Affinity Purification and Kinetic Analysis of Mutant Forms of Yeast NAD⁺-specific Isocitrate Dehydrogenase*

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Polyhistidine tags were added to the carboxyl termini of the two homologous subunits of yeast NAD⁺-specific isocitrate dehydrogenase (IDH). The tag in either the IDH1 or IDH2 subunit permits one-step affinity purification from yeast cellular extracts of catalytically active and allosterically responsive holoenzyme. This expression system was used to investigate subunit-specific contributions of residues with putative functions in adenine nucleotide binding. The primary effect of simultaneous replacement of the adjacent Asp-279 and Ile-280 residues in IDH1 with alanines is a dramatic loss of activation by AMP. In contrast, alanine replacement of the homologous Asp-286 and Ile-287 residues in IDH2 does not alter the allosteric response to AMP, but produces a 160-fold reduction in $V_{\text{max}}$ due to a 70-fold increase in the $K_{\text{m}}$ value for NAD⁺. These results suggest that the targeted aspartate/isoleucine residues may contribute to regulator binding in IDH1 and to cofactor binding in IDH2, i.e. that these homologous residues are located in regions that have evolved for binding the adenine nucleotide components of different ligands. In other mutant enzymes, an alanine replacement of Asp-191 in IDH1 eliminates measurable catalytic activity, and a similar substitution of the homologous Asp-197 in IDH2 produces pleiotropic catalytic effects. A model is presented for the primary function of IDH2 in catalysis and of IDH1 in regulation, with crucial roles for these single aspartate residues in the communication and functional interdependence of the two subunits.

The oxidative decarboxylation reaction catalyzed by mitochondrial NAD⁺-specific isocitrate dehydrogenase (IDH) is believed to be a rate-limiting step in the tricarboxylic acid cycle. The enzyme isolated from Saccharomyces cerevisiae has been described as responsive to energy charge due to allosteric activation by AMP and inhibition by ATP and NADH (1). Mammalian enzymes exhibit similar regulatory properties, but utilize ADP as a positive regulator (2).

The catalytically active form of IDH from yeast is an octamer composed of two subunits in a 1:1 ratio (3, 4). Independent nuclear genes encoding the IDH1 and IDH2 subunits have been cloned, and gene disruption studies show that both subunits are essential for catalytic activity (5, 6). Disruption of either or both of the IDH1 and IDH2 genes results in an inability to grow with acetate as a carbon source, a phenotype shared with several other tricarboxylic acid cycle mutants in yeast (7, 8). The deduced amino acid sequences and amino-terminal sequence analyses indicate that the mature IDH1 and IDH2 polypeptides contain 349 and 354 amino acid residues, respectively, and share 42% residue identity. The yeast polypeptides share ~32% sequence identity with Escherichia coli NAD⁺-specific isocitrate dehydrogenase (9), a homodimeric enzyme analyzed in several crystallographic studies (10–12).

Equilibrium binding and kinetic analyses (13, 14) conducted with purified yeast IDH led to the characterization of four binding sites for the substrate isocitrate, two for the cofactor NAD⁺, and two for the positive allosteric regulator AMP. Two possible models of subunit function and interaction were proposed based on these results. The individual subunits may have specialized functions in catalysis or regulation, with each containing complete independent binding sites. Alternatively, both subunits may contain half-binding sites, with interactions between IDH1 and IDH2 required to form complete binding pockets. Recent results from limited mutagenesis studies (15) favor the former model. In that work, evolutionarily conserved serine residues in each subunit were replaced with alanine residues. The homologous residue in bacterial NAD⁺-specific isocitrate dehydrogenase is the site for phosphorylation and inactivation in vivo (9), and replacement with aspartate produces a catalytically inactive enzyme (16). An S92A replacement in IDH2 was found to produce a 60-fold reduction in $V_{\text{max}}$ but no effect on cooperativity with respect to isocitrate or on AMP binding, whereas an S92A replacement in IDH1 produces only a 6-fold decrease in $V_{\text{max}}$ with complete loss of activation by AMP and a 2-fold decrease in cooperativity. These results suggested a primary role for IDH2 in catalysis and for IDH1 in regulating the catalytic properties of IDH2.

