Role of α-Asp[181], β-Asp[192], and γ-Asp[190] in the Distinctive Subunits of Human NAD-Specific Isocitrate Dehydrogenase†

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ABSTRACT: Human NAD-dependent isocitrate dehydrogenase (IDH) is allosterically activated by ADP by lowering the K_m for isocitrate. The enzyme has three subunit types with distinguishable sequences present in the approximate ratio 2α:1β:1γ and, per tetramer, binds 2 mol of each ligand. To evaluate whether the subunits also have distinct functions, we replaced equivalent aspartates, one subunit at a time, by asparagines; each expressed, purified enzyme was composed of one mutant and two wild-type subunits. The aspartates were chosen because β-Asp[192] and γ-Asp[190] had previously been affinity labeled by a reactive ADP analogue and α-Asp[181] is equivalent based on sequence alignments. The α-D181N IDH mutant exhibits a 2000-fold decrease in V_{max}, with increases of 15-fold in the K_ms for Mn(II) and NAD and a much smaller change in the K_m for isocitrate. In contrast, the V_{max} values of the β-D192N and γ-D190N IDHs are only reduced 4−5-fold as compared to wild-type enzyme. The K_m for NAD of the β-D192N enzyme is 9 times that of the normal enzyme with little or no effect on the affinity for Mn(II) or isocitrate, while the K_ms for coenzyme and for Mn(II) of the γ-D190N enzyme are 19 and 72 times, respectively, that of the normal enzyme with a much smaller effect on the K_m for isocitrate. Finally, all three mutant enzymes fail to respond to ADP by lowering the K_m for isocitrate, although they *do* bind ADP. Thus, these aspartates are close to but not in the ADP site and are required for communication between the ADP and isocitrate sites. These results demonstrate that α-Asp[181] is the only one of these aspartates essential for catalysis. β-Asp[192] is a determinant of the enzyme's affinity for NAD, as is γ-Asp[190], while γ-Asp[190] also influences the enzyme's affinity for metal ion. We conclude that the NAD and ADP sites are shared between α- and β- and α- and γ-subunits, and the Mn(II) site is shared between α- and γ-subunits, while the α-subunit is essential for catalysis. Although α-Asp[181], β-Asp[192], and γ-Asp[190] may have derived from a common progenitor, these aspartates of the three subunits have evolved distinct functions.

Isocitrate dehydrogenases constitute a group of enzymes that catalyze the conversion of *threo*-D-isocitrate to α-ketoglutarate in the presence of NADP (cytosolic and mitochondrial enzyme) or NAD (mitochondrial enzyme) (*1*, *2*). The mammalian NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41) is a mitochondrial, tetrameric enzyme consisting of three types of subunits in the approximate ratio 2α:1β:1γ with respective molecular weights of 37, 39, and 39 kDa (*3*, *4*).

The oxidative decarboxylation reaction catalyzed by mitochondrial NAD-dependent isocitrate dehydrogenase is the conversion of isocitrate to α-ketoglutarate and CO_2, as NAD is reduced to NADH. The NADH, in turn, is used in the formation of the energy storage compound ATP. The enzyme is under strict cellular control, being activated by ADP and inhibited by the ultimate products of the reaction, NADH and ATP; thus, when the cell does *not* require energy, the inhibition of the enzyme by ATP decreases the rate of oxidation of isocitrate, whereas when the cell requires energy, the conversion of isocitrate to α-ketoglutarate is promoted by the ADP activation. Previous studies in our laboratory on porcine NAD-dependent IDH showed that the enzyme binds tightly 2 mol per enzyme tetramer of each of the following: isocitrate, Mn(II), NAD, ADP, NADH, and NADPH (*5*, *6*). Additionally, dimers consisting of either α- and β-subunits or α- and γ-subunits were found to have considerable catalytic activity and to be capable of binding the substrates and ADP, but not to be activated by ADP (*7*). Based on these structure−function studies, at least two different subunits are required for catalytic activity, although all four subunits must be present for full catalytic and regulatory activity (*7*). However, it has not been clear whether all ligands are shared between two subunits or whether certain subunits harbor unique sites.

Several affinity labeling studies of the NAD-dependent isocitrate dehydrogenase have been conducted using the reactive ADP analogues (*8*−*11*). The most extensive studies were performed with 2-BDB-TADP[1] (*10*, *11*). It was found

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[1] Abbreviations: DTT, dithiothreitol; IDH, isocitrate dehydrogenase; IPTG, isopropyl β-D-1-thiogalactopyranoside; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; 2-BDB-TADP, 2-(4-bromo-2,3-dioxobutylthio)adenosine 5′-diphosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; WT, wild type.

