Evaluation by Mutagenesis of the Importance of 3 Arginines in α, β, and γ Subunits of Human NAD-dependent Isocitrate Dehydrogenase*

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Mammalian NAD-dependent isocitrate dehydrogenase is an allosteric enzyme, activated by ADP and composed of 3 distinct subunits in the ratio 2α1β1γ. Based on the crystal structure of NADP-dependent isocitrate dehydrogenases from Escherichia coli, Bacillus subtilis, and pig heart, and a comparison of their amino acid sequences, α-Arg88, β-Arg99, and γ-Arg97 of human NAD-dependent isocitrate dehydrogenase were chosen as candidates for mutagenesis to test their roles in catalytic activity and ADP activation. A plasmid harboring cDNA that encodes α, β, and γ subunits of the human isocitrate dehydrogenase (Kim, Y. O., Koh, H. J., Kim, S. H., Jo, S. H., Huh, J. W., Jeong, K. S., Lee, I. J., Song, B. J., and Huh, T. L. (1999) J. Biol. Chem. 274, 36866–36875) was used to express the enzyme in isocitrate dehydrogenase-deficient E. coli. Wild type (WT) and mutant enzymes (each containing 2 normal subunits plus a mutant subunit with α-R88Q, β-R99Q, or γ-R97Q) were purified to homogeneity yielding enzymes with 2α1β1γ subunit composition and a native molecular mass of 315 kDa. Specific activities of 22, 14, and 2 mol of NADH/mol of enzyme tetramer were measured, respectively, for WT, β-R99Q, and γ-R97Q enzymes. In contrast, mutant enzymes with normal α and β subunits and α-R88Q mutant subunit has no detectable activity, demonstrating that, although β-Arg99 and γ-Arg97 contribute to activity, α-Arg88 is essential for catalysis. For WT enzyme, the Kₘ for isocitrate is 2.2 mM, decreasing to 0.3 mM with added ADP. In contrast, for β-R99Q and γ-R97Q enzymes, the Kₘ for isocitrate is the same in the absence or presence of ADP, although all the enzymes bind ADP. These results suggest that β-Arg99 and γ-Arg97 are needed for normal ADP activation. In addition, the γ-R97Q enzyme has a Kₘ for NAD 10 times that of WT enzyme. This study indicates that a normal α subunit is required for catalytic activity and α-Arg88 likely participates in the isocitrate site, whereas the β and γ subunits have roles in the nucleotide functions of this allosteric enzyme.

Mammalian NAD-dependent isocitrate dehydrogenase (three-D-isocitrate-NAD⁺ oxidoreductase (decarboxylating), EC 1.1.1.41) is an allosteric mitochondrial enzyme regulated by ADP, which activates by lowering the Kₘ for the substrate isocitrate without changing the Vₘₐₓ (1). It is a hetero-oligomer, composed of three distinct types of subunits, present in the ratio 2α1β1γ. The subunits have molecular weights of 37,000, 39,000, and 39,000 for α, β, and γ, respectively, whereas their isoelectric points are distinctive (2, 3).

The pig heart NAD-dependent isocitrate dehydrogenase has been shown to bind tightly, per mole of enzyme tetramer, 2 mol of every ligand (isocitrate, Mn²⁺, NAD, ADP, NADH, and NADPH), indicating that there are half as many sites as subunits (4, 5). These observations have raised the question as to whether the subunits have specialized functions for particular ligands or if there is a functional similarity among the different subunits, and two subunits contribute to a given ligand site. Ehrlich and Colman (6) showed, by separating and recombining the subunits of the pig heart enzyme, that αβ and αγ dimers exhibit substantial catalytic activity, whereas, the separate β monomer, γ monomer, and α dimer are essentially inactive. This study indicated the importance of association of α with either β or γ subunits to generate a catalytic species.

No crystal structure has yet been determined for any NADP-dependent isocitrate dehydrogenase; however, the crystal structures of the bacterial NADP-specific isocitrate dehydrogenase of Escherichia coli (7–9) and of Bacillus subtilis (10) are known. Recently, a crystal structure was solved for a mammalian NADP-enzyme:porcine NADP-specific isocitrate dehydrogenase complexed with Mn²⁺ and isocitrate (11, 32). In these enzymes, the Arg¹¹⁰ of E. coli (8), Arg¹¹⁰ of B. subtilis (10), and Arg¹⁰³ of pig heart (11) interact with the α and β carboxylates of isocitrate bound to the active site. In contrast to the mammalian NAD-dependent isocitrate dehydrogenases, these NAD-specific enzymes are not allosterically regulated by ADP.

Among the human enzyme α, β, and γ subunits, alignment of the amino acid sequences of all 3 subunits indicates 34% identity plus 23% close similarity. If these three subunits are aligned with the E. coli enzyme, there is 13% identity plus 29% similarity; whereas only 5% identity plus 22% similarity is observed when the human NAD enzyme is compared with both the E. coli and pig heart NADP-dependent enzymes. However, there are certain amino acids that are conserved, including those known to interact with the isocitrate in the E. coli, B. subtilis, and pig heart NADP-enzymes. Fig. 1 shows a comparison of an important region of amino acid sequence alignment of the human NAD-isocitrate dehydrogenase subunits with the E. coli, B. subtilis, and pig heart NADP enzymes. Despite the relatively low amino acid sequence identity among these enzymes, the human NAD-enzyme α-Arg⁸⁸, β-Arg⁹⁹, and γ-Arg⁹⁷ can be aligned with Arg¹¹⁰ of E. coli, Arg¹¹⁰ of B. subtilis, and Arg¹⁰¹ of the pig heart NADP enzymes; all 3 arginines are conserved.