In this study, we extend analyses of subunit function in yeast IDH with the goal of modifying allosteric properties. Mutagenesis is based on homology to cofactor binding determinants in bacterial NAD⁺-specific isocitrate dehydrogenase and other decarboxylating dehydrogenases with the idea that adenine nucleotide binding is involved in both cofactor and regulator affinity. Also, we examine the role of a conserved aspartate residue identified by Huang and Colman (17) by chemical modification of pig heart NAD⁺-specific isocitrate dehydrogenase with an adenine nucleotide analogue.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—The ΔIDH1ΔIDH2 disruption mutant used in this study was previously constructed (6) using deletion/disruption methods with yeast strain S173-6B (MATa leu2-3,112 his3-1 ura3-52 trp1-289) (18). Transformations were conducted using the lithium acetate method of Ito et al. (19). Yeast strains were cultivated in rich YP medium (1% yeast extract, 2% Bacto-peptone) or in minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) with appropriate supplements of 20 μg/ml each to satisfy auxotrophic requirements for growth. Carbon sources were glucose, glycerol plus lactate, or acetate added to 2%.

Recombinant DNA Methods—DNA manipulations including ligation, amplification, purification, mutagenesis, and sequence analysis followed the methods described by Sambrook et al. (20).

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† The abbreviation used is: IDH, isocitrate dehydrogenase.
To obtain histidine-tagged IDH enzymes, oligonucleotide-directed mutagenesis was performed to introduce five sequential histidine codons to precede the termination codon in IDH1 or IDH2 genes carried on plasmid pRS316 (21). This centromere-based plasmid (designated pDH1/IDH2) has been shown to produce normal cellular levels of IDH1 and IDH2 in ΔDH1ΔDH2 transformants (22). The template for mutagenesis was single-stranded pDH1/IDH2, and the oligonucleotides were a 52-mer for IDH1 (5'-TATTCTTTTCC). Colony hybridization was used to identify GCAGTCATCAAGAGATTACATCACCATCACCATTAATAAAAGTCC-CCATCACCATTAATGAAAACAATTCCCC) and a 56-mer for IDH2 (5'-CGACGCACGAGAGATATCACCATCACCATTAATAAAAGTCC-CCATCACCATTAATGAAAACAATTCCCC) and a 56-mer for IDH2 (5'-CGACGCACGAGAGATATCACCATCACCATTAATAAAAGTCC-CCATCACCATTAATGAAAACAATTCCCC). Colony hybridization was used to identify E. coli transforms containing plasmids with the inserted histidine codons, and the designed sequence changes were confirmed by nucleotide sequencing. The resulting plasmids were designated pDH1His/IDH2 and pDH1/IDH2His

| I | Encoding histidine-tagged wild-type enzymes | II | Encoding mutant enzymes | III | Encoding histidine-tagged mutant enzymes |
|---|--------------------------------------------|---|-------------------------|---|----------------------------------------|
| pDH1His/IDH2 | pDH1/IDH2D197A | pDH1/IDH2D286A,D287A |
| pDH1/IDH2His | pDH1/IDH2D286A,D287A | pDH1/IDH2His/IDH2D197A |
| pDH1/IDH2D279A,D280A/IDH2 | pDH1/IDH2D191A/IDH2 |
| pDH1/IDH2D279A,D280A | pDH1/IDH2D191A |

For purification of histidine-tagged enzymes, yeast strains were cultivated in 500 ml of YM medium with glycerol plus lactate as carbon sources. The cells were harvested at a culture density of A600nm = 1.8–2.0 and lysed with glass beads as described previously (8). The cellular protein extracts (10 ml) were diluted with an equal volume of buffer A (50 mM NaH2PO4, pH 7.5, 300 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride) containing 40 mM imidazole, pH 7.5. The diluted extract was combined with 0.5 ml of Ni2+-nitrilotriacetic acid superfine resin (QIAGEN Inc.) and rocked at 24 °C for 30–90 min. The mixture was loaded onto a 1 × 5-cm column, and the flow-through fraction was collected. The column was washed three times with 3 ml of buffer A containing 20 mM imidazole, pH 7.5, and binding proteins were eluted with 1 ml of buffer A containing 200 mM imidazole, pH 7.5. The eluate was diluted 10-fold with buffer A, and the column absorption/elution procedure was repeated. The eluate from the second column was the source of purified protein used for kinetic and electrophoretic analyses.