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NADP PIG IDH QKKWP-LYMSTKNTILKAY**D**^{220}GRFKD

NAD IDH α-Human NNHRSNVTAVHKANIMRMS**D**^{181}GLFLQ

NAD IDH β-Human KKGRGKVTAVHKANIMKLG**D**^{192}GLFLQ

NAD IDH γ-Human ESGRKKVTAVHKANIMKLG**D**^{190}GLFLQ

NADP E.coli IDH ANDRDSVTLVHKGNIMKFT**E**^{238}GAFKD

FIGURE 1: Comparison of the amino acid sequences of a selected region of the α-, *β*-, and *γ*-subunits of human NAD-dependent isocitrate dehydrogenase (in black), with those of the pig mitochondrial NADP-dependent isocitrate dehydrogenase (in blue) and the *E. coli* NADP-specific isocitrate dehydrogenase (in orange).

that incorporation of 2-BDB-TADP leads to 76% inactivation of IDH, along with loss of ADP activation. These functional changes appear to be caused by incorporation of 2 mol of reagent/enzyme tetramer. The major labeled peptide associated with those changes was LGDGLFLQ, in which the modified amino acid was an aspartate corresponding to Asp^{192} of the *β*-subunit and Asp^{190} located on the *γ*-subunit of the human IDH (*11*, *12*). As shown in Figure 1, all three subunits of the human NAD IDH (in black letters) are similar in sequence in this region, but only *β*- and *γ*-subunits are *identical* around this aspartate. In the present study, we focused attention on these three equivalent negatively charged aspartates of the α-, *β*-, and *γ*-subunits.

The cDNA encoding each of the three subunits has been cloned into one plasmid allowing the expression of all of the subunits in the same *Escherichia coli* cells and providing a convenient system for studying the role of these aspartic acid residues by site-directed mutagenesis (*4*). Asp^{181} of the α-subunit, Asp^{192} of the *β*-subunit, and Asp^{190} of the *γ*-subunit (Figure 1) were replaced (one at a time) by the neutral asparagine. Thus, three mutant enzymes were constructed, expressed, and purified, each of which has a mutation in one subunit type (α-D181N, *β*-D192N, or *γ*-D190N) with the other two subunit types being wild type. Kinetic characterization of the newly constructed IDH variants as well as their ADP binding properties are presented herein. These studies allow distinctions in function to be made among the α-, *β*-, and *γ*-subunits, and of the corresponding aspartic acid in each.

EXPERIMENTAL PROCEDURES

Materials. ADP, GDP, *β*-NAD, DL-isocitrate, triethanolamine chloride, citrate, TRIS, DTT, ampicillin, chloramphenicol, cellulose phosphate, and ammonium sulfate were purchased from Sigma. DEAE-cellulose (DE-52) was bought from Whatman. Ultragel AcA34 was obtained from Industrie Biologic Française. BL21-Codon Plus competent cells and QuikChange XL Site-Directed Mutagenesis Kit were obtained from Stratagene. QIAprep Spin Miniprep Kit was purchased from Qiagen. Amicon Ultra-15, YM-10 centricons, Amicon stirred cell, and YM-10 membranes were obtained from Millipore. Slide-A-Lyzer mini dialysis units were purchased from Pierce. Other high-grade chemicals were obtained from Fisher Scientific.

Site-Directed Mutagenesis. The template for PCR mutagenesis was the pHIDHα$\beta_2\gamma$ expression vector carrying the entire human isocitrate dehydrogenase gene, as we have described (*13*). Site-directed mutants of isocitrate dehydrogenase were obtained by using the following primers: 5′-GCGGATGTCAAACGGGCTTTTTCTACAAAAATGC-3′ (α-D181N forward), 5′-GCATTTTTGTAGAAAAAGCCC-GTTTGACATCCGC-3′ (α-D181N reverse), 5′-GAAACT-TGGGAACGGGTTGTTCCTGC-3′ (*β*-D192N forward), 5′-GCAGGAACAACCCGTTCCCAAGTTTC-3′ (*β*-D192N reverse), 5′-GAAACTGGGCAACGGGCTTTTCCTCC-3′ (*γ*-D190N forward), 5′-GGAGGAAAAGCCCGTTGCCCA-GTTTC-3′ (*γ*-D190N reverse). Each mutation was introduced in one subunit at a time using the QuikChange kit, following the manufacturer's protocol. For every mutant plasmid, the nucleotide sequence encoding each of the three subunit types was confirmed by DNA sequencing (Allen Laboratory, University of Delaware). Plasmids containing the mutations were transformed into *E. coli* BL21-Codon Plus (DE3)-RP and stored at −80 °C in 85% glycerol (*14*).

Protein Expression and Purification. E. coli cells harboring the desired mutant or wild-type IDH were grown overnight at 37 °C in 400 mL of LB containing 100 *μ*g/mL ampicillin and 50 *μ*g/mL chloramphenicol. This starter culture was used to inoculate 10 L of LB medium in four Erlenmeyer flasks. The cells were further grown in a shaker at 37 °C, 200 rpm, to an OD_{600} nm of 1.0−1.4. At this point, the temperature was lowered to 25 °C, the shaking rate was reduced to 130 rpm, and overexpression of IDH was induced with the addition of IPTG to a final concentration of 0.5 mM. The growth was continued for 20−22 h. The cells were collected by centrifugation at 10000*g* for 10 min (Sorvall RC2-B), and the pellet (typically 25−30 g) was frozen at −80 °C.

