obtained for human NAD-IDH (11). These results also indicate that the mutagenesis, expression, and purification procedures described in this paper yield complete recombinant human IDH enzymes with subunits assembled in the correct composition.

**Determination of Specific Activities of Wild Type and Mutant Recombinant Enzymes**—The specific activities of wild-type and mutant enzymes were determined, in most cases, under the standard assay conditions, which include 20 mM isocitrate and 1 mM MnSO\(_4\). The concentrations of isocitrate and Mn\(^{2+}\) were increased to assay those mutant enzymes that did not show activity with the standard assay, with the results reported in Table 1. At each position, aspartic acid was replaced by asparagine. Only in the case of D206N is the specific activity the same or higher than that of wild-type enzyme, suggesting that α-Asp-206 is not involved in the catalytic activity of the enzyme. In contrast, the D230N and D234N mutants exhibited almost no activity even when assayed at 70 mM isocitrate and 60 mM MnSO\(_4\), indicating that α-Asp-230 and α-Asp-234 are essential for catalytic activity.

In the case of the pig heart mitochondrial NADP-specific isocitrate dehydrogenase, it has been found that cysteine is an acceptable (albeit less effective) substitute for critical aspartates in the manganese-isocitrate binding site (19). Therefore, for human NAD-IDH, we replaced α-Asp-230 and α-Asp-234 with cysteine. The results shown in Table 1 indicate a specific activity of α-D230C as high as 6.6% of that of wild type when assayed at unusually high concentrations of Mn\(^{2+}\), whereas α-D234C has the measurable specific activity of 0.9%, as compared with that of WT, under standard assay conditions. Clearly, cysteine is a better substitute than asparagine for the naturally occurring aspartate at these positions.

Because the NADP-specific isocitrate dehydrogenases have been shown to have three aspartates in the region of the metal-isocitrate site that are important for their function (7, 8, 19), we anticipated that the NAD-dependent isocitrate dehydrogenase might also have three critical aspartates. Since α-D206N is highly active, α-Asp-206 was not a candidate for a functionally important aspartate; therefore, we evaluated by mutagenesis the corresponding aspartates in the β and γ subunits. Table 1 shows that under standard assay conditions, the specific activity of β-D217N is reduced to one-third that of wild type, implying that β-Asp-217 has some influence on catalytic function. More notable, however, is the effect of replacing γ-Asp-215: γ-D215N exhibits only 17% of the specific activity of the wild-type enzyme, and that activity is observed only when the Mn\(^{2+}\) concentration is elevated 20-fold over that in the standard assay. The γ-Asp-215 appears to contribute to binding of metal ion by the enzyme.

Determination of Kinetic Constants for Metal Ion and NAD of Recombinant Enzymes—Table 3 shows that among the α subunit mutants, D206N exhibits Michaelis constants for Mn\(^{2+}\) and NAD, as well as a \(V_{\text{max}}\) value similar to those of wild type, implying a lack of involvement of Asp-206 in the metal or coenzyme binding functions of the enzyme. (Although the \(V_{\text{max}}\) of α-D206N is a little higher than that of wild type, the values of \(k_{\text{cat}}/K_m\) are slightly lower for the mutant enzyme (Tables 3 and
**Asp in Mn$^{2+}$ Sites of Human NAD-isocitrate Dehydrogenase**

**TABLE 3**

Michaelis-Menten constants for Mn$^{2+}$, Cd$^{2+}$, and NAD of recombinant wild-type and mutant NAD-IDH