**Kinetic Assays**—NAD+-specific isocitrate dehydrogenase activity was measured spectrophotometrically as described previously (4). Initial velocities are expressed as units/mg, with 1 unit of activity defined as the isocitrate-dependent production of 1 μmol of NADH/min at 24 °C. Vmax values are the maximal rate/mg of purified IDH and were obtained by Hanes plot analyses (24) of primary data. For measurements of isocitrate S0.5, assays contained 40 mM Tris-HCl, pH 7.5, 4 mM MgCl2, 0.25 mM NAD+, and N-isoctate at concentrations ranging from 0 to 10 mM (N-isoctate concentrations were calculated as 50% of the total isocitrate). Assays of AMP activation contained 100 μM AMP. For measurements of NAD+ S0.5, assays contained 40 mM Tris-HCl, pH 7.5, 4 mM MgCl2, 10 mM N-isoctate, 100 μM AMP, and NAD+ at concentrations ranging from 0 to as high as 10 mM for some mutant enzymes. All assays were conducted at 24 °C and were initiated by the addition of enzyme.

**RESULTS**

**Construction of Histidine-tagged IDH and Characterization of Affinity-purified Enzyme**—Previous kinetic analyses of wild-type and mutant forms of *S. cerevisiae* IDH were performed using enzymes purified from whole cell or mitochondrial extracts by several column chromatography steps (3, 4, 26). To expedite and simplify purification, we have adapted an affinity column method based on histidine tagging (27). Since IDH is an octamer containing four each of the IDH1 and IDH2 polypeptides, we have introduced histidine tags into each subunit. As described under "Experimental Procedures," independent oligonucleotide-directed mutagenesis steps were conducted using a centromere-based pRS316 plasmid carrying both complete
The slower migration of histidine-tagged subunits is apparent in comparison of lanes 3 and 6.

To determine if the introduction of histidine tags alters catalytic or allosteric parameters, several kinetic analyses were performed using the affinity-purified enzymes. As summarized in Table I, in the absence of AMP, $S_{0.5}$ values for isocitrate of 0.62 and 0.76 mM were measured, and corresponding Hill coefficients of 3.85 and 3.93 were calculated for the IDH1His/IDH2 and IDH1/IDH2His enzymes, respectively. These are similar to previously published values for IDH purified from yeast (1, 15), indicating that the apparent affinity for isocitrate and the high cooperativity of substrate binding are preserved in the affinity-purified enzymes. More important, for the experiments below, allosteric activation by AMP is also retained. In the presence of 100 mM AMP, the $S_{0.5}$ values for isocitrate decrease 9-fold for both IDH1His/IDH2 and IDH1/IDH2His enzymes, comparable to 5–6-fold decreases reported for the conventionally purified enzyme (1, 15). Also, previous studies indicated that activation by AMP produced a slight decrease in cooperativity, with a reduction of the Hill coefficient to 2.5 (26). The decrease in cooperativity is negligible for the histidine-tagged enzymes (Table I), perhaps due to conformational stability afforded by rapid purification. These results show that this expression/purification system is appropriate for analyses of mutant enzymes with residue replacements designed to alter catalytic or regulatory properties.