To purify the enzyme, the frozen pellet (typically 15 g) was thawed under tap water and resuspended in 50 mL of ice-cold 12 mM citrate-Tris, pH 7.2, 10% glycerol, 0.2 mM Mn(II), and 0.1 mM DTT containing 4 mg of lysozyme and 0.5 mL of 100 mM PMSF, and sonicated 5 × 1 min with 1 min intervals with a large probe at 30−40 kHz and 475 W (Cell Disruptor W-220F, Heat Systems-Ultrasonics, Inc.). The lysate was centrifuged for 30 min at 27000*g* to remove the cell debris. Ammonium sulfate was added to the supernatant to 20% w/v, and the precipitate was removed by centrifugation, as described above. The IDH enzyme was precipitated by the addition of ammonium sulfate to a final concentration of 36% w/v. Upon centrifugation, the pellet was resuspended in 20 mL of 12 mM citrate-Tris buffer, pH 7.7, 20% glycerol, 0.2 mM $MnSO_4$, and 0.1 mM DTT.

The protein solution was dialyzed against 4 L of the same buffer, with one change, for at least 8 h each. This solution was applied to a DE 52 column (2.5 × 17 cm), which had been equilibrated with the same buffer, and unbound proteins were eluted overnight. The NAD-dependent IDH was then eluted in a linear gradient formed from 200 mL of 12 mM citrate-Tris and 200 mL of 50 mM citrate-Tris buffer, both at pH 7.7 and containing 20% glycerol, 0.2 mM $MnSO_4$, and 0.1 mM DTT. Fractions exhibiting NAD-isocitrate dehydrogenase activity (or containing two bands at approximately 40 kDa on an SDS−PAGE gel) were pooled and concentrated in an Amicon stirred cell with an YM-10 membrane.

The concentrate (10−20 mL) was dialyzed overnight against 3 L of 12 mM sodium citrate buffer, pH 5.7, containing 20% glycerol and 0.1 mM DTT and was applied to a cellulose phosphate column (2.5 × 15 cm) that had been equilibrated in the same buffer. Unbound proteins were removed in the same buffer; a 200 mL linear gradient (100

mL of 12 mM sodium citrate and 100 mL of 50 mM sodium citrate, pH 5.7, 20% glycerol, and 0.1 mM DTT) was started when the A_{280} reached the baseline. The enzyme eluted at the end of the gradient and was collected in tubes containing $MnSO_4$ (a final concentration of 1 mM). Upon concentration in the Amicon stirred cell, the purity of the enzyme was assessed by SDS−PAGE.

If necessary, the protein was further purified using Ultragel AcA34 resin (2 × 82 cm) equilibrated using 50 mM PIPES, pH 7.0, 10% glycerol, 0.2 mM $MnSO_4$, and 0.1 mM DTT. The protein solution (∼1 mL concentrated in an Amicon Ultra-15 concentrator, 10 000 molecular weight cutoff) was loaded on the column and eluted with the same buffer at 0.25 mL/min. Upon concentration in Amicon Ultra-15, the protein was aliquoted and stored at −80 °C until needed. The purity of the enzyme was confirmed by SDS−PAGE and N-terminal amino acid sequencing. When necessary, the buffer was exchanged using Slide-A-Lyzer microdialysis units (10 000 molecular weight cutoff).

Enzymatic Assay of IDH. IDH and mutant enzymes were assayed for catalytic activity with DL-isocitrate and NAD in 33 mM Tris acetate buffer, pH 7.2. As isocitrate is converted to α-ketoglutarate + CO_2, NAD is reduced to NADH. The reaction rate can be measured by monitoring the formation of NADH at 340 nm ($\epsilon = 6220\ M^{-1}\ cm^{-1}$) in a cell with a 1 cm light path. The standard conditions used were 33 mM Tris acetate buffer, pH 7.2, 20 mM DL-isocitrate and 1 mM NAD. The specific activity is defined as micromoles of NADH produced per minute per milligram of enzyme, when assayed under standard conditions. Specific concentrations of DL-isocitrate, NAD, Mn(II), ADP, and GDP are given in the text. All assays were performed on an Agilent 8453 spectrophotometer equipped with a constant-temperature cell holder and a Haake K20 water bath. The enzyme concentrations were determined from the absorbance at 280 nm with the value $E_{280}^{1\%} = 6.45$ (*5*). All assays were performed at 25.0 ± 0.1 °C unless stated otherwise. Kinetic parameters were obtained by fitting the experimental data into the Michaelis−Menten equation using the program SigmaPlot (Wavemetrix). The values of K_m and V_{max} are presented along with their standard errors.

Circular Dichroism. Spectra to determine the secondary structure were recorded on a Jasco J-710 spectropolarimeter in a 0.1 cm path length quartz cuvette. The protein was exchanged into 25 mM triethanolamine hydrochloride buffer, pH 7.0, 10% glycerol, 0.2 mM $MnSO_4$ as described above. Typically, the protein concentration was 0.3 mg/mL, as determined by the Bio-Rad protein assay using the wild-type isocitrate dehydrogenase as the standard. The mean molar ellipticity [θ] (deg cm^2 $dmol^{-1}$) was calculated from the equation $[\theta] = \theta/10nCl$, where θ is the measured ellipticity, C is the concentration of enzyme subunits (average molecular weight 37 722 Da), l is the light path length (0.1 cm), and n is the number of residues per average subunit ($n = 346$) (*13*).