Although there have been numerous biochemical and kinetic
studies of NAD-dependent isocitrate dehydrogenases isolated from bacteria, yeast, and animal tissues, the precise function of the individual subunits has not yet been determined for these allosteric mammalian enzymes. To elucidate the functional roles of mammalian enzyme subunits, Kim et al. (12) developed a co-expression system for the three subunits of the human NAD-isocitrate dehydrogenase in E. coli. They made a construct in which human isocitrate dehydrogenase (IDH) α, β, and γ subunits were inserted into an expression vector to produce a complete and active human NAD-dependent isocitrate dehydrogenase. In this paper, to evaluate the functional roles of α-Argγβ88, β-Argγβ89, and γ-Argγβ97 in catalysis and ADP activation, we have separately mutated the corresponding arginines of each of the three subunits of the human enzyme to neutral glutamine by site-directed mutagenesis. Enzyme containing one mutant subunit and two wild type subunits was then expressed. We report here the results of these first mutagenesis studies on a mammalian NAD-specific isocitrate dehydrogenase. A preliminary version of this work has been presented (13).

EXPERIMENTAL PROCEDURES

Materials—ADP, β-NAD, Nα-isocitrate (trissodium salt), triethanolamine chloride, citrate (monohydrate), Coomassie Brilliant Blue-R, n-dithiothreitol, ampicillin (monosodium salt), cellulose phosphate, rotenone, and ammonium sulfate were obtained from Sigma, Manganese sulfate, SDS, acrylamide/bis-acrylamide mixture, yeast extract, thymopine, isopropyl-1-thio-β-D-galactopyranoside, acetonitrile, and other high grade chemicals were purchased from Fisher Scientific. Matrix Gel Blue-A and Centricon YM-10 microconcentrator tubes were the products of Millipore Corp. The C, reverse phase column was from Vydac and the low molecular weight standard proteins kit was from Amersham Biosciences. The Superose-12 gel filtration column, higher molecular weight gel filtration calibration kit, and pT7-7 expression vector were obtained from Amersham Biosciences. Restriction enzymes and T4 DNA ligase were purchased from POSCOHEM (Sungnam, Korea). Glycine was obtained from Bio-Rad and pT7Blue T-vector from Novagen. DEAE-cellulose (DE-52) was bought from Whatman.

Site-directed Mutagenesis and Construction of Plasmids—The complete recombinant human NAD-dependent IDH was expressed in E. coli deficient E. coli using the plasmid pHIDHαβγ (7.0 kb) for expression of wild type, α, ββ, and γ subunits, as described in Kim et al. (12). Point mutations were introduced into a single subunit, one at a time, by an overlap extension (PCR)-based site-directed mutagenesis (14). Subsequently, the DNA encoding one mutant subunit and two wild type subunits were assembled into the expression vector.

The oligonucleotide primers used in constructing the mutant plasmids (with the nucleotide location in the enzyme subunit shown in parentheses) are as follows: P1 (sense), 5′-AGACATAGACTGCGGTGCATGCCGTTCA-3′ (m02-99, NheI); P2, 5′-CTGGATCACCAGCAGC-3′; P3 (antisense), 5′-GTCGACAGTTCGTTCCGCCAGC-3′ (337–357); P4 (antisense), 5′-GTGGATCATTTAGGACTCTGATTGTCAGTAAGTCG3′- (1116–1147, BamHI); P5 (sense), 5′-AGACCACACCTGATCTAGGTTGG3′ (248–264, EcoNl); P6 (sense), 5′-GATATGGCCGGTCGAGCTGATATTG-3′ (385–408; P7 (antisense), 5′-CAATTCTACCAGCGGCGCATTAC3′ (385–408; P8 (antisense), 5′-CTGGACACCTGGCGTCAGCGA3′ (746–769, Sphl); P9 (sense), 5′-TCGGGCCGCGAGTCTGCTGTAAGTCG-3′ (194–213, Sphl); P10 (sense), 5′-CAGACATCTGTTAACACCGTTCGAGAC3′ (396–420); P11 (antisense), 5′-GTCAGGGTCTCTTGGAGGATTGGTG3′ (396–420); P12 (antisense), 5′-ACAGGGCTGCGCCCCCAGAC3′ (862–881, Apal). The primer sequences, with the restriction sites in bold letters and the restriction enzymes in parentheses, are shown. The underlined codons are those mutated from arginine to glutamine. To construct the IDHα(Q88 β89 γ97) mutant, two overlapping DNA fragments were produced by amplification of the human pHIDHβγβ plasmid DNA as template, using two sets of PCR primers, namely, P1/P3 and P2/P4 as a first PCR step. The two overlapping DNA fragments, 0.28 and 0.81 kb with the IDHα(Q88) mutation, were then mixed and ligated in a second PCR step, using as end primers, the P1/P4 set. Subsequently, the mutant IDHα DNA (1.1 kb) obtained from PCR ligations was subcloned into pT7Blue T-vector. The occurrence of the desired mutation was confirmed by DNA sequencing in both directions. The IDHα DNA was excised with NdeI/BamHI digestion and then inserted into the NdeI/BamHI site of the expression vector pT7-7 (Amersham Biosciences) to construct the mutant pHIDHαβγβ plasmid. To produce a complete plasmid DNA harboring IDHα(Q88 β89 γ97) DNA, with wild type IDHβγ and IDHγ subunits, the IDHβ of pHIDHαβγβ plasmid, with wild type IDHβ and IDHγ subunits, the IDHβγ and IDHγ DNA from plasmids pHIDHβγ and pHIDHγ, respectively, were removed and each was sequentially added to the mutant pHIDHαβγ plasmid, as previously described (12).

For the construction of the IDHαββγ(Q99) mutant, two overlapping DNA fragments of IDHαββ DNA (0.16 and 0.39 kb) were amplified by PCR using two primer sets (P5/P7 and P6/P8). By using the P5/P8 primer set, two partial IDHαβ DNA fragments obtained from the first PCR step were then ligated in a second PCR step. The resulting 0.53-kb mutant IDHαββγ DNA, after digestion with EcoNl/Sphl, was subcloned into pT7Blue T-vector. The EcoNl/Sphl-cleaved wild type IDHα DNA in a pHIDHαβ plasmid to produce a mutant pHIDHαββγ plasmid DNA (12).