**Construction and Expression of Mutant Forms of IDH**—With the goal of identifying and altering residues involved in allosteric regulation by AMP, we based strategies for mutagenesis on two different considerations. First, using the approach of chemical modification, Huang and Colman (17) identified a tryptic peptide containing a covalently modified aspartate residue following treatment of pig heart NAD$^+$-specific isocitrate dehydrogenase with an adenine nucleotide analogue, 2-(4-bromo-2,3-dioxobutylthio)adenosine 5'-diphosphate. The amino acid sequence around the modified aspartate is conserved in several other adenine nucleotide-binding proteins (28, 29) and in the IDH1 and IDH2 polypeptides (Fig. 3A). Thus, the homologous aspartate residues of the yeast subunits in positions 191 and 197, respectively, were targeted for replacement with alanine residues. Second, crystallographic studies of NAD$^+$- and NADP$^+$-specific dehydrogenases have indicated that many of the residues interacting with these cofactors are similar, with striking exceptions, as expected, being residues that interact with the ribose 2'-phosphate in the NADP$^+$-specific enzymes. The adjacent Lys-344 and Tyr-345 residues with this function are two of seven residue replacements introduced into E. coli NADP$^+$-specific isocitrate dehydrogenase (12) align with adjacent aspartate and iso-leucine residues in the highly homologous cofactor-binding site of NAD$^+$-specific 3-isopropylmalate dehydrogenase (30). Also, K344D and Y345I are two of seven residue replacements introduced into NADP$^+$-specific isocitrate dehydrogenase to change cofactor specificity from NADP$^+$ to NAD$^+$ (31). As shown in Fig. 3B, the amino acid sequences of the yeast IDH1 and IDH2 subunits are highly homologous to that of NAD$^+$-specific isocitrate dehydrogenase in a region containing several residues (underlined) involved in cofactor binding in the bacterial enzyme. Several of these residues are conserved in IDH2, and fewer in IDH1. In this region, both IDH subunits contain adjacent aspartate and isoleucine residues characteristic of NAD$^+$ specificity at positions occupied by Lys-344 and Tyr-345 of NADP$^+$-specific isocitrate dehydrogenase. Reasoning that these residues could be informative about NAD$^+$ binding or, alternatively, could be part of a region adapted during evolution for binding the adenine nucleotide moiety of an allosteric regulator, Asp-279 plus Ile-280 in IDH1 and Asp-286 plus Ile-287 in IDH2 were tar-
Codon changes in the IDH1 and IDH2 genes were introduced by oligonucleotide-directed mutagenesis as described under "Experimental Procedures." Small restriction fragments containing the desired mutations were completely sequenced and used to replace the corresponding restriction fragments in pIDH1/IDH2. Four plasmids, each containing one altered subunit gene and the other unaltered gene, were constructed and designated pIDH1D191A/IDH2, pIDH1/IDH2D197A, pIDH1D279A,I280A/IDH2, and pIDH1/IDH2D286A,I287A. This set of plasmids (Fig. 1, set II) provides controls to assess effects on the cellular specific activity of IDH in cells containing only the desired mutation. For purification and kinetic analyses, another set of four plasmids (Fig. 1, set III) was constructed by subcloning. These plasmids contain each mutant subunit allele in combination with the histidine-tagged but otherwise wild-type version of the other subunit allele. They are designated pIDH1D191A/IDH2His, pIDH1His/IDH2D197A, pIDH1D279A,I280A/IDH2His, and pIDH1His/IDH2D286A,I287A.

Both sets of plasmids were transformed into the ΔIDH1ΔIDH2 disruption mutant. Resulting Ura" transformants were isolated and cultivated in rich medium with glycerol plus lactate as a permissive carbon source. Immunoblot analysis conducted with whole cell protein extracts demonstrates equivalent cellular levels of expression of IDH1 and IDH2 in all transformants (data not shown). Enzymatic activities are measurable for all of the cellular extracts from transformants except those containing the IDH1D191A enzyme. Specific activities associated with the IDH1D191A enzyme are 3–4-fold lower and activities associated with the IDH2D197A or IDH2D286A,I287A enzymes are 20-fold lower than control values for histidine-tagged wild-type enzymes. These values for cellular activity were found to roughly correlate with restoration of growth on plates with acetate as a carbon source (data not shown), i.e. little or no growth is observed for strains expressing mutant IDH2 enzymes or the IDH1 D191A enzyme, whereas slow growth relative to control transformants expressing the wild-type enzyme is observed for strains expressing the IDH1D279A,I280A enzyme. In all cases, levels of activity and growth with acetate are very similar for strains transformed with plasmid pairs encoding either tagged or nontagged wild-type subunits, indicating no impact of the carboxyl-terminal histidine extensions on these properties.

Affinity Purification of Mutant IDH Enzymes—For kinetic analyses, the four histidine-tagged mutant enzymes were purified from yeast transformants using nickel-charged columns as described under "Experimental Procedures" and "Construction and Expression of Mutant Forms of IDH." The purities obtained were similar to those for wild-type enzymes containing either histidine-tagged subunit; however, overall yields of the mutant enzymes were lower, ranging from 24 to 42%. Denaturing electrophoresis and Coomassie Blue
staining (Fig. 2) resolved two major bands in each purified mutant enzyme, with reduced migration of the IDH2 subunits in the IDH1D191A/IDH2His and IDH1D279A,I280A/IDH2His enzymes (lanes 4 and 5, respectively) and of the IDH1 subunits in the IDH1His/IDH2D197A and IDH1His/IDH2D286A,I287A enzymes (lanes 7 and 8, respectively). The IDH2 band in the purified IDH1His/IDH2D197A enzyme (lane 7) migrated slightly faster than other forms of IDH2, presumably due to the specific amino acid substitution.