Native Molecular Weight Determination. The gel filtration column (Ultragel AcA34, 2 × 82 cm) was equilibrated using 50 mM PIPES, pH 7.0, 10% glycerol, 0.2 mM $MnSO_4$, and 0.1 mM DTT. Protein standards (thyroglobulin A, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; and bovine serum albumin, 67 kDa) were loaded on the column at ∼2−5 mg/mL and eluted with the same buffer at 0.20 mL/min. The void volume (V_0) was determined using Blue Dextran 2000 ($V_0 = 47$ mL). The molecular weight of the native wild-type and mutant enzymes was determined from a plot of $\log(M_r)$ vs K_d $((V_e - V_0)/(V_t - V_0))$, where V_e is the elution volume and V_t is the total column volume (258 mL).

Amino Acid Sequence Determination. The N-terminal amino acid sequence was determined using an Applied Biosystems Protein/Peptide Sequencer (model Procise) equipped with an online microgradient delivery system (model 140 C) and a Macintosh computer (model 610). The N-terminal amino acid sequence of the first 10 amino acids of the mature human NAD-IDH α-subunit is TGGVQTVT-LI; that of the β-subunit is ASRSQAEDVR; while that of the γ-subunit is FSEQTIPPSA. As expressed in *E. coli* and purified, the NAD-IDH γ-subunit completely retains its initiating Met, and the β-subunit completely loses the initial Met, while the α-subunit only partially loses the initial Met. Thus, to calculate the amount of any amino acid at a particular position of the α-subunit, it is necessary to use the sum of that amino acid in two successive cycles of the amino acid sequencer. For γ-subunit, the N-terminal sequence of the mature protein is found at positions 2−11. The amount of the α-subunit was calculated from the average of amino acids 1−4 and 7−10, that of the β-subunit from amino acids 1−4 and 6−10, and that of the γ-subunit from amino acids 1−3 and 6−10 (to avoid the overlap of amino acids present in more than one subunit in the same cycle).

ADP Binding Studies. Binding studies were performed by ultrafiltration at room temperature (20−25 °C) in 50 mM PIPES buffer, pH 7.0, 20% glycerol, and 0.3 mM $MnSO_4$ (*14*). Centricons with YM-10 membranes were equilibrated with the above buffer, followed by equilibration with an ADP solution of a given concentration until the absorbance at 259 nm in the eluate and retentate was the same. To determine the amount of bound ADP, the protein-ADP solution (0.5−1 mL) was incubated for 5 min at room temperature followed by centrifugation for 5 min at 3000*g*. The initial filtrate was discarded, and the solution was centrifuged for an additional 10 min to yield a measure of the free [ADP]. Bound ADP was calculated from the difference between the total concentration of ADP in the enzyme solution and the concentration of free ADP based on $A_{259\ nm}$ ($\epsilon_{259} = 15\ 400\ M^{-1}\ cm^{-1}$).

RESULTS

Mutagenesis, Protein Expression, and Purification. Plasmids containing the site-directed mutants were successfully expressed in BL21-Codon Plus (DE3)-RP, albeit with slightly lower yields compared to the native enzyme. Purification followed the established protocol described by Soundar et al. (*13*, *14*) with minor modifications. The mutants of IDH bound to the resins under the same conditions as the native enzyme and eluted at similar volumes in the citrate gradient. Under standard conditions (20 mM DL-isocitrate, 1 mM NAD, and 1 mM $MnSO_4$) the native enzyme exhibited a specific activity of 29−41 μmol/min/mg of NADH produced, while the β-D192N and γ-D190N variants of IDH showed significantly lower activity (4.5 and 2.3 μmol/min/mg, respectively). α-D181N IDH was inactive under the same

Table 1: Comparison of the Subunit Ratio of the WT and Mutant IDHs[a]

	enzyme subunit ratio		
enzyme	α	β	γ
WT	2.16	0.97	1.00
α-D181N	2.24	1.03	1.00
β-D192N	2.36	1.00	1.00
γ-D190N	2.22	0.97	1.00

[a] The subunit compositions were obtained from N-terminal amino acid sequencing of the whole protein (amino acids 1−4 and 7−10 for α-subunit; 1−4 and 6−10 for β-subunit; 1−3 and 6−10 for γ-subunit), as specified under Experimental Procedures.