Similarly, two overlapping DNA fragments (0.23 and 0.46 kb) for mutant IDHγ(Q97) were amplified by PCR using P9/P11 and P10/P12 primer sets. After a second PCR ligation of these two DNA fragments using a P9/P12 primer set, a partial DNA fragment (0.68 kb) of the IDHγ(Q97) mutant was obtained. After digestion with Apal/SacI, it replaced the Apal/SacI-excised wild type IDHγ DNA in a pHIDHαβγ plasmid to generate a mutant pHIDHαββγ(Q97) plasmid (12).

Transformation and Overexpression of Recombinant IDH Proteins in E. coli Cells—The wild type and mutant recombinant plasmids were used to transform E. coli EB 108 (DE3 3) cells, deficient in isocitrate dehydrogenase activity, prepared as previously described (14). Three bacterial colonies with positive inserts were subcultured by growing overnight at 37 °C in LB media supplemented with ampicillin (0.05 mg/ml). Aliquots of E. coli cells harboring wild type and mutant plasmid were maintained in stock solution at ~80 °C in 40% glycerol. E. coli cells harboring either wild type or mutant plasmid from glycerol stocks were subcultured overnight in 200 ml of LB media containing ampicillin. Four-liter flasks, each containing 2 liter of LB media with 0.05 mg/ml ampicillin (to a total volume of 8 liters), were inoculated with freshly grown E. coli cells (2% v/v) and were grown at 37 °C, at 220 rpm, until the growth reached a mid-log phase or cell density of A600 nm = 0.4. The cultures were then placed in chilled water to lower culture temperature to room temperature, which was induced in cells by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 μM and the plates were shaken at the lower speed of 140 rpm with minimal aeration at 25 °C for 22 h. The cell cultures were then centrifuged at 5000 × g for 10 min and, to improve the effectiveness of cell lysis, the cells were suspended in the remaining supernatant and homogenized by two cycles of sonicating the cell suspensions for 20 seconds each. The cell suspensions were then centrifuged at ~8000 × g for 10 min and the clear supernatant was separated. The cell-free supernatant and isocitrate dehydrogenase activity were determined in this crude extract.

Assay for NAD-dependent Isocitrate Dehydrogenase Activity—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 35 μM Tris acetate buffer, pH 7.2, 20 mM NAD, 1 mM MnSO4, and 1 mM NAD as the final concentrations. During the purification, the protein concentration was determined from the A280 nm after correcting for the ratio of A280/A260 (15). For the pure preparations, enzyme concentrations in milligrams/ml were calculated using E1%1cm 280 nm = 6.45 (4). The specific activity was expressed as micromoles of product/min under the assay conditions. The crude extract and ammonium sulfate fractions, to assay isocitrate dehydrogenase activity, the NADH oxidase inhibitor, rotenone (10 μM in 100% ethanol), was added to the 1-mL standard assay solution to give a final concentration of 2.5 μM rotenone.

**SDS-PAGE of Wild Type and Mutant Enzymes**—To determine the purity of the different components during the purification procedure, aliquots of fractions were analyzed in 15% polyacrylamide gels containing 0.1% SDS in a discontinuous pH electrophoresis system (16). The preparation of stacking and resolving gel, electrophoresis running conditions, protein staining, and destaining solutions were followed as described (17). Pure mammalian NAD-dependent isocitrate dehydrogenase was recognized by the appearance of two close bands in approximately equal intensity, with an upper band (β, γ subunits) of 39,000 Da and a lower band of 37,000 Da (α subunits) (3, 18).

**Purification of Wild Type and Mutant Isocitrate Dehydrogenases from Crude Extract**—The wild type IDH was isolated from crude extract by ammonium sulfate fractionation: the solution was brought to 30% saturation with ammonium sulfate and centrifuged. The supernatant was then brought to 50% ammonium sulfate and centrifuged. The resultant precipitate was dissolved in 12 mM sodium citrate buffer, pH 7.4, containing 20% glycerol, 0.2 mM MnSO4, and 0.1 mM DTT (Buffer A). This crude extract was dialyzed against 6 liters of Buffer A (3 × 13 cm), which had previously been equilibrated with Buffer A. The column was eluted with the same buffer (525 ml) until A280 nm reached baseline. The bound enzyme was eluted from DE-52 in a linear gradient consisting of 100 ml of Buffer A and 100 ml of 50 mM sodium citrate buffer, pH 7.4, containing 20% glycerol, 0.2 mM MnSO4, and 0.1 mM DTT.

**Native Molecular Mass Determination by Gel Filtration Chromatography**—Wild type and mutant enzymes, 0.4 mL of 2.0 mg/ml were applied to a Superose-12 (1 × 30 cm) column of a fast performance liquid chromatography system (Amersham Biosciences). The enzymes had been dialyzed and the column was equilibrated and eluted with 50 mM HEPES buffer, pH 7.0, containing 20% glycerol, 0.2 mM MnSO4, and 0.1 mM DTT. The proteins were eluted at a flow rate of 0.5 ml/min and 0.5-ml fractions were collected. The column had a total volume of 24.0 ml (V0) and a void volume of 7.14 ml (Vv) determined with blue dextran 2000. The elution volume (Ve) for each protein was measured from A280 nm of the fractions. The native molecular mass of the wild type and mutant enzymes was determined from the molar ratios of amino acids in these cycles upon sequencing the whole protein.

**Amino Acid Sequence Determination**—The NH2-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 810).

**Determination of Subunit Composition and Separation of Subunits by High Performance Liquid Chromatography (HPLC)**—Wild type or mutant enzyme, 0.2 ml of 1 mg/ml was denatured by addition of 0.05 ml of 20% SDS and the solution was incubated at 35 °C for 24 h. The sample was diluted to 1.0 ml with 55 ml of a mixture containing 0.1% trifluoroacetic acid (CH3CN, 95:5), and applied to a C18 reverse phase column (Vydac) using a Varian 5000 liquid chromatograph system. The column was previously equilibrated with 0.1% trifluoroacetic acid in water and the proteins (1 ml fractions) were eluted with the same solution for 10 min followed by a linear gradient to 0.1% trifluoroacetic acid in CH3CN in 200 min. The fractions collected in each distinct peak were pooled and subjected to amino acid analysis and to A280 nm determination. The subunits in these protein samples were identified by comparing their NH2-terminal sequences with those of the known sequences of the enzyme subunits (12, 19). Subunit composition was determined by determining the area under each peak obtained from a whole protein sample applied to HPLC separation. Because the amino acids at positions 1, 6, and 7 are different in the subunits, the subunit composition of wild type and mutant enzymes was also determined from the molar ratios of amino acids in these cycles upon sequencing the whole protein.