| Enzyme      | $K_m$,Mn$^{2+}$ | $K_m$,NAD | $V_{max}$ (with Mn$^{2+}$) | $k_{cat}$/$K_m$,Mn$^{2+}$ | $k_{cat}$/$K_m$,NAD | $K_m$,Cd$^{2+}$ | $V_{max}$ (with Cd$^{2+}$) |
|-------------|----------------|-----------|--------------------------|--------------------------|-------------------|----------------|--------------------------|
| Wild type   | 0.22 ± 0.02    | 70 ± 10   | 22.5 ± 0.1               | 30.0                     | 136               | 0.429         | 62 ± 0.07                |
| a-D206N     | 0.32 ± 0.02    | 140 ± 10  | 27.6 ± 0.11              | 36.8                     | 115               | 0.263         | ND                       |
| a-D230C     | 7.13 ± 0.50    | 1100 ± 130| 1.87 ± 0.11              | 2.49                     | 0.35              | 0.002         | 6.19 ± 0.55              |
| a-D234C     | 0.46 ± 0.06    | 40 ± 10   | 0.20 ± 0.01              | 0.27                     | 0.58              | 0.007         | 0.06 ± 0.01              |
| β-D217N     | 0.10 ± 0.02    | 680 ± 40  | 14.7 ± 1.0               | 19.6                     | 0.029             | 0.03 ± 0.01   | 0.13 ± 0.01              |
| γ-D215N     | 21.1 ± 1.9     | 190 ± 20  | 8.83 ± 0.07              | 11.8                     | 0.56              | 0.062         | 1.75 ± 0.15              |

*To calculate the mol of dimeric enzyme/mg of protein, a molecular weight of 80,000 was used for NAD-IDH (1.25 × 10$^{-5}$ mol of dimeric enzyme/mg of protein).

The constant was determined using varying concentrations of NAD under standard conditions except that the isocitrate concentration was 60 mM and the MnSO$_4$ concentration was 20 mM.

This mutant enzyme was assayed using standard assay solutions, which includes 70 mM isocitrate.

The kinetic constants of this mutant for NAD were determined except for 70 mM isocitrate plus 25 mM Mn$^{2+}$ under otherwise standard conditions.

**TABLE 4**

Kinetic constants for isocitrate and ADP activation of wild type and mutant NAD-IDHs

As described under “Experimental Procedures,” the activities were measured under standard assay conditions, which includes various concentrations of metal or coenzyme and to determine the effect of ADP, 1 mM as final concentration of ADP was maintained unless otherwise indicated.

| Enzyme      | $K_m$,isocitrate | $K_m$,isocitrate + ADP | $V_{max}$,isocitrate | $V_{max}$,isocitrate + ADP | $k_{cat}/K_m$,isocitrate |
|-------------|-----------------|------------------------|----------------------|-----------------------------|--------------------------|
| Wild type   | 2.2 ± 0.30      | 0.32 ± 0.04            | 22.0 ± 0.97          | 21.6 ± 1.1                  | 13.3$^a$                 |
| a-D206N     | 3.5 ± 0.13      | 0.46 ± 0.03            | 27.1 ± 1.02          | 29.4 ± 1.4                  | 10.3$^a$                 |
| a-D230C     | 20. ± 0.9       | 6.50 ± 0.05            | 1.32 ± 0.11          | 1.23 ± 0.11                 | 0.09$^a$                 |
| a-D234C     | 3.4 ± 0.13      | 0.01 ± 0.01            | 0.18 ± 0.01          | 0.19 ± 0.01                 | 0.07$^a$                 |
| β-D217N     | 5.1 ± 0.05      | 0.33 ± 0.04            | 13.7 ± 0.01          | 14.3 ± 1.1                  | 3.58$^a$                 |
| γ-D215N     | 4.1 ± 0.3       | 2.16 ± 0.23            | 9.0 ± 0.9            | 9.4 ± 0.8                   | 0.29$^a$                 |

*To calculate the mol of dimeric enzyme/mg of protein, a molecular weight of 80,000 was used for NAD-IDH (1.25 × 10$^{-5}$ mol of dimeric enzyme/mg of protein).

Determined at standard assay conditions except for 20 mM MnSO$_4$, ADP (3 mM) was added to the above assay mixture to determine the effect of ADP.

Standard assay conditions were used except for 10 mM NAD.

The assays were conducted under standard conditions, except for 25 mM Mn$^{2+}$, with or without 5 mM ADP.

