Subunit Interactions of Yeast NAD\(^+\)-specific Isocitrate Dehydrogenase*

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Yeast mitochondrial NAD\(^+\)-specific isocitrate dehydrogenase is an octamer composed of four each of two nonidentical but related subunits designated IDH1 and IDH2. IDH2 was previously shown to contain the catalytic site, whereas IDH1 contributes regulatory properties including cooperativity with respect to isocitrate and allosteric activation by AMP. In this study, interactions between IDH1 and IDH2 were detected using the yeast two-hybrid system, but interactions between identical subunit polypeptides were not detected with this or other methods. A model for heterodimeric interactions between the subunits is therefore proposed for this enzyme. A corollary of this model, based on the three-dimensional structure of the homologous enzyme from *Escherichia coli*, is that some interactions between subunits occur at isocitrate binding sites. Based on this model, two residues (Lys-183 and Asp-217) in the regulatory IDH1 subunit were predicted to be important in the catalytic site of IDH2. We found that individually replacing these residues with alanine results in mutant enzymes that exhibit a drastic reduction in catalysis both *in vitro* and *in vivo*. Also based on this model, the two analogous residues (Lys-185 and Asp-222) of the catalytic IDH2 subunit were predicted to contribute to the regulatory site of IDH1. A K189A substitution in IDH2 was found to produce a decrease in activation of the enzyme by AMP and a loss of cooperativity with respect to isocitrate. A D222A substitution in IDH2 produces similar regulatory defects and a substantial reduction in \(V_{\text{max}}\) in the absence of AMP. Collectively, these results suggest that the basic structural/functional unit of yeast isocitrate dehydrogenase is a heterodimer of IDH1 and IDH2 subunits and that each subunit contributes to the isocitrate binding site of the other.

NAD\(^+\)-specific isocitrate dehydrogenase is thought to be important for regulatory control of mitochondrial energy metabolism primarily because of kinetic responses to adenine nucleotides in *in vitro* assays. For example, catalytic activation of this tricarboxylic acid cycle enzyme from Saccharomyces cerevisiae by AMP (i.e. a response to low levels of ATP) was described several decades ago (1). A correlate of allosteric regulation is multisubunit structure, and, based on sedimentation velocity and gel filtration experiments, an octameric structure was proposed for yeast isocitrate dehydrogenase (2, 3). However, a nonequivalence of subunits was suggested by equilibrium binding studies that demonstrated twice the number of isocitrate binding sites relative to binding sites for Mg\(^{2+}\) or NAD\(^+\) (4).

In more recent studies, the yeast enzyme was shown to be composed of two nonidentical subunits, IDH1 \((M_r = 38,001)\) and IDH2 \((M_r = 37,755)\), with both being equally represented in the holoenzyme (5). The two subunits share 42% identity at the level of amino acid sequence. Both subunits were shown to be essential for holoenzyme activity, since disruption of either or both genes encoding the subunits results in yeast strains that exhibit no detectable NAD\(^+\)-specific isocitrate dehydrogenase activity and that are unable to grow with acetate as a carbon source (6, 7). The acetate growth phenotype is shared with yeast mutants containing disruptions in genes encoding other tricarboxylic acid cycle enzymes including malate dehydrogenase (8) and citrate synthase (9).

The yeast IDH1 and IDH2 subunits also share 32% sequence identity with *Escherichia coli* isocitrate dehydrogenase, which requires NADP\(^+\) as cofactor. The bacterial enzyme is a homodimer and contains two identical isocitrate-Mg\(^{2+}\) and NADP\(^+\) binding sites per dimer (12). The *E. coli* enzyme exhibits no allosteric regulation but is instead regulated by phosphorylation. This covalent modification of a specific serine residue located in the isocitrate binding pocket inactivates the enzyme under physiological conditions requiring reduced flux through the tricarboxylic acid cycle (11, 12). The target serine residue of the *E. coli* enzyme is conserved in both IDH1 and IDH2, and mutagenesis was previously performed to determine the function of the corresponding residues in each yeast subunit (13). An S98A substitution in IDH2 produced a mutant enzyme that exhibited a 60-fold decrease in \(V_{\text{max}}\) but retained AMP activation and cooperativity. In contrast, an S92A substitution in IDH1 produced a mutant enzyme with primary kinetic defects in regulatory properties of the enzyme, including loss of both AMP activation and cooperativity with respect to isocitrate. Based on these results, different functions were assigned to the subunits, with IDH2 being primarily responsible for catalysis and with IDH1 playing the primary role in regulation. In support of these assignments, residues comprising the catalytic site of the bacterial enzyme are more highly conserved in IDH2 than in IDH1 (7). We therefore postulate that both subunits contain isocitrate binding sites, but while the “active” site in IDH2 is catalytic, the active site in IDH1 has evolved to bind isocitrate for the purpose of cooperative control rather than catalysis.