**Kinetic Studies of Wild Type and Mutant Enzymes**—For Km determinations, the concentration of either coenzyme, Dl-isocitrate or MnSO4,
was varied, whereas the other substrates were maintained at the standard assay concentration. For the isocitrate $K_m$ determination, the substrate concentration varied between 0.05 and 20 mM; for the Mn concentration, the metal ion concentration was 0.02–5.0 mM; whereas, for the NAD $K_m$ determination, 0.01–8.0 mM coenzyme was used. For the ADP activation studies, 1 mM ADP and 1.65 mM MnSO$_4$ were included, whereas the concentration of m-isocitrate varied. For measurements with the γ-R97Q mutant enzyme, 5 mM NAD was included (instead of 1 mM) in the determination of the $K_m$-isocitrate and in the ADP activation studies. To measure the ADP concentration dependence of $V_{max}$ for the γ-R97Q enzyme, the ADP concentration was varied from 5 μM to 2 mM under the standard conditions at pH 7.2 except that the MnSO$_4$ concentration was 1.65 mM.

**RESULTS**

*Mutagenesis, Expression, and Purification of Wild Type and Mutant Enzymes*—The human NAD-dependent isocitrate dehydrogenase, with glutamine substituted for arginine at positions 88 in α, 95 in β, or 97 in γ subunit, was generated by an oligonucleotide-directed PCR method and expressed in E. coli using the protein expression vector pHisDHαβγ (12, 14). There are human IDHβ, (1.6 kbp) and IDHβγ (1.3 kbp) cDNA sequences corresponding to two isoforms of β subunit (12), of which the βγ subunit has been used here. Each subunit (with the introduction of one point mutation at a time) was assembled into the expression vector, while maintaining the other two subunits as wild type, to express a complete recombinant enzyme. The desired mutation that had been introduced in the plasmid DNA was confirmed by nucleotide sequencing analysis. The cDNA encoding wild type or mutant enzymes was expressed in IDH-deficient E. coli, and the enzymes were purified as described under “Experimental Procedures.”

The yield of purified human wild type and mutant enzymes was 9–36 mg from 8 liters of cell culture. The highest specific activity of the wild type human enzyme purified by this procedure was 22 μmol/min/mg, comparable with that of the NAD-specific isocitrate dehydrogenase purified from pig hearts (4). Whereas α-R88Q mutant enzyme preparation has no detectable activity (i.e., <6.4 × 10$^{-3}$ μmol/min/mg), the β-R99Q and γ-R97Q mutant enzymes exhibited specific activities of 14 and 2 μmol/min/mg, respectively, as shown in the Table II. The purity of the enzyme preparations was assessed by SDS-PAGE. Fig. 2 shows that all four of the purified human enzyme samples exhibit only the two bands characteristic of mammalian NAD-dependent isocitrate dehydrogenase (3, 18), with an upper band of 39 kDa (β, γ subunits) and a lower band of 37 kDa (two α subunits). The two bands exhibit approximately equal intensity as was shown for the pig heart NAD-dependent IDH enzyme (3, 18). Subunits α, β, and γ have distinct sequences in the region of the amino terminus.

**Fig. 2.** SDS-PAGE of purified wild type and mutant enzymes. Wild type, lane 2; α-R88Q, lane 3; β-R99Q, lane 4; and γ-R97Q, lane 5. Lanes 1 and 6 show standard proteins: phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). The same amount of each purified IDH sample (10 μg) was applied to each gel lane and electrophoresis was conducted under the conditions described in “Experimental Procedures.” In IDH, the upper band (39 kDa) contains both β and γ subunits, whereas the lower band (37 kDa) contains α subunits.

Edman degradation of each of the enzyme preparations reveals the presence of only the sequences of the α, β, and γ subunits of human NAD-dependent isocitrate dehydrogenase (12, 19). Circular dichroism spectra of expressed enzymes—Circular dichroism spectra of the wild type and mutant isocitrate dehydrogenases were determined to ascertain whether the mutations have caused an appreciable conformational change. The CD spectra of the three mutant enzymes are superimposable on the spectrum of the wild type enzyme. All the spectra exhibit minima at 208 and 220 nm characteristic of proteins with appreciable amounts of α-helix. These results indicate that the mutations do not result in a detectable change in the secondary structure of the enzyme.

**Determination of Molecular Mass of Native Enzymes by Gel Filtration**—The wild type and mutant enzymes were subjected to gel filtration on a Superose-12 column equilibrated and eluted in 50 mM HEPES, pH 7.0, buffer containing 20% glycerol, 0.2 mM MnSO$_4$, and 0.1 mM DTT. Fig. 3 shows that the wild type and mutant enzymes elute at about the same elution volume and each enzyme exhibits only one peak, indicating that these proteins have similar molecular weights under native conditions. As determined from a plot of log $M_r$ against $K_p$, using standard proteins, the molecular masses of the wild type and three mutant enzymes are ~315 kDa. These data indicate that the native enzyme exists in solution as an octamer. The native molecular weight for human NAD-dependent isocitrate dehydrogenase as measured here is comparable with those of the purified enzymes from beef heart (20) and pig heart (2, 21); these enzymes have been reported to have molecular masses of 333 to 340 kDa as determined by gel filtration.