Table 2: Kinetic Constants for Mn(II) and NAD of WT and Mutant IDH Enzymes

enzyme	K_m^{Mn} (mM)[a]	K_m^{NAD} (mM)[b]	V_{max} (μmol/mg/min)	V_{max} (+ADP) (μmol/mg/min)
WT	0.067 ± 0.013	0.080 ± 0.002	41.8 ± 1.9	40.8 ± 1.6
α-D181N	1.09 ± 0.16	1.2 ± 0.1	0.018 ± 0.001	nd
β-D192N	0.14 ± 0.01	0.71 ± 0.03	8.8 ± 0.1	9.4 ± 0.4
γ-D190N	4.8 ± 0.1	1.5 ± 0.2	10.2 ± 1.2	12.2 ± 1.2

[a] Experiments were carried out in 33 mM Tris-acetate, pH 7.2, 20 mM isocitrate with or without 1 mM ADP. NAD concentrations were as follows: WT and β-D192N, 10 mM; α-D181N and γ-D190N, 15 mM. The values of K_m and V_{max} are given along with their standard errors. [b] Assay conditions: 33 mM Tris-acetate, pH 7.2, 80 mM isocitrate, 12 mM Mn(II), in the presence of 1 mM ADP (when noted); nd = not determined.

reaction conditions (i.e., activity less than 10^{-4} μmol/min/mg). The enzyme preparations were pure as indicated by polyacrylamide gel electrophoresis in the presence of SDS (data not shown). The purified proteins exhibit two bands on SDS−PAGE; the upper band represents the β- and γ-subunits (molecular weight 39 kDa), whereas the lower band contains α-subunits (molecular weight 37 kDa) (*3*, *13*, *14*). No significant differences between the wild-type and mutant enzymes were noted.

Determination of Molecular Weight of Native Enzymes by Gel Filtration. Gel filtration was used to determine the molecular weights of the wild-type and mutant enzymes (data not shown). Wild type, α-D181N, β-D192N, and γ-D190N all had elution peaks at the same volume, corresponding to 238 kDa. These results suggest that there is an equilibrium between a tetramer (calculated MW of 152 kDa) and octamer (MW of 304 kDa), as has been observed for the porcine NAD-dependent isocitrate dehydrogenase (*15*).

Determination of Subunit Composition by Amino Acid Sequencing. The amino acid sequences of α-, β-, and γ-subunits differ in the first 10 amino acids (*12*, *14*), as indicated in Experimental Procedures. Therefore, the N-terminal amino acid sequence of the isolated enzymes was obtained in order to determine the subunit composition of the wild-type and mutant enzymes. The yields of amino acids 1−10 of the mature protein were determined (with avoidance of those positions at which the same amino acid from more than one subunit appears in the same cycle), and the average ratio of α- and β-subunits relative to γ-subunit is presented in Table 1. The ratios of the α-, β-, and γ-subunits of the wild-type and three mutant enzymes all approximate 2:1:1. Thus, replacement of the aspartate residues did not result in major changes in the overall subunit composition.

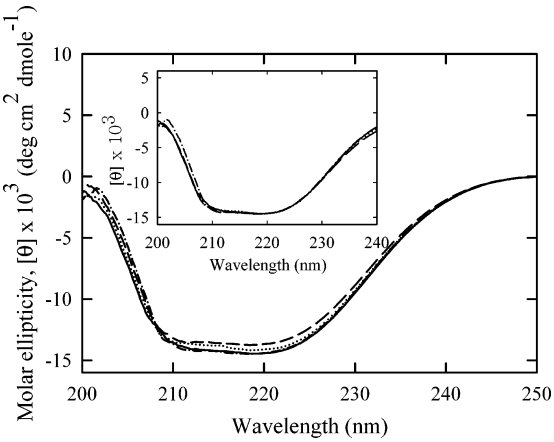

FIGURE 2: CD spectra of wild-type IDH (—, solid line), α-D181N IDH (− − −, dashed line), β-D192N IDH (•••, dotted line), and γ-D190N IDH (− • −, dot-dashed line). Spectra were recorded in 25 mM triethanolamine hydrochloride buffer, pH 7.0, containing 10% glycerol plus 0.2 mM $MnSO_4$, and the protein concentration was 0.3 mg/mL.

Circular Dichroism Spectra of Wild-Type and Mutant Enzymes. To evaluate whether the mutations caused changes in the secondary structure, we recorded CD spectra of the wild-type and mutant enzymes, which are shown in Figure 2. The spectra of α-D181N, β-D192N, and γ-D190N are very similar to that of the wild type and, when normalized at 225 nm, they are superimposible, indicating that no secondary structural changes are observed upon mutation of these three aspartates.

Kinetic Parameters for Mn(II) and NAD. Isocitrate dehydrogenase catalyzes the NAD-dependent conversion of isocitrate to α-ketoglutarate + CO_2. As the specific activities of the IDH mutants were lower than that found for the wild-type enzyme, it was necessary to determine whether this observation was due to altered affinity for the substrates or to a change in V_{max}. Mn(II) is known to be required for productive isocitrate binding to porcine IDH (*5*). Table 2 shows that both α-D181N and γ-D190N exhibit large increases (16−72-fold) in the K_m for Mn(II), whereas the β-D192N has only about a 2-fold increase in the K_m for Mn-(II), as compared to wild-type enzyme. These results indicate the involvement of α-Asp^{181} and γ-Asp^{190} in Mn(II) binding. Further kinetic experiments were conducted using 12 mM Mn(II), as higher concentrations of Mn(II) resulted in apparent inhibition of the enzymes. This metal ion concentration is saturating for all mutants except for γ-D190N IDH, which is close to saturation. The determination of the kinetic parameters for NAD was performed at 12 mM Mn(II) and 80 mM isocitrate (Table 2). A significant increase in the Michaelis−Menten constant for NAD was observed in all mutants, ranging from 9- to 19-fold. Most striking, however, is the 2000-fold decrease in V_{max} for α-D181N, while the β- and γ-subunit mutants exhibit V_{max} values that are only 4−5 times lower than that of the wild-type enzyme. The α-D181N mutant is nearly inactive, suggesting that α-Asp^{181} is directly involved in (or required for) the catalytic reaction.