Some subsequent mutagenesis studies have supported these designations of subunit function (14). In these studies, specific adjacent residues thought to be important for cofactor specificity were altered. These changes in IDH2 (D286A plus I287A) resulted in a dramatic decrease in activity due to a reduction in affinity for NAD\(^+\). However, parallel residue replacements in IDH1 (D279A plus I280A) eliminated AMP activation of the
enzyme. These results supported the designations of IDH2 as the catalytic subunit and of IDH1 as the regulatory subunit, and we postulate that homologous adenine nucleotide binding domains have evolved for binding of the cofactor by IDH2 and for binding of the allosteric activator by IDH1.

In contrast with results described above, other recent experiments have revealed that catalytic and regulatory functions are not strictly confined to distinct subunits. Based on a resi
due in pig heart isocitrate dehydrogenase that was previously implicated in the binding of an adenine nucleotide analog (15), the corresponding conserved aspartate residues in IDH1 and IDH2 were altered. Mutation of the conserved aspartate residue (D197A) in IDH2 reduced AMP activation, cooperativity of substrate binding, and $V_{\text{max}}$. Altering the homologous residue in IDH1 (D191A) produced an inactive enzyme (14).

To understand the functions of IDH subunits in more detail, the current study further examines the interactions and specific functions of subunits in yeast isocitrate dehydrogenase. Based on previous results showing that both IDH1 and IDH2 subunits copurify when only one is affinity-tagged (14) and that each residual subunit appears to be monomeric in the absence of the other subunit (6, 7), we have investigated the possibility that the basic structural/functional unit of the enzyme is a heterodimer of an IDH1 and an IDH2 subunit. As a corollary, based on the bacterial enzyme model for homodimeric subunit interactions, we have examined the potential contribution of a few residues from each yeast subunit to the isocitrate binding site of the other subunit. In light of results presented in this paper, we now interpret the kinetic effects of the D197A replacement in IDH2 and of the D191A replacement in IDH1 as indicative of contributions of each residue to the active site of the other subunit in the heterodimer.

EXPERIMENTAL PROCEDURES
Yeast Strains and Growth Conditions—In expression studies, the yeast haploid strain S173-6B (MATa, leu2–3, 112, his3–1, ura3–52, trp1–289) was used as the parental wild-type control. Strains containing gene disruptions (ΔIDH1::LEU2, ΔIDH2::HIS3, or ΔIDH1::LEU2/ΔIDH2::HIS3) were constructed using the parental strain as described previously (6, 7). Yeast strains used in two-hybrid analyses were constructed using the parental strain as described previously (6, 7), the corresponding conserved aspartate residues in IDH1 and IDH2 were altered. Mutation of the conserved aspartate residue (D197A) in IDH2 reduced AMP activation, cooperativity of substrate binding, and $V_{\text{max}}$. Altering the homologous residue in IDH1 (D191A) produced an inactive enzyme (14).