**Determination of Subunit Composition of Enzymes by Reverse Phase Chromatography**—The SDS-denatured wild type and mutant enzymes were separated and eluted from a C$_4$ column equilibrated with 0.1% trifluoroacetic acid in water, using a gradient in acetonitrile, as illustrated in Fig. 4. The subunits of porcine NAD-dependent IDH have previously been shown to elute in the order: γ then β then α under similar conditions (22). (The microheterogeneity observed in Fig. 4, within the region designated as β subunit and within the region designated as α subunit, corresponds to the observation on isoelectric focusing of several bands within a given subunit group, each of which has the same NH$_2$-terminal amino acid sequence. This microheterogeneity has been attributed to differences in the extent of amidation of the acidic amino acid residues (3.). To confirm the identification of the enzyme subunits, the separated subunits were subjected to NH$_2$-terminal
sequencing. Table I shows the subunit composition of wild type and mutant human NAD-dependent isocitrate dehydrogenase. In each case, the ratio of \( \alpha:\beta:\gamma \) is approximately 2:1:1, as has been shown for the NAD-dependent isocitrate dehydrogenase isolated from pig hearts (2, 3). Thus the expression system described in this paper yields complete enzyme with each subunit type present in the correct proportion.

**Determination of Specific Activities and Kinetic Constants for Mn\(^{2+}\) and NAD\(^{-}\)**—Table II, first column, shows the specific activities of the purified wild type and mutant NAD-dependent IDH enzymes under standard assay conditions, as described under “Experimental Procedures.” No activity was detected for the \( \alpha\)-R88Q mutant enzyme either in crude extract or with the purified enzymes (i.e. activity was \( \sim 6.4 \times 10^{-3} \) \( \mu \)mol/min/mg); these results indicate the Arg\(^{97}\) in the \( \alpha \) subunit is essential for activity. The mutants \( \beta\)-R99Q and \( \gamma\)-R97Q have measurable catalytic activity, but the specific activity is lower than that of the wild type enzyme. This result demonstrates that Arg\(^{97}\) of \( \beta \) and Arg\(^{97}\) of \( \gamma \) subunits contribute to catalytic activity. The kinetic constants determined for Mn\(^{2+}\) and NAD of wild type and mutant enzymes are shown in Table II. For the \( \beta\)-R99Q mutant enzyme, the \( K_m \) values for Mn\(^{2+}\) and NAD are similar to those of wild type enzyme, indicating that Arg\(^{97}\) in \( \beta \) subunit is not needed for Mn\(^{2+}\) or NAD binding. For the \( \gamma\)-R97Q mutant enzyme, the \( K_m \) for Mn\(^{2+}\) of the \( \gamma\)-R97Q mutant is similar to that of wild type enzyme, indicating that Arg\(^{97}\) in the \( \gamma \) subunit is not required for Mn\(^{2+}\) binding. However, the \( K_m \) for NAD is about 10-fold higher in the \( \gamma \) subunit mutant as compared with wild type enzyme, indicating that \( \gamma\)-Arg\(^{97}\) contributes to the apparent affinity for NAD. The \( V_{\text{max}} \) value of 3.7 \( \mu \)mol/min/mg (obtained by extrapolating to infinitely high concentration of NAD) is the maximum activity that could be measured for the \( \gamma\)-R97Q mutant and is higher than its “specific activity” obtained with the 1 mM NAD concentration in the standard assay.

**Kinetic Parameters for Isocitrate and ADP Activation of IDH Enzymes**—ADP is known to activate mammalian allosteric NAD-dependent IDH enzymes by decreasing the \( K_m \) for isocitrate without changing the \( V_{\text{max}} \) (1). Table III shows that for the human wild type enzyme, the \( K_m \) for isocitrate and the 7-fold decrease in \( K_m \)-isocitrate in the presence of 1 mM ADP is comparable with that determined for the pig heart enzyme (1, 3).

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**Fig. 3.** Gel filtration of purified wild type and mutant enzymes. Approximately 0.8 mg of each protein was applied to a Superose-12 column of a fast performance liquid chromatography system, and proteins were eluted in 50 mM HEPES, pH 7.0, buffer containing 20% glycerol, 0.2 mM MnSO\(_4\), and 0.1 mM DTT. The gel filtration profiles for wild type (■), \( \alpha\)-R88Q (□), \( \beta\)-R99Q (△), and \( \gamma\)-R97Q (◊) isocitrate dehydrogenases are shown. The standard proteins used are: thyroglobulin A (669 kDa, A), ferritin (440 kDa, B), catalase (232 kDa, C), aldolase (158 kDa, D), bovine serum albumin (67 kDa, E), and chymotrypsigen A (25 kDa, F). The void volume (\( V_v \)) was determined using blue dextran 2000. Inset, plot of log molecular mass of standard proteins (■) as a function of their \( K_m \) values, as defined under “Experimental Procedures” in the section on gel filtration. The average \( K_m \) of human NAD-dependent isocitrate dehydrogenase is indicated by ◊.

**Fig. 4.** Reverse phase chromatography of wild type and mutant enzymes. The protein (0.2 mg) was treated with 4% SDS, as described under “Experimental Procedures.” The \( \alpha \), \( \beta \), and \( \gamma \) subunits were separated by HPLC (Ca column). The column was initially eluted for 10 min with 0.1% trifluoroacetic acid in water (1 ml/min), followed by a linear gradient from the initial solvent to 100% acetonitrile containing 0.07% trifluoroacetic acid in 200 min. The elution of proteins monitored at \( A_{220} \) (——) and the gradient (—) are shown.

**Table I**

| Enzyme subunit ratio | Enzyme |
|----------------------|--------|
|                      | \( \alpha \) | \( \beta \) | \( \gamma \) |
| Wild type            | 1.88    | 1.08    | 1.04    |
| \( \alpha\)-R88Q     | 1.92    | 0.96    | 1.12    |
| \( \beta\)-R99Q      | 1.80    | 1.04    | 1.16    |
| \( \gamma\)-R97Q     | 1.84    | 1.00    | 1.16    |
The assay for specific activity was carried out as described under “Experimental Procedures,” in 33 mM Tris acetate buffer, pH 7.2, containing 20 mM NAD, 1 mM isocitrate, 1 mM MnSO₄, and 1 mM NAD as final concentrations. The $K_m$-Mn$^{2+}$ and $K_m$-NAD of wild type and mutant enzymes were determined under the same standard conditions, with varying concentrations of either MnSO₄ or NAD, at pH 7.2. The data were analyzed using SigmaPlot and are reported with standard errors.

