The human NAD-dependent isocitrate dehydrogenase (IDH) is a heterotetrameric mitochondrial enzyme with 2α:1β:1γ subunit ratio. The three subunits share 40–52% identity in amino acid sequence and each includes a tyrosine in a comparable position: αY126, βY137, and γY135. To study the role of the corresponding tyrosines of each of the subunits of human NAD-IDH, the tyrosines were mutated (one subunit at a time) to Ser, Phe, or Glu. Enzymes were expressed with one mutant and two wild-type subunits. The results of characterization of the mutant enzymes suggest that βY137 is involved in NAD binding and allosteric activation by ADP. The αY126 is required for catalytic activity and likely acts as a general acid in the reaction. The γY135 is also required for catalytic activity and may be involved in proper folding of the enzyme. The corresponding tyrosines in the three dissimilar subunits of NAD-IDH thus have distinctive functions.

Mammalian NAD-dependent isocitrate dehydrogenase (NAD-IDH) is a mitochondrial tricarboxylic acid cycle enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate, while reducing NAD to NADH. It is a heterotetramer with three subunits in the ratio 2α:1β:1γ (1), with molecular masses of ~37,000, 39,000, and 39,000 Da, respectively (2, 3). The amino acid sequences of the β and γ subunits of the human are 52.4% identical, whereas α and β subunits are only 40.4% and α and γ subunits are only 41.6% identical. This enzyme is allosterically regulated by ADP, which decreases the $K_m$ for isocitrate by ~38-fold (4). NAD-IDH has two binding sites per tetramer for each ligand: isocitrate, NAD, Mn$^{2+}$, and ADP (2, 5), raising the question of the function of each of the subunits.

We have recently shown that homozygous mutations exclusively of the β subunit of human NAD-IDH are a cause of Retinitis Pigmentosa, a hereditary degeneration of the retina that leads to blindness in patients (6). Characterization of these two types of mutant enzymes in lymphoblast cell extracts revealed a ~300-fold increase in $K_m$ for NADH and partial or complete loss of allosteric activation by ADP. The involvement of this enzyme in causing Retinitis Pigmentosa increases the importance of studying NAD-IDH in more detail.