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Yeast Two-hybrid Constructs and Analyses—Polymerase chain reaction (PCR) was used to synthesize IDH1 and IDH2 gene fragments that lacked codons for the mitochondrial targeting sequences. PCR primers also introduced BamHI restriction sites 5' and 3' of the coding sequences for the mature proteins. The PCR products were subcloned into vectors pGOT1 and pGAD424 (CLONTECH Laboratories, Inc.), and the inserts were sequenced by the Center for Advanced DNA Technologies, San Antonio, TX, to ensure that no errors were introd
uced by PCR. The BamHI fragments were then subcloned into two other sets of two-hybrid vectors: pAS2–1 and pACT2 (CLONTECH Laboratories, Inc.) and pGBK-C2 and pGAD-C2 (17). The fusion constructs were then transformed singly and in all possible binding/activation domain pairs into the appropriate host strains: the CLONTECH vector constructs into strain Y190 and the pGBK-C2 and pGAD-C2 fusion constructs into PJ69–4. Transformsants were selected for their ability to activate the reporter genes. Y190 transformants were analyzed using a β-galactosidase colony lift filter assay, which was performed using CLONTECH Laboratories Protocol PT1030–1. A control strain contained a plasmid encoding the wild type GAL4 protein. PJ69–4 transformants were tested for their ability to grow on plates in the absence of adenine or histidine after incubation for 3 to 5 days at 30 °C.

Affinity Purification Tests for Identical Subunit Interactions—A 2.3-kilobase pair XbaI/HindIII fragment containing the IDH2 gene with codons for a C-terminal histidine tag was subcloned from pIDH1/IDH2His into pRS316. The resulting construct (pRS316 IDH2His) was transformed into the ΔIDH1 host strain. Immunoblot analysis was used to verify that this strain expressed two versions of IDH2, one native and one histidine-tagged. Similarly, a 4.0-kilobase pair EcoRI fragment containing the IDH1 gene with codons for a histidine tag was subcloned from pIDH1His/IDH2 (14) into pRS316. The resulting construct (pRS316 IDH1His) was transformed into the ΔIDH2 strain. This transformant contained two types of IDH1, one histidine-tagged and one native. The two strains were grown in 500-ml cultures of YM medium with glycerol/lactate as the carbon source to induce expression of the isocitrate dehydrogenase subunits (15). The cells were harvested, and extracts were used for affinity purification as described previously (14) with the following modifications. Buffer A contained only 20 mM imidazole, and the column absorption/elution procedure was not repeated. Column flow-through, wash, and eluant fractions were combined with equal volumes of loading buffer and electrophoresed on a SDS 12% polyacrylamide gel. The gel was transferred to a polyvinylidene difluoride membrane and blocked in 5% bovine serum albumin overnight. For analysis of fractions from the ΔIDH1 transformant expressing IDH2His, the membrane was incubated in a 1:500 dilution of IDH2 antisera (5), and protein was detected by autodiagnostics after incubation of the membrane with 125I-labeled protein A (19). Analysis of fractions from the ΔIDH2 transformant was conducted using enhanced chemiluminescence (Amersham Pharmacia Biotech ECL Kit and protocol).

Construction and Purification of Mutant Enzymes—Mutagenesis was performed using the Transform Site-Directed Mutagenesis Kit from CLONTECH Laboratories and the following primers to introduce planned substitutions: IDH1 K183A (5'-CAGCCTGGTCATCGCCGCAA-TATCATG), IDH1 D217A (5'-CTGCCACTTGGCAGCAGCTCATTGAC), IDH2 K189A (5'-TTTGCGTAGCCACTCCCTTATCCGAGCAG), and IDH2 D222A (5'-GAAAAGTCAA CTATTGCCAACAGTGTGTTA-AGG). Another primer was used to simultaneously eliminate a unique BamHI site in the vector for selection of the mutant plasmids. All mutations were confirmed by sequencing. A 1.0-kilobase pair ReoI/EcoRI fragment containing each mutation in IDH1 was subcloned into pRS316 (14). Similarly, a 1.5-kilobase pair BamHI/HindIII fragment containing each mutation in IDH2 was subcloned into pIDH1/IDH2His (14). Each of the four resulting plasmids and the two wild-type plasmids (pIDH1/IDH2 and pIDH1/IDH2His) were transformed into a ΔIDH1/ΔIDH2 host strain. Transformant strains were grown in 0.5–1.0 liters of YM medium with glycerol/lactate as the carbon source. Affinity-tagged enzymes were purified by chromatography using NTA–NTA resin (Qiagen) as described previously (14). Concentrations of purified proteins were determined by the method of Lowry (20). To ensure that holozyme was purified from all strains, samples of ~15 µg of each purified enzyme were electrophoresed on a 10% polyacrylamide/SDS gel and stained with Coomassie Blue (19).