### Table II

| Enzyme      | Specific activity | $K_m$-Mn$^{2+}$ | $K_m$-NAD | $V_{max}$ (NAD)$^a$ | $V_{max}$ (1 mM ADP)$^a$ |
|-------------|------------------|-----------------|-----------|---------------------|--------------------------|
|             | μmol/min/mg      | μM              | μM        | μmol/min/mg         | μmol/min/mg              |
| Wild type   | 21.7             | 0.22 ± 0.02     | 73 ± 12   | 22.0 ± 0.9          |                          |
| α-R88Q      | 0.00             |                 |           |                     |                          |
| β-R99Q      | 13.5             | 0.22 ± 0.02     | 47 ± 3    | 13.7 ± 0.2          |                          |
| γ-R97Q      | 2.2              | 0.39 ± 0.06     | 820 ± 89  | 3.7 ± 0.1           |                          |

$^a$ These values were obtained by extrapolating to infinitely high concentrations of NAD.

$^b$ $<6.4 \times 10^{-3}$ μmol/min/mg.

### Table III

**Kinetic characteristics for isocitrate and ADP activation of wild type and mutant isocitrate dehydrogenases**

The enzyme activity was determined using standard assay conditions as described under “Experimental Procedures,” except that the concentrations of isocitrate were varied in the absence or presence of 1 mM ADP. The data were analyzed by SigmaPlot, and the standard errors are given.

| Enzyme      | $K_m$ isocitrate | $V_{max}$ isocitrate (1 mM ADP) | $K_m$ ADP | $V_{max}$ ADP (1 mM ADP) |
|-------------|------------------|---------------------------------|-----------|--------------------------|
|             | μM               | μmol/min/mg                     | μM        | μmol/min/mg              |
| Wild type   | 2.2 ± 0.3        | 0.32 ± 0.04                     | 22.0 ± 1.1| 21.6 ± 1.1               |
| α-R88Q      | 0.9 ± 0.1        | 0.77 ± 0.09                     | 13.1 ± 0.2| 13.6 ± 0.2               |
| β-R99Q      | 2.0 ± 0.5        | 1.80 ± 0.19                     | 2.3 ± 0.2 | 6.7 ± 0.2                |
| γ-R97Q      | 1.8 ± 0.3        | 1.49 ± 0.12                     | 3.4 ± 0.1 | 6.6 ± 0.1               |

$^a$ The $K_m$ and $V_{max}$ for isocitrate in the absence or presence of 1 mM ADP were determined at 5 mM NAD concentration under otherwise standard assay conditions at pH 7.2.

23). For the β-R99Q mutant enzyme, the $K_m$ for isocitrate is not appreciably different from that of the wild type enzyme, indicating that Arg$^{29}$ in the β subunit is not required for isocitrate binding. Because the addition of 1 mM ADP does not affect the $K_m$ for isocitrate, Arg$^{29}$ of the β subunit is critical for ADP activation; however, one cannot distinguish from this result whether this Arg$^{29}$ is required for ADP to bind or to activate the enzyme. For the γ-R97 mutant enzyme, the $K_m$-isocitrate, $V_{max}$ and ADP activation were determined both at 1 mM NAD and at the saturating 5 mM NAD. The results show that $K_m$-isocitrate remains the same as that of wild type enzyme, indicating that Arg$^{37}$ in the γ subunit is not involved in isocitrate binding. Addition of 1 mM ADP has no effect on the $K_m$ for isocitrate, but there is a 2-fold increase in $V_{max}$ observed at both concentrations of NAD, showing that Arg$^{37}$ in the γ subunit influences the ADP activation of the γ-R97Q mutant enzyme. In contrast to wild type or the β-R99Q mutant enzyme, the γ-R97Q enzyme, there is an effect on $V_{max}$ although there is no influence of ADP on $K_m$-isocitrate; therefore, it is apparent that ADP can still bind to the mutant enzyme.

**Determination of ADP Binding of Enzymes by Ultrafiltration**—Because the kinetic data do not yield information on ADP binding by the α subunit mutant enzyme (which lacks detectable catalytic activity), or β subunit mutant enzyme (because this nucleotide fails to affect the kinetics), we tested the ability of wild type and mutant enzymes to directly bind ADP using an ultrafiltration technique that is equivalent to equilibrium dialysis. Table IV shows the number of moles of ADP bound per mol of enzyme tetramer when 80–120 μM total ADP was added to the enzyme (at subunit concentration of 25 μM). For wild type enzyme, the result indicates that up to 2 mol of ADP/mol of enzyme tetramer are bound, similar to the results previously reported for pig heart native enzyme (6). The α-R88Q mutant is still capable of binding ADP, indicating that the Arg$^{29}$ mutation in α subunit does not prevent the ADP binding. The results indicate that the β-R99Q and γ-R97Q mutants can also bind nucleotide at 80–120 μM total ADP, although less ADP is bound by these mutant enzymes than by wild type enzyme at the same total concentrations of ADP. If it is assumed that the mutant enzymes can also bind up to 2 mol of ADP/mol of enzyme tetramer, it can be estimated that the dissociation constant for enzyme-ADP is 15.9 ± 7.7, 73.4 ± 11.1, 234 ± 97, and 513 ± 156 μM for wild type, α-R88Q, β-R99Q, and γ-R97Q enzymes, respectively. (Values of greater precision cannot be determined by the method used when the affinity between enzyme and ligand is this weak.) Because ADP increases 2-fold the $V_{max}$ of the γ-R97Q enzyme, the affinity between this mutant enzyme and ADP can also be estimated kinetically from the ADP concentration dependence of $V_{max}$, the dissociation constant for the γ-R97Q enzyme-ADP complex is estimated as 302 ± 44 μM by this method. All three mutant enzymes retain the ability to bind ADP, albeit with weaker affinity than wild type; however, substitution of Arg by Gln at β-Arg$^{29}$ and γ-Arg$^{37}$ clearly changes the allosteric effect of ADP on this isocitrate dehydrogenase.