High-resolution crystal structures are available for the homodimeric NADP-dependent IDH of pig and Escherichia coli (7–11). The individual subunits of human NAD-IDH are only about 25–34% identical in amino acid sequence to the E. coli NADP-IDH (Fig. 1A) and about 12–18% identical to the pig NADP-IDH. Although there is a low % identity among these enzymes, the isocitrate-binding site is well conserved, including E. coli Arg-119, Arg-153, Tyr-160, and Lys-230. A previous study of pig NADP-IDH showed that Tyr-140 interacts with the β-carboxylate of isocitrate and acts as a general acid in the reaction (8). The Tyr-160 mutants of E. coli NADP-IDH showed a decreased $k_{cat}$ (12, 13) and the crystal structure revealed that the tyrosine is appropriately positioned to donate a proton to the carbanion following decarboxylation (11). Partial alignment of the amino acid sequences of the human NAD-IDH and pig and E. coli NADP-IDH, shown in Fig. 1B, indicates that Tyr-160 of the E. coli and Y140 of the pig NADP-IDH align with Tyr-126 of the α subunit, Tyr-137 of the β-subunit and Tyr-135 of the γ subunit of the human NAD-IDH. In this study, to distinguish the role of each subunit, we engineered and characterized recombinant human NAD-IDH mutants of the conserved Tyr, each of which has one mutated subunit and two wild-type subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-goat IgG, ammonium sulfate, citric acid, adenosine 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. Manganese sulfate, sodium dodecyl sulfate, acrylamide/bis-acrylamide, yeast extract, isopropyl thio-β-D-galactopyranoside (IPTG), Tris base, glycine, and other high-grade chemicals were purchased from Fisher. Ultrafiltration tubes (Amicon Ultra YM-10) were from Millipore Corp. DEAE-cellulose (DE-52) and cellulose phosphate were obtained from Whatman International. Ultragel (ACA 34) gel filtration resin was from LKB Co. The QuikChange XL site-directed mutagenesis kit and E. coli XL-10 Gold cells were purchased from Stratagene Inc. Low molecular weight standard proteins were from Amersham Biosciences and QIA Prep Spin miniprep kits were purchased from Qiagen Co. Primary antibody (goat, polyclonal) against the human NAD-IDH γ subunit (IDH3G (C-19)) was from Santa Cruz Biotechnology, Inc. ECL Western blotting substrate was from Pierce. Nitrocellulose membrane was from GE Healthcare. Western blotting filter paper was from Thermo Scientific.
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Site-directed Mutagenesis—The pHIDHαβγ vector carrying all three subunits of human NAD-IDH was used as a template for the mutagenesis (14, 15). The following forward primers were used for the site-directed mutagenesis: αY126S: 5'-CC ATT CGA GAG AAC ACA GAA GGA GAA TCC AGT GAA ATT GAG CAT GTG-3'; αY126F: 5'-CC ATT CGA GAG AAC ACA GAA GGA TCC AGT GAA ATT GAG CAT GTG-3'; αY126E: 5'-CC ATT CGA GAG AAC ACA GAA GGA TCC AGT GAA ATT GAG CAT GTG-3'; βY137S: 5'-CAG ACA GAA GGG GAG TCC AGC TCT CTG GAA CAT GAG AGT GC-3'; βY137F: 5'-GTC CGG GAG AAC ACA GAA GGG GAG TCC AGC TCT CTG GAA CAT GAG AGT GC-3'; βY137E: 5'-GTC CGG GAG AAC ACA GAA GGG GAG TCC AGC TCT CTG GAA CAT GAG AGT GC-3'; γY135F: 5'-CC ATT CGA GAG AAC ACA GAA GGG GAG TCC AGC TCT CTG GAA CAT GAG AGT GC-3'. The mutated codon is underlined in each primer. Only one subunit was mutated at a time using the QuikChange kit. For each mutant plasmid, the mutation was confirmed by DNA sequencing (Allen Laboratory, University of Delaware). Plasmids containing the desired mutation were transformed into E. coli XL-10 Gold cells.

Protein Expression and Purification—E. coli XL-10 Gold cells harboring wild-type or mutant NAD-IDH plasmids were grown overnight in 250 ml of LB medium with 0.1% ampicillin at 37 °C and 220 rpm. Overnight culture (30 ml) was used to inoculate five 6-liter flasks each containing 2 liters fresh LB-ampicillin medium. The culture was allowed to grow until A600 of 1.0–1.5 was reached. NAD-IDH expression was then induced with 1 mM IPTG. The cells were grown overnight at 25 °C and 150 rpm and collected by centrifuging the culture at 5000 g for 10 min. The collected cells were resuspended in 12 mM citrate-Tris, pH 7.2 containing 10% glycerol, 0.2 mM MnSO4, and 0.1 mM DTT, and frozen at −80 °C.

NAD-IDH was purified as described by Soundar et al. (15, 16). The frozen pellet from 10 liters of cell culture was thawed and lysed under conditions described previously. The cell lysate was centrifuged, and the supernatant was subjected to precipitation.
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by 30–50% ammonium sulfate at 4 °C. The enzyme was then passed through a series of DE-52 (equilibrated at pH 7.7) and cellulose phosphate (at pH 5.7) columns. Purity was assessed by SDS-PAGE (17). If required, the enzyme was concentrated to ~1 ml in Amicon Ultra tubes (YM-10) and passed through the Ultrogel gel filtration column as described before (15, 16). In all cases, fractions exhibiting NAD-IDH activity or two bands at ~40 kDa by SDS-PAGE were pooled and concentrated in Amicon Ultra (Ultracel 10K) tubes.

The α126E mutant was purified similarly except that the cellulose phosphate column was omitted from the purification procedure. Instead, the fractions pooled after the DE-52 column were concentrated to ~1 ml and loaded on the Ultrogel gel filtration column. Purity was assessed by SDS-PAGE (17).