Kinetic Analyses of Purified Enzymes—Isocitrate dehydrogenase activity was measured as described previously (5, 14). Values for $V_{\text{max}}$ and $K_m$ were determined by Hanes analysis (21) of initial velocity data. One unit of isocitrate dehydrogenase activity was defined as production of 1.0 µmol of NADH/min. All assays were performed at 24 °C and initiated by the addition of isocitrate. Data shown in plots are from a single representative experiment, whereas tabulated data represent results from three independent experiments. Hill plots include data points between 10 and 90% of $V_{\text{max}}$.

RESULTS
Yeast Two-hybrid Analyses—For analysis of subunit interactions in yeast isocitrate dehydrogenase using the yeast two-hybrid system, PCR was used to amplify IDH1 and IDH2 coding regions lacking mitochondrial targeting sequences. The amplified genes were sequenced to ensure that no errors were introduced by PCR and ligated into three different sets of yeast two-hybrid plasmids. Two vector pairs, pG8T9 with pGAD424 and pAS2–1 with pACT2, were utilized. The first in each pair contains the nucleotide sequence for the GAL4 DNA-biding

1 The abbreviations used are: PCR, polymerase chain reaction; NTA, nitrilotriacetic acid.
Affinity Purification Test for Identical Subunit Interactions—Affinity purification was previously used to demonstrate that IDH1 and IDH2 interactions are significant. Those studies showed that active holoenzyme could be purified using Ni²⁺-NTA column chromatography following the introduction of five consecutive histidine residues at the carbonyl terminus of either subunit (14). Here we used a version of this histidine tag method to further investigate identical subunit interactions. For this, two new yeast strains were created as described under “Experimental Procedures.” One strain has the IDH1 gene disrupted and contains a centromere-based plasmid to provide single copy expression of IDH2 with a histidine tag; the reciprocal strain has the IDH2 gene disrupted and expresses a histidine-tagged version of IDH1 supplied by a plasmid. As a result, each strain has two versions of the same subunit, one that is native and one that contains a histidine tag. Ni²⁺-NTA affinity chromatography was performed using whole cell extracts from each strain. Flow-through, wash, and eluant fractions were reserved from each column. As shown in Fig. 1, immunoblot analyses of column fractions demonstrate that native subunits do not copurify with otherwise identical affinity-tagged subunits. For example, analysis of the IDH2 subunits from the ΔIDH1 strain reveals that only the faster migrating native version of the subunit is found in the column flow-through fraction (Fig. 1A, lane 1), and neither form of the subunit is detected in the wash fraction (Fig. 1A, lane 2). Only the higher molecular weight histidine-tagged IDH2 subunit is observed in the eluant from the column (Fig. 1A, lanes 3 and 4). Similar results are observed for the ΔIDH2 strain expressing the IDH1 subunits, with the bulk of the native subunit and a small portion of the affinity-tagged species appearing in the column flow-through fraction (Fig. 1B, lane 1). Again, only the affinity-tagged form of IDH1 is observed in the fraction eluted with high concentrations of imidazole (Fig. 1B, lanes 3–5).

Thus, results from both two-hybrid and affinity purification methods demonstrate that subunit polypeptides show no substantial homomeric interactions, at least in the absence of the other subunit, and suggest that the basic structural unit of the enzyme is a heterodimer of an IDH1 and an IDH2 subunit.

**Table I**

| Yeast two-hybrid assays | Construct pair | Reporter gene assay | Construct pair | Reporter gene assay |
|-------------------------|----------------|---------------------|----------------|---------------------|
| Heterodimer tests       | pGBT9-IDH1 + pGAD424-IDH2 | β-gal + | pGBT9-IDH1 + pGAD424-IDH1 | β-gal + |
|                         | pGBT9-IDH2 + pGAD424-IDH1 | β-gal + | pGBT9-IDH2 + pGAD424-IDH2 | β-gal + |
|                         | pAS2–1-IDH1 + pACT2-IDH2 | β-gal + | pAS2–1-IDH1 + pACT2-IDH1 | β-gal + |
|                         | pGBT9-IDH1 + pACT2-IDH2 | β-gal + | pGBT9-IDH1 + pACT2-IDH1 | β-gal + |
|                         | pGBT9-IDH2 + pACT2-IDH1 | β-gal + | pGBT9-IDH2 + pACT2-IDH2 | β-gal + |
|                         | pAS2–1-IDH1 + pGAD424-IDH2 | β-gal + | pAS2–1-IDH1 + pGAD424-IDH1 | β-gal + |
|                         | pAS2–1-IDH2 + pACT2-IDH2 | β-gal + | pAS2–1-IDH2 + pACT2-IDH1 | β-gal + |
|                         | pGBP-C2-IDH1 + pGAD-C2-IDH2 | Ade⁻/His + | pGBP-C2-IDH1 + pGAD-C2-IDH2 | Ade⁻/His + |
|                         | pGBP-C2-IDH2 + pGAD-C2-IDH1 | Ade +/His + | pGBP-C2-IDH2 + pGAD-C2-IDH1 | Ade +/His + |