Kinetic Parameters for Isocitrate. Changes in the affinity for DL-isocitrate mirror those for Mn(II). For β-D192N there is no effect on K_m for isocitrate, while the α-D181N and γ-D190N enzymes exhibit a 3−5-fold increase in the K_m for isocitrate (Table 3). The striking decrease in the V_{max} for

Table 3: Kinetics for Isocitrate of WT and Mutant IDH Enzymes[a]

enzyme	K_m^{IC} (mM)[a]	K_m^{IC} (+ADP) (mM)	K_m^{IC} (+GDP) (mM)	V_{max}^{IC} (μmol/mg/min)	V_{max}^{IC} (+ADP) (μmol/mg/min)
WT	2.4 ± 0.4 3.2 ± 0.3[b]	0.69 ± 0.10 1.9 ± 0.2[b]	1.5 ± 0.3	44.6 ± 3.0	42.4 ± 1.5
α-D181N	9.1 ± 1.9	9.0 ± 0.8	nd	0.024 ± 0.002	0.026 ± 0.001
β-D192N	2.9 ± 0.3	2.9 ± 0.2	5.9 ± 0.4	7.9 ± 0.3	9.6 ± 0.2
γ-D190N	13 ± 2	15.3 ± 0.6	15.3 ± 1.2	11.4 ± 0.6	15.6 ± 1.6

[a] All experiments were carried out in 33 mM Tris acetate, pH 7.2. Other conditions: WT and β-D192N, 1 mM $MnSO_4$ (1.45 mM in the presence of ADP or GDP) and 10 mM NAD; α-D181N and γ-D190N, 12 mM $MnSO_4$ and 15 mM NAD. When noted, the experiment was run in the presence of 1 mM ADP or GDP. V_{max} in the presence of GDP was identical, within error, to that without any nucleotide added to the assay. The K_m and V_{max} values are given with their standard errors. [b] Reaction conditions were identical to those for α-D181N and γ-D190N: 12 mM $MnSO_4$ and 15 mM NAD.

Table 4: ADP Binding to WT and Mutant Isocitrate Dehydrogenase[a]

	mol of ADP bound per mol of enzyme tetramer at the total ADP concn	
enzyme	50 μM	150 μM
WT	0.63 ± 0.27	1.50 ± 0.50
α-D181N	0.15 ± 0.03	0.55 ± 0.01
β-D192N	0.27 ± 0.02	0.83 ± 0.11
γ-D190N	0.67 ± 0.01	1.95 ± 0.27

[a] Binding studies were performed at room temperature in 50 mM PIPES, pH 7.0, 20% glycerol, 0.3 mM $MnSO_4$.

the α-D181N mutant is also seen in Table 3, as are the smaller V_{max} decreases for the β- and γ-subunit mutants.

Effects of ADP on Kinetic Parameters. ADP is known to increase the affinity of IDH for isocitrate as exhibited by a decrease in K_m for this substrate, without changing V_{max} (*10*). Indeed, the Michaelis−Menten constant for DL-isocitrate is lowered 3.5-fold in the wild-type IDH under the reaction conditions used (Table 3). In contrast, the substitution of asparagine for aspartate in α-D181N, β-D192N, and γ-D190N abolishes this allosteric effect of ADP on K_m (Table 3). In the presence of GDP which is not an activator of the wild-type IDH but complexes Mn(II), the kinetic parameters are similar to those without added ADP. Thus, the ADP allosteric effect cannot be attributed to the trivial effect of chelation of Mn(II).

The increase of K_m for NAD in the mutant enzymes along with the loss of response to ADP might suggest that the ADP binding site partially overlaps with that of NAD. To test this hypothesis, we determined the K_m for NAD in the presence of 1 mM ADP. If the ADP and NAD sites overlap, one would expect to see an increase in K_m for NAD in the presence of ADP. For WT enzyme, the K_m for NAD was 0.080 mM in the absence and 0.085 mM in the presence of ADP. Thus, the K_m value for NAD does not change when ADP is added. These results exclude the overlapping of the binding sites of these two nucleotides.