**Protein Expression and Western Blot for the γY135F Mutant—** Cells harvested from 2 liters of γY135F mutant and wild-type NAD-IDH cultures were resuspended in 35 ml of 12 mM citrate-Tris, pH 7.2, 10% glycerol, 0.2 mM MnSO₄, and 0.1 mM DTT. The cells were lysed and centrifuged to remove the pellet as described before. Various amounts of the supernatant were loaded on a SDS-PAGE gel and electroblotted on a nitrocellulose membrane, as described by Huang and Colman (18). The gel and the nitrocellulose membrane were soaked briefly in 10 mM CAPS, pH 11.0, 10% methanol (transfer buffer) and sandwiched between filter papers soaked in the same buffer. The transfer was conducted overnight in the Xcell II blot module from Invitrogen in the transfer buffer at 100 mA and 4 °C.

The blot was fixed with 5% milk in 0.1% Tween in Tris-buffered saline, pH 7.6 (TBST), pH 7.6 for 1 h. The blot was then soaked in 1:2000 dilution of the 1st antibody (goat polyclonal antibody) in 5% milk for 1 h at room temperature. The blot was then washed with TBST and soaked in 1:2000 dilution of the horseradish peroxidase-conjugated 2nd antibody (anti-goat IgG) for 20 min. The blot was washed with TBST and 1:1 solution of the ECL substrate (Pierce) was added to the blot. The blot was soaked in the solution for 5 min before viewing under the Fluorochem 8800 (Alpha Innotech). The area of each band was compared using the software ImageJ 1.40g.

**N-terminal Sequencing**—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 molar ratio was calculated as described by Bzymek and Colman (19).

**Determination of Enzyme Activity**—The standard conditions for assaying the enzyme activity were at 25 °C in 1 ml of 33 mM Tris acetate, pH 7.2, 20 mM DL-isocitrate, 1 mM NAD, and 1 mM Mn²⁺. The specific activity is defined as μmol of NADH produced/min/mg of enzyme, when assayed under standard conditions, with the formation of NADH being monitored by A₃₄₀nm (15). The α126E mutant was assayed in 30 mM MES at pH 6.1 and compared with the wild type at the same pH. The enzyme concentrations were determined from the A₂₈₀nm (E₄₃₀nm = 6.45) (2). The kinetic parameters were determined by fitting the data in the Michaelis-Menten equation using the program SigmaPlot.

**pH Profile**—For determination of the pH-profile, enzyme activity of the wild-type and mutants was measured in 1 ml at various pH values using the following buffers: sodium acetate (pH 4.4–5.8), MES (pH 5.4–6.6), and Pipes (pH 6.2–7.4) and triethanolamine hydrochloride (pH 6.8–7.6). All the buffers had a final concentration of 30 mM. The pH dependence of Vmax was determined for the wild-type and each mutant with 20 mM isocitrate, 1 mM NAD, and 1 mM Mn²⁺, unless otherwise stated.

**Circular Dichroism**—Secondary structure of the wild-type and mutant enzymes was determined using Aviv circular dichroism spectrometer (Model 400) in a 0.1 cm pathlength quartz cuvette. The enzymes were first dialyzed against 2 liters of 25 mM triethanolamine hydrochloride, pH 7.4, containing 10% glycerol and 0.2 mM MnSO₄. The molar ellipticity was determined using average molecular weights and average number of residues per subunit, as described before (15).

**RESULTS**

**Site-directed Mutagenesis, Protein Expression, and Purification**—Plasmids containing the wild-type and mutant NAD-IDH were successfully expressed in E. coli XL-10 Gold cells. All the mutants of IDH, except the γY135F and α126E, were purified using the established protocol described by Soundar et al. (15, 16). As assessed by SDS-PAGE (Fig. 2), the wild-type and mutant NAD-IDH enzymes were pure, exhibiting two protein bands, with the upper band reflecting the β and γ subunits (39 kDa) and the lower band representing the α subunit (37 kDa) (1, 15, 20).