### Table IV

| Enzyme         | mol of ADP bound/mol of enzyme tetramer at the total ADP concentration |
|----------------|-----------------------------------------------------------------------|
|                | 80 μM | 100 μM | 120 μM          |
| Wild type      | 1.48 ± 0.01 | 1.68 ± 0.08 | 1.92 ± 0.48 |
| α-R88Q         | 1.12 ± 0.01 | 1.28 ± 0.32 | 1.04 ± 0.08 |
| β-R99Q         | 0.64 ± 0.16 | 0.80 ± 0.01 | 0.48 ± 0.16 |
| γ-R97Q         | 0.40 ± 0.08 | 0.40 ± 0.08 | 0.48 ± 0.01 |

**DISCUSSION**

Both NADP- and NAD-dependent isocitrate dehydrogenases catalyze the conversion of isocitrate to α-ketoglutarate and carbon dioxide, although only the eukaryotic NAD-dependent enzymes are allosterically regulated. Despite the very low amino acid sequence identity plus similarity for the human NAD-dependent enzyme as compared with the NADP-dependent enzymes from bacterial as well as mammalian sources, certain critical amino acids involved in isocitrate binding are well conserved. Based on the known crystal structures of the NADP-dependent enzymes from *E. coli* (7–9), *B. subtilis* (10), and pig heart (11), we chose as candidates for mutagenesis one arginine from each subunit of the human NAD-isocitrate dehydrogenase that was equivalent to Arg$^{101}$ of the pig heart NADP enzyme: α-Arg$^{29}$, β-Arg$^{29}$, and γ-Arg$^{37}$ (Fig. 1). Arg$^{101}$ of the porcine enzyme is less than 2.9 Å away from the negatively charged α and β carboxylates of bound isocitrate (11), where it may help to position the isocitrate correctly for the catalytic reaction. The aim of the present study was to evaluate in the NAD-dependent isocitrate dehydrogenase whether the corresponding arginine in each type of subunit was important in catalysis, or whether, in some of the subunits, the arginine had a different function.

For each of the three mutant enzymes we constructed, only
one subunit contained an amino acid substitution, whereas the other two subunit types were wild type. Thus, the effect of replacing the equivalent arginine on each subunit could be separately examined. Neutral glutamines were used as replacements for the positively charged arginines, because they are similar in size and are therefore expected to produce minimal effects on the overall structure of the mutant enzyme.

In fact, the circular dichroism spectra of the three mutant enzymes are similar to that of wild type enzyme, indicating that none of the mutations causes any appreciable effect on the secondary structure of the enzymes. Furthermore, all expressed mutant enzymes have the same native molecular mass as wild type enzyme, as indicated by gel filtration. The average molecular mass observed (315 kDa) corresponds to that of an octamer, as has previously been reported for the porcine and bovine NAD enzymes (20, 21). Most significantly, the expression procedure did not affect the subunit composition of the enzymes, because both mutant and wild type human enzymes have similar ratios of 2:1:1 of αβγ subunits, the same characteristic ratios documented earlier for complete porcine NAD enzyme (3).

We now find that intact, purified mutant human enzyme, with only Arg88 of the α subunit replaced by Gln, is totally inactive, indicating that Arg88 in the α subunit is essential for catalytic activity. Although this mutant enzyme is inactive, it retains its ability to bind the allosteric activator, ADP, but with an affinity for the nucleotide that is a little less than that of wild type enzyme. These results show that Arg88 in the α subunit is not required for ADP binding. Because Arg88 is positively charged, it is likely that it interacts with the negatively charged α and β carboxylates of isocitrate as does Arg101 in the metal-isocitrate crystalline complex of the porcine NAD-isocitrate dehydrogenase (11). The role of Arg101 of the porcine NADP enzyme was supported by our mutagenesis study in which replacement of Arg101 by Gln caused an 18-fold increase in the $K_m$ for isocitrate as well as a decrease of $V_{max}$ to 4% of that of the wild type enzyme (24).

The importance for catalytic activity of Arg88 in the α subunit was presaged by affinity labeling studies of the NAD-dependent pig heart isocitrate dehydrogenase in our laboratory. The compound adenosine 5′-O-(4-bromo-2,3-dioxobutylthio)phosphate was shown to react at Arg88 and Arg98 residues in the α subunit concomitant with inactivation and, based on the ligands that protect against inactivation by the reagent, we concluded that the target site was the isocitrate substrate site (25). The same residues in the α-subunit (Arg88 and Arg98) were also labeled by 8-(4-bromo-2,3-dioxobutylthio)NAD; in that case, protection by both isocitrate and NADPH against inactivation led to the conclusion that the isocitrate and allosteric NADPH sites were overlapping (22, 23). Both of these studies with the pig NAD enzyme not only demonstrated that the modification of Arg88 in the α subunit led to loss of activity, but also that the α subunit contained the isocitrate binding site. These affinity labeling studies of the pig NAD enzyme are undoubtedly relevant to the human enzyme, because the α subunit sequence is highly conserved among the mammalian species: a comparison of the α subunit sequences of monkey, human, bovine, and pig shows that there is 94% identity plus similarity (22), and Arg88 of the α subunit is among the residues completely conserved among these species (22).

In contrast to the mutation in the α subunit, the mutant enzyme with β-R97Q as the only alteration retains about 63% of the $V_{max}$ of wild type enzyme. Clearly, Arg99 in the β subunit is not required for catalysis, although it has some influence on activity. Furthermore, the $K_m$ values for isocitrate, Mn$^{2+}$, and NAD are similar to the values for wild type enzyme, indicating that β-Arg99 does not contribute to the apparent affinity of the enzyme for active site ligands. The most striking change in the kinetic properties of the β subunit mutant enzyme is the loss of response to the ADP activator. Because this β subunit mutant is still able to bind ADP (albeit more weakly than wild type enzyme), it may be that β-Arg99 facilitates communication between ADP bound to the β subunit and isocitrate bound to the α subunits to effect a lowering of the $K_m$ for isocitrate.