4.) In contrast, for a-D230C, the $K_m$ for Mn$^{2+}$ is elevated 32-fold, whereas that for Cd$^{2+}$ is increased 10-fold in comparison with wild type, suggesting that Asp-230 may interact directly with the metal ion. (It was earlier shown that the mammalian NAD-IDH would accept several divalent metal ions, including Mn$^{2+}$, Mg$^{2+}$, Co$^{2+}$, and Cd$^{2+}$, with similar specific activities (20–22)). With an increase in $K_m$,NAD of ~16-fold for D230C as compared with wild type, Asp-230 may also participate in coenzyme binding. Furthermore, D230C exhibits an extrapolated $V_{max}$ of only ~8% that of wild type, indicating that α-Asp-230 is a determinant of the maximum catalytic rate.

For the a-D234C mutant, whereas the $K_m$ values for Mn$^{2+}$ and NAD are similar to those of wild type, the $K_m$ for Cd$^{2+}$ is 10-fold lower than that of wild type, suggesting that the cysteine at position 234 interacts strongly with Cd$^{2+}$. However, the extrapolated $V_{max}$ with either Mn$^{2+}$ or Cd$^{2+}$ as divalent metal ion is only 1% that of wild type, implying that α-Asp-234 is needed for catalytic function.

For the β-D217N mutant, whereas the $K_m$ for Mn$^{2+}$ is unchanged and $V_{max}$ is ~65% that of wild type, the $K_m$ for NAD is elevated ~10-fold, indicating that Asp-217 may contribute to the binding of the coenzyme (Table 3). Surprisingly, D217N exhibits much stronger affinity for Cd$^{2+}$, as indicated by a 20-fold lower $K_m$ value compared with wild type. However, in this case, the $V_{max}$ (with Cd$^{2+}$) is only 4% of that of the same enzyme when Mn$^{2+}$ is the cation, suggesting an unusual and detrimental interaction of Cd$^{2+}$ with this mutant enzyme.

Notably, the γ subunit mutant, D215N, exhibits a 100-fold elevation in its $K_m$,Mn$^{2+}$ and a significant increase in the $K_m$ for Cd$^{2+}$. These results indicate that γ-Asp-215 is likely to be another ligand for the Mn$^{2+}$.
the pig heart mitochondrial NADP-dependent isocitrate dehydrogenase (10, 19). The pK of a water molecule bound to a metal ion depends on the metal ion to which it is bound, with the pK of Mg$^{2+}$ > Mn$^{2+}$ > Co$^{2+}$ (26, 27). To determine whether the pH dependence of $V_{\text{max}}$ for the NAD-IDH could be due to the ionization of the metal-bound α-OH of isocitrate in the enzyme-substrate complex, pK$_{\text{aex}}$ values were determined for wild-type NAD-IDH using Co$^{2+}$, Mn$^{2+}$, or Mg$^{2+}$ as the essential divalent metal ions. In each case, the $V_{\text{max}}$ versus pH curve was the same when measured at two different high concentrations of metal ion, isocitrate, and NAD (as described under “Experimental Procedures”), indicating that the enzyme was saturated with substrate over the entire range of pH. Table 5 shows the values for pK$_{\text{aex}}$ for human wild-type NAD-dependent isocitrate dehydrogenase in the presence of Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$. The pK$_{\text{aex}}$ changes with the metal ion used in the order Mg$^{2+}$ > Mn$^{2+}$ > Co$^{2+}$. These results are consistent with the pK$_{\text{aex}}$ reflecting the deprotonation of the metal-bound α-hydroxyl of isocitrate bound to the enzyme.