Under standard assay conditions (20 mM DL-isocitrate, 1 mM NAD, and 1 mM MnSO₄ at pH 7.2) the enzymes showed the specific activities recorded in Table 1. The wild-type enzyme exhibited a specific activity of 26.3 μmol of NADH produced/min/mg of enzyme. The α126S, α126F, and α126E mutants had no activity under the standard conditions, suggesting that the Tyr in the α subunit is essential for the catalytic function. In contrast, the β137S, β137F, and β137E showed lower specific activities of 3.1, 12.2, and 7.9 μmol/min/mg, respectively, indicating that this Tyr in the β subunit may contribute to, but is not required for, activity.

**Western Blot on γY135F Mutant**—The γY135F mutant could not be purified using the conventional methods followed for wild-type and the other IDH mutants. There was also no detect-
TABLE 1
Specific activities of recombinant wild-type and mutant NAD-IDH
Activities of purified enzymes were measured under standard conditions in 33 mM citrate-Tris, pH 7.2, containing 20 mM isocitrate, 1 mM NAD, and 1 mM MnSO4.

| Sample          | Specific activity μmol/min/mg |
|-----------------|------------------------------|
| Wild-type       | 26.3                         |
| αY126S          | 0                            |
| αY126F          | 0                            |
| αY126E          | 0                            |
| βY137S          | 3.1                          |
| βY137F          | 12.2                         |
| βY137E          | 7.9                          |
| Wild-type*      | 0.049                        |
| γY135F*         | 0                            |

* As measured in the cell lysate.

TABLE 2
Subunit molar ratio of wild-type and mutant NAD-IDH

| Enzyme subunit ratio | α  | β  | γ  |
|----------------------|----|----|----|
| Wild type            | 2.23 | 1.02 | 1  |
| αY126S               | 2.29 | 0.98 | 1  |
| αY126F               | 1.79 | 0.85 | 1  |
| αY126E               | 1.88 | 0.83 | 1  |
| βY137S               | 2.06 | 1.15 | 1  |
| βY137F               | 2.14 | 0.90 | 1  |
| βY137E               | 2.00 | 0.93 | 1  |

Effect of pH on the Observed Vmax—Previous studies with the homodimeric pig NADP-IDH demonstrated that, in contrast to the wild-type enzyme, the Y140E enzyme exhibits an increase in activity as the pH decreases (8). To determine whether the corresponding tyrosines in the heterodimeric human NAD-IDH behave similarly, the pH dependence of Vmax was measured for the wild-type and mutant enzymes. As shown in

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**TABLE 3**

Kinetic parameters for wild-type and βY137 mutants at pH 7.2

| Sample       | $K_{m,Mn^{2+}}$ (μM) | $K_{m,isocitrate}$ (μM) | $K_{m,isocitrate}$ in presence of 1 mM ADP (μM) | $K_{m,NAD}$ (μM) | $V_{max}$ (μmol/min/mg) |
|--------------|----------------------|-------------------------|-----------------------------------------------|------------------|------------------------|
| Wild type    | 0.12 ± 0.01          | 2.0 ± 0.2               | 0.05 ± 0.01                                   | 0.06 ± 0.01      | 26.3 ± 0.5             |
| βY137S*     | 0.18 ± 0.03          | 1.2 ± 0.2               | 0.92 ± 0.38                                   | 0.63 ± 0.13      | 8.4 ± 1.5              |
| βY137F      | 0.18 ± 0.03          | 1.8 ± 0.3               | 0.28 ± 0.03                                   | 0.12 ± 0.02      | 12.3 ± 0.7             |
| βY137E      | 0.11 ± 0.02          | 3.4 ± 0.6               | 0.09 ± 0.03                                   | 0.09 ± 0.02      | 7.9 ± 0.7              |

*Experiments were carried out with 6 mM NAD keeping the other conditions constant.