The mutant enzyme featuring γ-R97Q retains about 17% of the maximum activity of wild type enzyme when saturated with the coenzyme, Mn$^{2+}$, and isocitrate. We conclude that Arg97 of the γ subunit makes some contribution but is also not essential for catalysis. Whereas the $K_m$ values for isocitrate and Mn$^{2+}$ are unchanged, the $K_m$ for NAD is 10 times higher than that of wild type enzyme. Therefore, Arg97 of the γ subunit must strengthen the affinity of the enzyme for the coenzyme. Perhaps the positively charged guanido group of arginine interacts with the negatively charged pyrophosphate moiety of NAD. We conclude that the γ subunit must contain at least part of the NAD binding site.

Another notable feature of the kinetics of the γ-R97Q mutant enzyme is that, similar to the β subunit mutant enzyme, it exhibits the same $K_m$ for isocitrate in the absence and presence of 1 mM ADP. In fact, 1 mM ADP increases the $V_{max}$ of the enzyme 2-fold (with an enzyme-ADP dissociation constant of about 300 μM) in contrast to the absence of an effect of ADP on $V_{max}$ for wild type, as well as the other mutant enzymes. Thus, the γ-R97Q mutant enzyme still can bind ADP. It appears that Arg97 of the γ subunit is a determinant of the mode of ADP activation; it may also modulate interaction between the ADP bound to the γ subunit and isocitrate bound to the α subunit.

Additional evidence for the location of the ADP sites on the β and γ subunits comes from affinity labeling of the ADP site of NAD-dependent isocitrate dehydrogenase by the reactive ADP analogue, 2-BDB-TADP (26). In this chemically modified enzyme, the $K_m$ for isocitrate is also insensitive to the addition of ADP. The modified amino acid was identified as the aspartate within the sequence LGDGLF, which was first located in the γ subunit (27) and later was also found to be in the β subunit (28). The target aspartates can now be identified as β subunit Asp192 and γ subunit Asp191 (22), indicating that β and γ are the two subunits that contain the ADP sites.

The earlier studies in which the individual subunits were separated as monomers and then recombined to αβγ dimers suggested that the β and γ subunits are functionally equivalent, although they are distinguishable in sequence (6). (The β and γ subunits are actually 53% identical in sequence and, in addition, have 17% strong similarities; they are much closer in sequence than either is to the α subunit.) The basic units of the enzyme, each containing one catalytic site and one ADP site per two subunits (4, 5), are proposed to be the αβγ dimers. These dimers are then assembled into tetramers and octamers. The allosteric yeast NAD-dependent isocitrate dehydrogenase, relatively distant in evolution from the human enzyme, is an octameric enzyme composed of only two types of subunits, termed IDH1 and IDH2. In a series of studies involving mutagenesis and gene disruption (e.g. Refs. 29–31), the laboratory of McAlister-Henn has attributed catalytic activity primarily to IDH2, and AMP activation to IDH1, but noted that there are important interactions between the subunits necessary for a functional enzyme.

The α, β, and γ subunits of the human NAD-dependent isocitrate dehydrogenase are clearly distinct but exhibit a resemblance in sequence: comparison of all three yields 34% identity plus 23% similarity. The present study, in which glutamine replaces a homologous arginine in each subunit in
separate mutant enzymes, revealed that α-Arg is essential for catalysis and likely participates in the isocitrate site; whereas β-Arg and γ-Arg contribute to the allosteric activation by ADP and (in the case of γ-Arg) the binding of NAD. We propose that in the course of evolution, gene duplication led to the diversity of subunits found in the mammalian NAD-dependent isocitrate dehydrogenase, and the arginine that was originally part of the isocitrate site was then recruited (in the new subunits) to play a role in the nucleotide functions of this allosteric enzyme.

REFERENCES
1. Cohen, P. F., and Colman, R. F. (1972) Biochemistry 11, 1501–1508
2. Ehrlich, R. S., Hayman, S., Ramachandran, N., and Colman, R. F. (1981) J. Biol. Chem. 256, 10560–10564
3. Ramachandran, N., and Colman, R. F. (1980) J. Biol. Chem. 255, 8859–8864
4. Ehrlich, R. S., and Colman, R. F. (1981) J. Biol. Chem. 256, 1276–1282
5. Ehrlich, R. S., and Colman, R. F. (1983) J. Biol. Chem. 257, 4769–4774
6. Ehrlich, R. S., and Colman, R. F. (1983) J. Biol. Chem. 258, 7079–7086
7. Hurley, J. H., Thorsness, P. E., Ramalingam, V., Helmers, N. H., Koshland, D. E., Jr., and Stroud, R. M. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 8635–8639
8. Hurley, J. H., Dean, A. M., Koshland, D. E., Jr., and Stroud, R. M. (1991) Biochemistry 30, 8671–8678
9. Stoddard, B. L., Dean, A. M., and Koshland, D. E., Jr. (1993) Biochemistry 32, 9310–9316
10. Singh, K. S., Matsuno, K., LaPorte, D. C., and Banaszak, L. J. (2001) J. Biol. Chem. 276, 26154–26163
11. Cecarelli, C., Grodsky, N. B., Aryaratne, N., Colman, R. F., and Bahnsen, B. J. (2002) J. Biol. Chem. 277, 43454–43462
12. Kim, Y. O., Koh, H. J., Kim, S. H., Jo, S. H., Hub, J. W., Jeong, K. S., Lee, I. J., Song, B. J., and Hub, T. L. (1999) J. Biol. Chem. 274, 36866–36875
13. Soundar, S., Park, J. H., Hub, T. L., and Colman, R. F. (2002) FASEB J. 16, A536
14. Tao, B. Y., and Lee, K. C. P. (1994) in PCR Technology: Current Innovations (Griffin, H. G., and Griffin, A. M., eds) p. 71, CRC Press, Cleveland, OH
15. Warburg, O., and Christian, W. (1941) Biochem. Z. 310, 384–387
16. Blackshear, P. J. (1984) Methods Enzymol. 104, 237–255
17. Soundar, S., and Colman, R. F. (1993) J. Biol. Chem. 268, 5264–5271
18. Ramachandran, N., and Colman, R. F. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 252–255