**DISCUSSION**

The α, β, and γ subunits of human NAD-dependent isocitrate dehydrogenase exhibit strong resemblance in amino acid sequence, yet their isoelectric points are distinct. Amino acid sequence comparison using ClustalW reveals 32% identity plus 35% similarity among the three subunits. The β and γ subunits have a stronger resemblance in sequence (53% amino acid identity plus 17% similarity) and are 12–15 amino acids longer than α. It is likely that the differences among subunits arose from gene duplication followed by divergent evolution; however, whether the sequence diversity is associated with specialized functions for the subunits has not yet been established. The minimum molecular weight for a complete functional enzyme is 160,000 (2), and, with its three types of distinguishable subunits (2 α, β, and γ), the enzyme has only two binding sites per enzyme tetramer for every ligand tested (4, 5). A major question for this allosteric enzyme is whether the enzyme active site is entirely located within the two α subunits per tetramer and the β and γ subunits have regulatory functions or, alternatively, whether the two active sites per tetramer are shared between dissimilar subunits (i.e. αβ and αγ). In this study, we sought to address the issue by identifying the subunit location of the aspartates, which are ligands of the required divalent metal ion.

The pig mitochondrial NADP-dependent isocitrate dehydrogenase, with a determined structure for the manganese-isocitrate complex (10), can serve as a guide to our study of the mammalian NAD-specific isocitrate dehydrogenase. The porcine NADP-IDH is a dimer of identical subunits, as are the NADP-IDHs of *E. coli* (7) and *B. subtilis* (9). Each manganese-isocitrate bound to crystalline porcine NADP-IDH is located close to the interface between the two subunits (10), and amino acids from both subunits contribute to binding and catalysis (19, 28). For manganese-isocitrate bound at the B subunit, the direct ligands of the hexacoordinate Mn$^{2+}$ are the carboxylate of Asp-252 from the A subunit and of Asp-275 from the B subunit, the α-hydroxyl and α-carboxylate of isocitrate, and two water molecules (10). Asp-279 of the B subunit is located a little further from the Mn$^{2+}$ but is within hydrogen bonding distance of the two water molecules that are coordinated to the metal ion (10, 19). The roles of Asp-252, Asp-275, and Asp-279 of the porcine NADP-specific isocitrate dehydrogenase have been demonstrated by site-directed mutagenesis (19, 29).

Here, we selected the target sites for mutagenesis in the human NAD-dependent isocitrate dehydrogenase because of their sequence alignment with the three critical aspartates of the porcine NADP-IDH. Asp-279 of the NADP-IDH corresponds to α-Asp-234 of NAD-IDH; Asp-275 of the NADP enzyme is equivalent to α-Asp-230; and Asp-252 of the opposite subunit of the NADP-IDH aligns with α-Asp-206, β-Asp-217, and γ-Asp-215. We replaced each of these amino acids with asparagine, because it lacks the negative charge of aspartate, which is important in binding metal cations but retains the size and shape of asparatate. In addition, at the two positions at which the asparagine replacement yielded a completely inactive enzyme (α-D230N and α-D234N), we substituted cysteine, because, with its -SH group, it is known to be a metal ligand in a variety of proteins.

The results for mutants of the NAD-dependent isocitrate dehydrogenase can be summarized as follows. At position α-230, although α-D230N has no detectable activity, α-D230C exhibits a low but measurable $V_{\text{max}}$ (1.9 μmol of NADH/min/mg of enzyme) as compared with WT (22 μmol NADH/min/mg enzyme). These results indicate that asparatate at this position is important for function, whereas cysteine is an acceptable, but poorer, alternative. The α-D230C mutant exhibits increases of about 32-fold and 10-fold in the $K_m$ values for Mn$^{2+}$ and isocitrate, respectively, which is consistent with α-Asp-230 acting as a direct ligand of the Mn$^{2+}$. In addition, the D230C enzyme has a 16-fold elevation in $K_m$ for NAD, which might indicate that normally α-Asp-230 participates in the binding of the coenzyme. However, an enzyme ligand of the Mn$^{2+}$ may also be close to the isocitrate α-hydrogen, which is transferred as a hydride to the nicotinamide of NAD. Thus, perturbation of the manganese-isocitrate binding by changing a ligand of the metal ion may indirectly have an adverse effect on the affinity of the enzyme for NAD. These characteristics are similar to those of mutants of Asp-275 of the porcine NADP-IDH (19, 29).