Initial enzyme assays of purified mutant enzymes were performed with saturating concentrations of substrates and cofactor. The values for $V_{\text{max}}$ presented in Table I were found to vary only slightly with different preparations of purified enzymes. A particular objective at this point was to determine if the IDH1D191A/IDH2His enzyme exhibits any activity after enrichment. It was found that a trace of activity could be measured, but only with high concentrations of enzyme and after allowing the reaction to proceed for several hours. This trace activity suggests that the enzyme may be structurally intact, but precludes further kinetic analysis. The corresponding replacement in the IDH1His/IDH2D197A enzyme produced a less severe 13-fold reduction in $V_{\text{max}}$ compared with that measured for IDH1His/IDH2. In contrast, the aspartate/isoleucine replacements in IDH1D279A,I280A/IDH2His produced a 2.5-fold reduction and in IDH1His/IDH2D286A,I287A a 160-fold reduction in $V_{\text{max}}$ relative to the corresponding histidine-tagged wild-type enzymes. Thus, replacement of these adjacent residues has a much greater effect on contributions of IDH2 than of IDH1 to activity.

Kinetic Analyses of Affinity-purified Enzymes—Three of the four purified mutant enzymes retain catalytic activity. To more precisely evaluate effects of specific residue substitutions, the catalytic parameters were compared with those for the corresponding histidine-tagged wild-type enzymes. Initial experiments examined activity as a function of isocitrate concentration in the absence or presence of a saturating concentration of AMP (Fig. 4 and Table I).

The single aspartate to alanine substitution in either subunit produces a dramatic effect on activity. As stated above, the IDH1D191A/IDH2His enzyme is effectively inactive. The activity of the IDH1His/IDH2D197A enzyme is also reduced, with a 13-fold decrease in $V_{\text{max}}$ relative to that measured for the IDH1His/IDH2His enzyme.
IDH2 enzyme. The isocitrate $S_{0.5}$ in the absence of AMP is not altered in this mutant enzyme. However, the D197A substitution in IDH2 nearly eliminates cooperativity in the presence or absence of AMP and produces a complete loss of activation by AMP. These results suggest that the targeted aspartate residues are critical for function, with Asp-191 in IDH1 being essential for catalytic activity and Asp-197 in IDH2 being crucial for allosteric regulation (cooperativity and AMP activation) of the holoenzyme.

Substitutions of adjacent aspartate/isoleucine residues with alanine residues in IDH1 and IDH2 produce very different effects on catalytic properties. The D279A/I280A substitutions in IDH1 result in an ~2.5-fold reduction in $V_{max}$. The isocitrate $S_{0.5}$ in the absence of AMP is only slightly affected, increased ~30% relative to that for the IDH1/IDH2$_{His}$ control enzyme, and cooperativity is unaffected. However, there is a dramatic reduction in AMP activation, with a modest 23% decrease for the mutant enzyme as compared with an 8.4-fold decrease in the isocitrate $S_{0.5}$ for the wild-type enzyme in the presence of 100 $\mu$M AMP. These results suggest, in contrast to those obtained for the enzyme containing a D197A substitution in IDH1, that cooperativity and allosteric regulation by AMP are separable catalytic properties.

The D286A/I287A substitutions in IDH2 produce the most dramatic measurable effect on catalytic capacity, with a reduction in $V_{max}$ of 160-fold relative to the wild-type control. However, regulatory properties including cooperativity and AMP activation are not dramatically altered. The mutant enzyme exhibits a slightly reduced isocitrate $S_{0.5}$ in the absence of AMP (73% of the wild-type enzyme), and this $S_{0.5}$ value decreases 7.5-fold relative to 9-fold for the wild-type enzyme in the presence of AMP. Thus, the decrease in $V_{max}$ cannot be attributed to effects on binding of either the substrate isocitrate or the regulator AMP.