* The first plasmid in each pair contains an IDH gene fusion with the GAL4 DNA binding sequence, and the second contains an IDH gene fusion with the GAL4 transcription activation domain sequence.

* Reporter gene activity was determined as outlined under “Experimental Procedures.” “β-gal” is β-galactosidase activity indicated by filter lift assays; “Ade” and “His” refer to growth on plates with minimal medium in the absence of adenine or histidine.
allelic variants of the other subunit in a ΔIDH1ΔIDH2 yeast strain. Mutant enzymes were purified by Ni\(^{2+}\)-NTA affinity chromatography as described under "Experimental Procedures." Samples of all purified enzymes were electrophoresed on a denaturing gel and stained with Coomassie Blue. As shown in Fig. 4, both subunits copurify during affinity chromatography of the mutant enzymes. Differences in electrophoretic mobility are due to the histidine tags (e.g. compare mobility of the more slowly migrating histidine-tagged form of IDH1 in lanes 1–3 versus that for the native IDH1 subunit in lanes 4 and 5) and presumably to amino acid substitutions in mutant subunits as also previously observed (14). Differences in mobility are observed for both subunits containing substitutions for aspartate residues, D222A in IDH2 (Fig. 4, lane 2) and D217A in IDH1 (Fig. 4, lane 5). None of the residue substitutions appears to have any gross conformational effect on holoenzyme structure, since both subunits copurify when only one subunit is affinity-tagged.

Measurements of \(V_{\text{max}}\) were conducted using affinity-purified enzymes. \(S_0.5\) values for isocitrate were measured in the presence or absence of 100 \(\mu\text{M}\) AMP, and corresponding Hill coefficients were calculated. \(S_0.5\) values and Hill coefficients for NAD\(^+\) were determined in the presence of AMP. Results of the kinetic studies are illustrated in Fig. 5 and summarized in Table II. As previously reported (14), the two histidine-tagged wild-type enzymes share similar kinetic properties. \(S_0.5\) values for isocitrate are similar and reduced 5–6-fold in the presence of 100 \(\mu\text{M}\) AMP. For both enzymes, Hill coefficients calculated in the presence or absence of AMP range from 3.2 to 3.9.

As predicted by the model described above, mutant enzymes containing IDH1 K183A or D217A substitutions (with IDH2 histidine-tagged) exhibit severe defects in catalytic activity. For these mutant enzymes, using as much as 19 \(\mu\text{g}\) of enzyme per 1-ml reaction produces a barely measurable change in absorbance unit per min. Thus, the full range of kinetic parameters for these enzymes could not be determined. However, based on the minimally measurable activities, the substitution of alanine for Asp-217 produces a greater catalytic defect than the same replacement for Lys-183. Respective velocities of 1000- and 300-fold lower than the \(V_{\text{max}}\) value of the corresponding wild-type enzyme.

As also predicted by the model, we find that the cooperativity with respect to isocitrate is lost in the mutant IDH2 enzymes containing K189A or D222A substitutions; i.e. Hill coefficients for isocitrate are reduced to values slightly greater than 1 (Fig. 5 and Table II). In addition, allosteric activation of catalytic activity by AMP is also eliminated by the substitutions for Lys-189 and Asp-222; i.e. \(S_0.5\) values for isocitrate are largely unaffected by the presence of AMP. Thus, \(S_0.5\) values for isocitrate measured in the absence of this activator are similar for mutant and wild-type enzymes, the \(S_0.5\) values measured in the presence of 100 \(\mu\text{M}\) AMP are, respectively, 7- and 9-fold lower than \(V_{\text{max}}\) values for the corresponding wild-type enzyme.