At position α-234, the asparagine mutant is inactive, and the α-D234C mutant has a specific activity only 1% that of the wild-type NAD-IDH. However, the $K_m$ values for Mn$^{2+}$, isocitrate, and NAD are normal. This pattern is similar to the properties of mutants of Asp-279 of the porcine NADP-IDH (19, 29), which
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is a “second shell” ligand of the metal ion. If $\alpha$-Asp-234 has a corresponding location in the human NAD-IDH, replacement of this aspartate can indirectly affect the orientation of the substrate at the active site by changing the interaction of amino acid 234 with water coordinated to the Mn$^{2+}$. Indirect effects of changing second shell ligands have been reported for other enzymes (30).

Mutation of the third conserved aspartate of the $\alpha$-subunit, $\alpha$-Asp-206, has little effect on the kinetic characteristics of NAD-IDH. The $\alpha$-D206N mutant has nearly normal $K_m$ values for Mn$^{2+}$, isocitrate, and NAD, and a $V_{\text{max}}$ that is not decreased compared with wild-type enzyme. Clearly, $\alpha$-Asp-206 is not involved either in the binding of substrates or in the catalytic reaction. These results contrast with the major changes in the kinetics of the porcine NADP-IDH upon mutation of Asp-252 (19), although the sequence alignment suggested that these aspartates were equivalent in the two enzymes. The explanation must lie in the fact that in the crystal structure of the pig NADP enzyme, Asp-275 and Asp-279 come from one subunit, whereas Asp-252 is contributed to the active site from the opposite subunit (10, 19). Thus, the actual amino acids of the NADP enzyme are likely to come from the opposite subunit (10, 19). Thus, the amino acids of the NADP enzyme are likely to come from the opposite subunit (10, 19).

The $\gamma$-D215N mutant exhibits a $K_m$ for Mn$^{2+}$ that is elevated 10-fold, with a 20-fold increase in the $K_m$ for isocitrate, and only a small perturbation in the $K_m$ for NAD. These characteristics expected if $\gamma$-Asp-215 is a direct ligand of Mn$^{2+}$, equivalent to Asp-252 of the NADP-IDH. Once this mutant enzyme is saturated with substrates, however, its $V_{\text{max}}$ is only decreased to $\sim$40% of the wild-type value, indicating that the major role of $\gamma$-Asp-215 is in binding to the metal ion, which, in turn, is coordinated to isocitrate.

In contrast, the corresponding $\beta$-D217N mutant exhibits a normal affinity for metal ion and for isocitrate, a $V_{\text{max}}$ that is only decreased to $\sim$65% of the normal value, but a striking 10-fold elevation in the $K_m$ for NAD. The $\beta$-Asp-217 functions as a determinant of the affinity between the enzyme and its coenzyme.

The NAD-dependent isocitrate dehydrogenase is regulated by ADP, which acts by lowering the $K_m$ for isocitrate (1, 4). All of the mutants examined in this study exhibit a decreased $K_m$ for isocitrate in the presence of ADP. Thus, none of these aspartates is required for the allosteric response to ADP. In a previous paper from this laboratory (11), we found that mutation of $\beta$-Arg-99 and $\gamma$-Arg-97 resulted in loss of the allosteric response to ADP, leading to the suggestion that the $\beta$ and $\gamma$ subunits are responsible for the regulation of the enzyme by nucleotides. The present study does not contradict the earlier conclusion. However, it indicates that the $\beta$ and $\gamma$ subunits have additional roles in the active site: contributing a ligand for Mn$^{2+}$ and a determinant of the NAD affinity.