**Kinetic Parameters for the Wild-type and αY126E Mutant at pH 6.1**—Although the αY126 mutants are inactive under standard conditions at pH 7.2, the αY126E has some activity at pH 7.2 (0.06 μmol/min/mg) in the presence of the higher 5 mM NAD. Because the pH profile of αY126E shows a higher $V_{max}$ at pH 6.1, the kinetic parameters for the αY126E mutant were determined at pH 6.1 and compared with those of the wild-type enzyme at the same pH. Table 4 shows that the $K_{m,isocitrate}$ is 30-fold higher than that of the wild-type enzyme and, in the presence of 1 mM ADP, the $K_{m,isocitrate}$ is reduced almost equally in the wild-type (3.6-fold) and the αY126E mutant (4.3-fold). $K_{m,NAD}$ for the αY126E mutant is 29-fold higher than that of the wild-type. The $V_{max}$ of the wild-type at pH 6.1 is 14.4 μmol/min/mg whereas that for the αY126E mutant is only 1.03 μmol/min/mg, suggesting a critical role for the residue in enzyme activity.

**DISCUSSION**

The α, β, and γ subunits of human NAD-IDH are clearly related, but each has a distinct amino acid sequence (Fig. 1A). However, the amino acid residues in the isocitrate-binding site of the pig and E. coli NAD-IDH, one of which is a conserved tyrosine, can be identified in all 3 subunits of the human NAD-IDH (Fig. 1B). This observation raises questions about the contribution of these residues, and hence of the subunits to enzyme function. Do they perform a similar or a different role, and is the active site shared between the subunits? The studies reported in this report, in which the tyrosine of one subunit at a time is replaced, provide insights into the role of the tyrosines in these three types of subunits.

In the case of the βY137 mutants, their kinetic properties revealed similar affinity toward Mn$^{2+}$ and toward isocitrate, as that of wild type; these results suggest that βY137 is not involved in binding either Mn$^{2+}$ or isocitrate. The relatively small changes in the $V_{max}$ also indicate that this residue is not essential for enzyme catalysis. The most striking changes in the βY137 mutants are their substantial increase in the $K_{m}$ for NAD, and their marked decrease in the ability of ADP to lower the $K_{m}$ for isocitrate. The βY137S and βY137F mutants exhibit a higher $K_{m}$ for NAD as compared with that of the wild type, with βY137S featuring the highest $K_{m}$ for NAD. The replacement of Tyr with Glu, however, causes almost no difference in the enzyme affinity toward NAD. Serine is considerably smaller than tyrosine and even though it has an aliphatic -OH, it is not surprising that Ser cannot interact with NAD as does Tyr at the same position. Phenylalanine is similar in size to tyrosine but lacks the phenolic -OH. Glutamate is similar in length to Tyr.

**FIGURE 5. pH profiles of wild-type and mutant enzymes.** A, wild-type and β subunit mutants: Wild-type (▲), βY137S (●); βY137F (○); βY137E (▼) in the following buffers: sodium acetate (pH 4.4–5.8), MES (pH 5.4–6.6), and PIPES (pH 6.2–7.4), and triethanolamine hydrochloride (pH 6.8–7.6) at standard substrate concentrations. B, α subunit mutants: αY126S (●); αY126F (○); αY126E (▼). The buffers used were same as in A, except 5 mM NAD was used to assay the αY126E mutant enzyme.

Fig. 5A, the pH profiles of the wild-type and βY137 NAD-IDH mutants are similar with apparent pK values (± S.E.) of 6.37 ± 0.05, 6.33 ± 0.10, 6.25 ± 0.04 and 6.45 ± 0.07 for the wild-type, βY137S mutant, βY137F mutant and βY137E mutant, respectively. The αY126S and αY126F mutants are both inactive throughout the pH range. In contrast, the αY126E mutant shows a rise in the observed $V_{max}$ as the pH decreases (Fig. 5B) with a pK of ~5.8 ± 0.25. This result suggests that the glutamate replacing αY126 (and presumably the natural Y126) must be protonated for the enzyme to be active and therefore the αY126E mutant of human NAD-IDH is similar to the Y140E mutant of pig NADP-IDH (8).
and has the polar -COO instead of the -OH but lacks the aromatic group. The observation that Glu is the best substitute for Tyr at βY137, indicates that the phenolic -OH or the -COO of Glu contributes to the affinity of the enzyme for NAD.