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Electrophoretic analysis of affinity-purified wild-type and mutant forms of yeast isocitrate dehydrogenases. Histidine-tagged enzymes were expressed and purified as described under “Experimental Procedures.” Samples containing ~15 µg of each purified enzyme were utilized for electrophoresis and Coomassie Blue staining. The purified enzymes include IDH1\(^{\text{His}}\)/IDH2 (lane 1), IDH1\(^{\text{His}}\)/IDH2\(^{\text{D222A}}\) (lane 2), IDH1\(^{\text{His}}\)/IDH2\(^{\text{K189A}}\) (lane 3), IDH1/IDH2\(^{\text{His}}\) (lane 4), IDH1\(^{\text{D217A/IDH2His}}\) (lane 5), and IDH1\(^{\text{K183A/IDH2His}}\) (lane 6). In each lane, the slower migrating band is IDH1, and the faster migrating band is IDH2.

Discussion

Our original prediction of differential catalytic and regulatory function for the two homologous subunits of yeast NAD\(^+\)-specific isocitrate dehydrogenase was based on alignment of amino acid sequences and three-dimensional modeling using the sequence and structure of the E. coli NAD\(^+\)-specific enzyme (22). At similar positions in the sequences and in similar modeled orientations, IDH2 has residues identical to all of those in the E. coli isocitrate/Mg\(^{2+}\) binding site illustrated in Fig. 2. IDH1, on the other hand, shares some identities with residues in the bacterial enzyme site but differs in four of nine positions (with Ala, Phe, Thr, and Asn occupying respective residue positions corresponding to bacterial Arg-129, Tyr-160, Asp-307, and Asp-311). Since IDH1 lacks two of the bacterial enzyme residues (Asp-307 and Asp-311) involved in coordination of Mg\(^{2+}\), it seems unlikely that the putative cooperative site would bind an isocitrate-Mg\(^{2+}\) complex, potentially explaining previous observations that the yeast enzyme has twice as many isocitrate as Mg\(^{2+}\) binding sites (4). We are currently investigating this possibility. This structural information led us to propose that IDH2 contains the catalytic isocitrate-Mg\(^{2+}\) binding site and that IDH1 binds isocitrate alone in a manner important for cooperativity but not for catalysis. Results of mutagenesis experiments (Refs. 13 and 14; summarized in Table III) consistent with these designations of catalytic versus regulatory function include (a) substitutions for active site serine residues corresponding to bacterial Ser-113 that reduce catalytic activity (IDH2 S98A) versus cooperativity and AMP activation (IDH1 S92A) and (b) substitutions for adjacent residues corresponding to bacterial residues known to be important for cofactor specificity (25) that eliminate apparent binding of NAD(H) by IDH2 (D286A/I287A) versus of AMP by IDH1 (D279A/I280A).

Results from both yeast two-hybrid assays and affinity chromatography (Ref. 14; this study) indicate that there is significant physical interaction between IDH1 and IDH2 subunits.
other words, Lys-189 presence of all necessary catalytic residues in this subunit. In contributions to the active site in each subunit are made by a nonidentical subunits of the yeast enzyme, other, results of this study suggest that the same is true for the from one subunit contribute to the isocitrate binding site of the IDH1 catalytic site. Reciprocally, the Lys-183 sites and instead contribute to the heteromeric cooperative activity of IDH1. Furthermore, based on the structural E. coli isocitrate dehydrogenase homodimer, which corresponds to the bacterial residue Asp-238 located near residues 234–236 that are known to be involved in intersubunit contacts (24).

whereas identical subunit polypeptides show no substantial interactions with these tests. This experimental evidence suggests that the basic structural/functional unit of this enzyme is a heterodimer, although other interactions clearly occur within the octameric holoenzyme. Furthermore, based on the structure of the E. coli isocitrate dehydrogenase homodimer, which shows that specific residues, Lys-230' and Asp-238' (Fig. 2), from one subunit contribute to the isocitrate binding site of the other, results of this study suggest that the same is true for the nonidentical subunits of the yeast enzyme, i.e. that reciprocal contributions to the active site in each subunit are made by a few residues from the other polypeptide chain. The essential nature of these reciprocal interactions would explain the absence of catalytic activity for IDH2 alone despite the apparent presence of all necessary catalytic residues in this subunit. In other words, Lys-189' and Asp-222' residues of IDH2 apparently do not function to complete homomeric catalytic active sites and instead contribute to the heteromeric cooperative active site of IDH1. Reciprocally, the Lys-183' and Asp-217' residues of IDH1 appear to be essential components of the IDH2 catalytic site.