It has been proposed that isocitrate dehydrogenase reactions are initiated by the abstraction of a proton, facilitated by an enzymic general base, from the C2-hydroxyl of isocitrate prior to the transfer of the hydride to the nicotinamide of the coenzyme (31). For the NAD-IDH wild type enzyme, the pH dependence of $V_{\text{max}}$ was thought to reflect the deprotonation of that enzymic group in the enzyme-substrate complex; since the $pK_{\text{ase}}$ was about 6.5 when determined with Mn$^{2+}$, the enzymic general base was postulated to be an aspartate or glutamate. However, there is another possibility that should be considered; the $pK$ of 6.5 could represent the ionization of the metal-coordinated C2-hydroxyl of isocitrate, despite the fact that this $pK$ is much lower than that of the hydroxyl of isocitrate when free in solution. Such an identification of the ionizable group has been made for the complex of manganese-isocitrate with the porcine NADP-IDH and is consistent with the crystal structure of this enzyme (10, 19). There are other enzymes for which an enzyme-metal–water complex exhibits a $pK$ much lower than that of the metal-water complex alone (32, 33). In the case of the NAD-dependent isocitrate dehydrogenase, the positively charged arginines in the region of the isocitrate site (11) may be responsible for the decreased $pK$. Characteristic of the ionization of a metal-coordinated water bound to an enzyme (34), or of a metal-coordinated hydroxyl group of a substrate bound to an enzyme (19), the $pK$ changes with the metal ion, decreasing from Mg$^{2+}$ to Mn$^{2+}$ to Co$^{2+}$. In the data for the wild-type human NAD-IDH presented in this paper, $pK_{\text{ase}}$ ranges from 6.78 with Mg$^{2+}$ to 6.51 with Mn$^{2+}$ to 6.16 with Co$^{2+}$, which is consistent with the designation of the enzyme-bound metal-coordinated $\alpha$-hydroxyl of isocitrate as the ionizable group influencing $V_{\text{max}}$.

An important issue addressed in this paper is the functional role of the three dissimilar subunits of human NAD-dependent isocitrate dehydrogenase. Previous studies from this laboratory (11, 35–37) using mutagenesis and affinity labeling have provided evidence that the $\beta$ and $\gamma$ subunits contain the allosteric ADP sites. The results presented in this paper constitute the first experimental demonstration that a metal-isocitrate site of human NAD-dependent isocitrate dehydrogenase has contributions from both $\alpha$ and $\gamma$ subunits ($\alpha$-Asp-230 and $\gamma$-Asp-215 provide ligands for the Mn$^{2+}$ site, and $\alpha$-Asp-234 is required for high catalytic activity, probably affecting the Mn$^{2+}$ through its water ligands). These results explain the earlier observations that the addition of $\gamma$ subunit to an $\alpha$ subunit results in formation of an $\alpha\gamma$ dimer with substantial regain in activity (6) and that the addition of $\gamma$ subunit to an $\alpha_2\beta$ complex enhances activity about 7-fold (35, 38). The enzyme has two Mn$^{2+}$ binding sites per tetramer (4). For one Mn$^{2+}$ site, we have identified three aspartates ($\alpha$-Asp-230, $\alpha$-Asp-234, and $\gamma$-Asp-215), whereas for the second Mn$^{2+}$ site, we have located only two aspartates associated with metal binding ($\alpha$-Asp-230 and $\alpha$-Asp-234), since the corresponding aspartate on the $\beta$ subunit does not seem to influence Mn$^{2+}$ interaction. Either the two Mn$^{2+}$ sites of the enzyme are not identical, or we have yet to find the third aspartate (on the $\beta$ subunit), which interacts with the metal ion. The data of this paper also constitute an experimental demonstration that the NAD binding site includes amino acids from both the $\alpha$ and $\gamma$ subunits ($\alpha$-Asp-230 and $\beta$-Asp-217). Thus, the catalytic site is not the exclusive function of the $\alpha$ subunit but requires interaction of all of the subunits.

Yeast NAD-dependent isocitrate dehydrogenase is another extensively studied eukaryotic allosteric enzyme that is activated by a purine nucleotide (in this case, by AMP). This enzyme is composed of two types of distinct subunits, IDH1 and IDH2, of which the IDH2 subunit has been designated as the catalytic subunit, whereas the IDH1 subunit is the regula-
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