ADP Binding to Wild-Type and Mutant Enzymes. Mutation of these three aspartate residues results in the loss of ADP activation, as indicated by the failure of ADP to decrease the K_m for isocitrate. Kinetic experiments do not reveal whether the loss of activation is due to the inability of the enzyme to bind ADP or to elimination of the allosteric influence of ADP on other sites. To test whether the mutant enzymes retain the ability to bind ADP, we determined the amount of ADP bound to the enzymes at several total ADP concentrations. Table 4 demonstrates that all the mutant enzymes are capable of binding ADP, although they vary in affinity for the nucleotide. The wild-type enzyme, as well as the γ-D190N enzyme, binds up to 2 mol of ADP per tetramer, similar to previously obtained results for porcine and human NAD-dependent IDH (*5*, *13*). Although the affinity for ADP is reduced in the α-D181N and β-D192N mutants, they *do* bind ADP to an extent that increases as the ADP concentration is elevated. These results suggest that loss of the allosteric effect of ADP on the K_m for isocitrate is due to disruption of the communication between the ADP and substrate sites in the mutant enzymes.

DISCUSSION

All of the mammalian NAD-dependent isocitrate dehydrogenases which have been examined are composed of three distinguishable subunits (e.g., 16).[2] For the human enzyme, alignment of the α-, β-, and γ-subunits reveals 34% amino acid sequence identity as well as 23% close similarity, while the β- and γ-subunits are even more closely related (53% amino acid sequence identity plus 17% close similarity). It is highly likely that the α-, β-, and γ-subunits evolved from a common progenitor and, as their sequences diverged, the functions of the subunits were differentiated. The challenge has been to understand the role of each of these distinct subunits. The availability of a convenient expression system for the human NAD-dependent IDH in *E. coli* allows for detailed studies regarding the role of particular amino acid residues in the structure and function of the human IDH. Based on previous affinity labeling studies we focused on β-Asp^{192} and γ-Asp^{190}, the targets of the reactive ADP analogue (*11*, *12*). Additionally, we sought to evaluate the role of α-Asp^{181}, which is equivalent to the aspartates in the β- and γ-subunits based on amino acid sequence alignment (Figure 1). In each case the negatively charged aspartate of one subunit was replaced by the neutral asparagine in order to maintain a similar side chain size, while eliminating the charge.

[2] Although many studies on the NAD-dependent isocitrate dehydrogenase have been conducted on other mammalian enzymes (particularly the porcine NAD-IDH), the information obtained from these studies is expected to be relevant for the human NAD-IDH because of the high degree of amino acid sequence identity among the mammalian IDHs. Each of the α-, β-, and γ-subunits is estimated to be at least 94−97% identical in amino acid sequence when the enzymes from several mammals are compared (e.g., 16).

The three human mutant and wild-type enzymes all exhibit similar average molecular weights (238 kDa), suggesting that all of these preparations exist as equilibrium mixtures of tetramer (with a calculated molecular weight of 152 kDa) and octamer (with a calculated molecular weight of 304 kDa). These results are in agreement with previous studies on the porcine enzyme, for which an average molecular weight of 224 kDa was determined based on analytical centrifugation, light scattering, and gel filtration (*15*). The subunit composition also does not change appreciably in the mutant as compared to the wild-type enzyme and remains in the approximate ratio of 2α:1β:1γ subunit. Furthermore, the circular dichroism spectra of the mutant enzymes are not detectably different from that of the wild-type enzyme, implying that replacement of these three aspartates does not appreciably alter the secondary structure of human NAD-dependent isocitrate dehydrogenase.

In contrast, these single mutations cause striking changes in the catalytic properties of the enzyme. In the α-subunit, substitution of Asn for Asp^{181} results in a 2000-fold decrease in V_{max}, rendering the complete enzyme almost inactive. *An intact* α*-subunit is clearly essential for catalysis*. This conclusion is consistent with a previous study demonstrating that mutation of Arg^{88} of the α-subunit eliminates all catalytic activity (*14*). It is also in agreement with the earlier observation that, while isolated β- and γ-subunits had no activity, there was detectable activity associated with the isolated α_2 dimer and substantial activity in the $\alpha\beta$ or $\alpha\gamma$ dimer (*7*). The α-Asp^{181} appears to play a role in the catalytic mechanism. It has been proposed that the oxidative decarboxylation of isocitrate is initiated by the deprotonation of the C_2 hydroxyl of isocitrate as facilitated by a general base provided by the enzyme (*17*, *18*). The side chain carboxylate of α-Asp^{181}, either directly or (more likely) through a network of enzyme-bound water, may function to accept the proton from the metal-bound isocitrate hydroxyl (*13*, *19*).

In addition to the marked decrease in V_{max}, the α-D181N mutant exhibits weaker affinity for Mn(II), NAD, and isocitrate. The K_m values for Mn and NAD are increased about 15-fold as compared to the wild-type enzyme, while the K_m for isocitrate is only elevated about 4-fold. Although α-Asp^{181} may have some influence on the binding of substrates, these effects are much smaller than the effect on V_{max} of replacement of α-Asp^{181}. Thus, the major role of Asp^{181} of the α-subunit is its involvement in catalysis.