The allosteric effect of ADP is completely lost in the βY137S mutant enzyme and is partially lost in the βY137F mutant, whereas the βY137E enzyme exhibits about the same allosteric effect as that of the wild-type enzyme. The same order of the effectiveness of the replacement amino acids (i.e. Ser < Phe < Glu) suggests that the same properties determine the allosteric effect of ADP on the enzyme. Previous studies have shown that mutations at other positions in the β subunit resulted in a higher $K_m$ for NAD and complete or partial loss of allosteric activation by ADP (6, 15, 16). None of these studies showed a major effect on the $V_{max}$ of the enzyme. Hence, we conclude that the β subunit is important for NAD binding and for the allosteric effect of ADP, but it is not critical for enzyme activity.

In the case of the α subunit mutants, the αY126S and αY126F are inactive, and the αY126E is inactive at pH 7.2 implying that Tyr-126 is required for enzyme activity. It is striking that the αY126E mutant is active at low pH indicating that, when protonated, Glu can partially substitute for Tyr. The αY126 could be acting as a general acid that protonates the enolate after decarboxylation to form α-ketoglutarate; this role has been proposed for Tyr-140 of the pig NADP-IDH (8). Previous studies have shown that replacement of different amino acids in the α subunit of human NAD-IDH (Arg-88, Asp-230, and Asp-234) yield inactive enzyme (15, 16). These reports, together with the results presented in this study, indicate that alpha is the catalytic subunit.

In the case of the γ subunit mutant, although there is no activity in the cell lysate of the mutant, it is clear that the protein is expressed. The lack of activity can be interpreted to indicate either that γY135 is essential for activity or that this tyrosine is important for the correct folding of the enzyme. Since the enzyme did not bind normally to the ion exchange resins generally used for the purification of NAD-IDH, it appears that there is a major change in the exposed ionic groups of the enzyme. Therefore it seems likely that γY135 plays a structural role (or a role in the correct folding of the enzyme). Other amino acids of the γ subunit have been implicated in isocitrate, Mn$^{2+}$, and NAD binding, as well as the allosteric effect of ADP (15, 19).

Earlier studies on the human and pig NAD-IDH demonstrated that there are only two binding sites per tetramer for each ligand (2, 5), suggesting that the subunits may have specialized functions. The α, β, and γ subunits are clearly related to each other but, as the subunits deviated in amino acid sequence, their roles in the enzyme became differentiated. The α subunit is required for catalytic reaction. The β subunit is important for optimal binding of the coenzyme and for the allosteric effect of ADP. The γ subunit may contribute to optimal affinity for isocitrate, Mn$^{2+}$ and NAD and to the allosteric effect of ADP, as well as promoting proper folding of the enzyme. This study shows that the NAD and Mn$^{2+}$ sites are shared between the α and β subunits. Earlier studies have indicated that the active sites are shared between α and β subunits and between α and γ subunits (16, 19). Thus, although catalysis by the simple NADP-dependent isocitrate dehydrogenase is carried out by a single subunit type, the more complex allosteric NAD-specific isocitrate dehydrogenase requires three distinct subunits.

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**TABLE 4**

**Kinetic parameters for wild-type and αY126E mutant at pH 6.1**

| Sample         | $K_m$min$^{-1}$ | $K_m$isocitrate | $K_m$isocitrate in presence of 1 mM ADP | $K_m$NAD | $V_{max}$ |
|----------------|-----------------|-----------------|----------------------------------------|----------|----------|
| Wild type$^a$  | 0.01 ± 0.001    | 7.5 ± 1.1       | 2.1 ± 0.6                               | 0.08 ± 0.01 | 14.4 ± 1.0 |
| αY126E$^b$     | 0.29 ± 0.09     | 0.3 ± 0.1       | 0.07 ± 0.02                             | 2.33 ± 0.85 | 1.09 ± 0.1 |

$^a$ Experiments were carried out in 1 ml of 30 mM MES at pH 6.1, 40 mM isocitrate, 5 mM NAD, 1 mM MnSO$_4$, resulting in a higher $V_{max}$ at pH 6.1 compared to that seen in the pH profile for the wild-type enzyme.

$^b$ Experiments were carried out in 1 ml of 30 mM MES at pH 6.1, 20 mM isocitrate, 5 mM NAD, 1 mM MnSO$_4$. 

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