Previous and current kinetic studies of mutant enzymes summarized in Table III support these hypotheses. Activity is dramatically reduced with alanine substitutions for residues with putative catalytic functions in substrate isocitrate binding (IDH2 Ser-96 (13) and IDH1 Lys-183' or Asp-217' (this study)) or in cofactor NAD(II) binding (IDH2 Asp-286/Ile-287 (14)). For these catalytically mutant enzymes, when kinetic properties are measurable, there is no deleterious effect on cooperativity or activation by AMP. We believe that the IDH1 mutant enzyme with an alanine substitution for Asp-191 (14) also belongs in this category. As described above, this residue was originally targeted for mutagenesis based on correspondence with an aspartate residue in the pig heart enzyme that is selectively modified by an adenine nucleotide analogue (15). Although this residue is not part of our model for the active sites, the kinetic properties of the IDH1 D191A enzyme are consistent with its designation as Asp-191’, i.e. with a function in the catalytic site of IDH2. This residue is located near IDH1 Lys-183’, and it corresponds to the bacterial residue Asp-238 located near residues 234–236 that are known to be involved in intersubunit contacts (24).

For mutant yeast enzymes with substitutions in residues with putative functions in regulatory binding of isocitrate (Table III; IDH1 Ser-92 (13) and IDH2 Lys-189’ or Asp-222’ (this study)), both cooperativity and reduction of S0.5 values by AMP are defective. Adjacent substitutions within the putative AMP binding region of IDH1 (Asp-279/Ile-280 (14)) produce a loss of activation but no effect on cooperativity. We also include in this regulatory category a previous mutant enzyme with a substitution for IDH2 Asp-197’ (14), the reciprocal of IDH1 Asp-191’. This residue is located near IDH2 Lys-189’, and the IDH2 D197A enzyme exhibits kinetic defects consistent with a contribution to the regulatory isocitrate binding site of IDH1.

Of particular interest are the effects of substitutions for “regulatory” residues on Vmax (Table III). None of these effects are as striking as the defects produced with substitutions for “catalytic” residues, but deleterious effects range from 2- to 18-fold decreases relative to Vmax values for corresponding wild-type enzymes. That we always see some effect on catalysis indicates that full catalytic activity may require regulatory functions. It was in fact predicted over 30 years ago (1) that either the regulatory isocitrate site or the AMP binding site must be occupied to obtain maximal catalytic activity. However, because effects on Vmax vary significantly for mutant enzymes that exhibit similar defects in cooperativity, it may be that some residues have dual functions or that portions of the polypeptide chains containing these residues may be located in proximity of both subunit active sites. IDH2 Asp-222’ is particularly interesting in this respect. This residue appears to be
important for regulatory properties presumably through contributions to the IDH1 active site. However, its replacement also has a deleterious effect on $V_{\text{max}}$ more so in the absence of AMP (a 4-fold decrease). One possibility is that IDH2 Asp-222 is important for functional communication between the active sites of the IDH1 and IDH2 subunits, particularly in the absence of allosteric activator.

Overall, it appears that the yeast IDH1 and IDH2 subunits have independently evolved for different functions but that physical interactions between the subunits, including residues that contribute to reciprocal subunit function, have been maintained. It is also clear that, while the bacterial and yeast isocitrate dehydrogenases differ substantially in terms of subunit composition, cofactor specificity, and regulation, significant structural motifs have been conserved. We predict that information obtained from the E. coli enzyme will continue to aid in functional analysis of the more complex S. cerevisiae homologue. Ongoing crystallographic analysis of the yeast enzyme should also clarify, by comparison with the bacterial structure, the basis and evolution of its allosteric properties.

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