No crystal structure is yet available for any NAD-dependent isocitrate dehydrogenases. However, structures have been determined for several NADP-specific isocitrate dehydrogenases including that of pig mitochondria (*19*) and of *E. coli* (*18*). Amino acid sequence comparisons of these two NADP-specific isocitrate dehydrogenases with the three subunit types of human NAD-IDH reveal very low sequence identity. There *are* certain critical amino acids that interact with Mn^{2+} or isocitrate that are conserved; these amino acids include the arginines that interact with isocitrate (*14*) and aspartates that are ligands of Mn^{2+} (*13*). In contrast, the alignment of the two NADP-enzymes, in the region around α-Asp^{181}, β-Asp^{192}, and γ-Asp^{190}, is not very impressive (see Figure 1). The pig NADP-IDH has Asp^{220}, and the *E. coli* enzyme has a glutamate (E^{238}) which may be equivalent to the aspartates we have replaced in the three subunits of human NAD-IDH in this study. Examination of the crystal structure of porcine NADP-IDH reveals that Asp^{220} is far from the Mn-isocitrate or coenzyme sites (*19*): the closest distance between a carboxylate oxygen of Asp^{220} and isocitrate, Mn(II), or NADP is ~18 Å, 12 Å, and 16 Å, respectively. Similar distances are observed in the *E. coli* enzyme between Glu^{238} and the active site. Since the aspartates which are the mutagenic targets in the present study are not likely to be close to the active site, the effects on catalysis must be indirect. An oxygen of the homologous Asp^{220} of the pig IDH is only 2.7 Å from the nitrogen of Lys^{270} which is on the helix harboring Asp^{275} which, in turn, is one of the ligands of the active site Mn(II) (*19*). An influence of Lys^{270} on Asp^{275} may be transmitted either via the extensive water network or via Asp^{273} which is close enough for an electrostatic interaction with Lys^{270}. Similarly, a substitution for Asp^{181} of the α-subunit of human NAD-IDH may have an influence, mediated through the protein or the enzyme-bound water, on one of the direct ligands of the Mn(II). The structural perturbation would have to be subtle, because it is not reflected in a change in the circular dichroism spectrum of the mutant enzyme.

In contrast to the α-D181N properties, substitution for Asp^{192} of the β-subunit or Asp^{190} of the γ-subunit causes only about a 4−5-fold decrease in V_{max}. Clearly these equivalent aspartates on the β- and γ-subunits are *not* required for catalysis. In the case of β-Asp^{192}, replacement by Asn does not affect the enzyme's affinity for Mn(II) or for isocitrate; rather, the largest change is the 9-fold increase in the K_m for NAD. An aspartate residue is often present in the binding pocket of NAD-dependent enzymes, where it forms hydrogen bonds to the 2′-OH and/or 3′-OH of ribose; examples include malate dehydrogenase, isopropylmalate dehydrogenase, and the K344D mutant of *E. coli* NADP-dependent isocitrate dehydrogenase which was designed for improved affinity for NAD (*20*−*22*). It is possible that β-Asp^{192} plays a similar role in human NAD-dependent isocitrate dehydrogenase.

In the γ-subunit mutant, γ-D190N, the K_m for Mn(II) is 72 times that of the wild-type NAD-IDH. Thus, γ-Asp^{190} may be a direct ligand of the metal ion, in addition to α-Asp^{230} and γ-Asp^{215}, which we reported previously (*13*). Since the γ-D190N mutant also has an elevated K_m for NAD (19 times that of the wild-type enzyme), γ-Asp^{190} may also contribute to the enzyme's affinity for its coenzyme.

All three mutant isocitrate dehydrogenases have lost their ability to lower the K_m for isocitrate in response to the wild-type enzyme's allosteric activator, ADP. We had expected this loss of allosteric regulation by ADP because affinity labeling by 2-BDB-TADP of β-Asp^{192} and γ-Asp^{190} resulted in insensitivity to ADP, and protection against 2-BDB-TADP was provided by ADP (*10*−*12*). However, the mutant enzymes with Asn replacing Asp retain the ability to bind ADP, although the nucleotide does not affect the K_m for isocitrate. We conclude that these aspartates are not directly within the ADP site but, rather, are close to the allosteric site where they influence communication between the ADP and the isocitrate sites. The isocitrate binding site has been located within the α-subunit by the effect of mutation of α-Arg^{88} (*14*). Allosteric activation by ADP must therefore require appropriate interactions between the α- and β-, or α- and γ-subunits of NAD-dependent isocitrate dehydrogenase. Although the native molecular weight and subunit

composition of the β-192N and γ-D190N mutant enzymes are normal, it appears that the detailed subunit interactions have been altered by elimination of these negatively charged amino acids.

In summary, despite high sequence homology among the α-, β-, and γ-subunits of human NAD-dependent isocitrate dehydrogenase, it appears that the three aspartates under investigation in this study have evolved different roles. The α-Asp^{181} is essential for catalysis, and may also facilitate the binding of substrates. The β-Asp^{192} is needed for optimal affinity of the enzyme for NAD, but is not critical for catalysis. The γ-Asp^{190} is a determinant of the enzyme's affinity for Mn(II), as well as for NAD, but is also not directly required for the catalytic reaction. Finally, at the corresponding positions of all three subunit types, the negatively charged aspartate is required to facilitate the site−site and subunit interaction needed for allosteric regulation by ADP. The results indicate that most of the ligand sites are shared between two subunits (NAD and ADP between α and β or α and γ; Mn(II) between α and γ), but only α-subunit is indispensable for catalysis.

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