One of our initial postulates was that adenine nucleotide-binding sites may independently evolve to accommodate both cofactor NADH and regulator AMP binding. Since the D279A/I280A substitutions in IDH1 primarily affect AMP activation, $S_{0.5}$ values for NAD$^+$ were measured for mutant and wild-type enzymes to determine if parallel D286A/I287A substitutions in IDH2 show a possible effect on cofactor binding. Activity was assessed as a function of NAD$^+$ concentration in the presence of saturating concentrations of isocitrate (10 mM) and AMP (100 $\mu$M). As shown in Table I, the $S_{0.5}$ for NAD$^+$ is not altered by the D279A/I280A substitutions in IDH1, but the $S_{0.5}$ value is increased a dramatic 70-fold by D286A/I287A substitutions in IDH2. Thus, the reduction in catalytic capacity for the latter enzyme is primarily due to effects on cofactor affinity. These results suggest that these adjacent aspartate/isoleucine residues in IDH2 are functionally equivalent to conserved residues involved in cofactor binding in other NAD$^+$-dependent dehydrogenases. The $S_{0.5}$ for NAD$^+$ is also increased 6.5-fold by the D197A substitution in IDH2, indicating pleiotropic effects on cofactor affinity as well as on cooperativity and AMP activation.

**DISCUSSION**

Using the cloned genes for the IDH1 and IDH2 subunits of NAD$^+$-specific isocitrate dehydrogenase from *S. cerevisiae*, we have introduced histidine codons and have shown that holoenzyme can be purified using affinity chromatography with carboxyl-terminal tags on either subunit. We have utilized this approach to examine potential adenine nucleotide-binding sites in yeast IDH.

The most informative mutant enzymes constructed in this study contain adjacent alanine substitutions for Asp-279 and Ile-280 in IDH1 or similar substitutions for homologous Asp-286 and Ile-287 in IDH2. The rationale for these substitutions is the similar positioning of adjacent aspartate/isoleucine residues within regions known to participate in cofactor binding in other NAD$^+$-specific dehydrogenases (30). Our results are consistent with participation of adjacent aspartate/isoleucine residues in both IDH subunits in adenine nucleotide binding. However, the effects of alanine substitutions are quite distinct. The substitutions in IDH1 produce an enzyme that is not responsive to activation by AMP, but that apparently retains full cofactor affinity. In contrast, the substitutions in IDH2 dramatically reduce the apparent affinity for NAD$^+$ and consequently $V_{max}$ but have no effect on allosteric activation. Thus, the adjacent aspartate/isoleucine residues in IDH1 may contribute to affinity for the adenine nucleotide moiety in AMP, whereas the homologous residues in IDH2 may contribute to affinity for the adenine nucleotide moiety in NAD$^+$.

These results are consistent with the previous assignment of subunit function based on mutagenesis to replace highly conserved serine residues in IDH1 and IDH2 (15), which correspond to Ser-113 of the *E. coli* enzyme, a residue with a critical function in binding isocitrate at the active site (10). Previous and current results suggest that IDH1 contains residues necessary for cooperative binding of isocitrate and AMP and thus appears to be the primary contributor to regulatory functions. IDH2, in contrast, appears to contribute primarily to catalytic binding of isocitrate and NAD$^+$. A schematic model for the distribution of binding sites is shown in Fig. 5. Several elements of this simple model supported by current and previous studies are as follows. (a) Most of the structural subunit interactions in the holoenzyme involve heterologous subunit interactions. This is suggested by the apparent monomeric state of either IDH1 or IDH2 isolated from yeast mutants lacking the other subunit (6). Also, preliminary results from the yeast two-hybrid system indicate strong interactions between IDH1 and IDH2, but no detectable interactions between identical subunits. $^3$ (b) The substrate and cooperative binding sites for isocitrate are separable by mutagenesis, as are the adenine nucleotide-binding sites for the regulator and cofactor. (c) In addition, the properties of AMP activation and cooperativity assigned as functions of IDH1 are separable by mutagenesis (IDH1$_{D279A,I280A/IDH2_{His}}$ enzyme) (Table I), as are the proper-

$^3$ W.-N. Zhao, E. Panisko, and L. McAlister-Henn, unpublished observations.
ties of isocitrate and NAD\(^{+}\) binding assigned to IDH2 (IDH1^{His}/IDH2^{D286A,I287A} enzyme) (Table I).

Although these data and this model indicate independent functions for the subunits in the basic heterodimeric structure, it is clearly necessary that regulatory functions of IDH1 be communicated to catalysis by IDH2. A possible interpretation of other results obtained in this study, as illustrated in Fig. 5, is that Asp-191 in IDH1 and the corresponding Asp-197 in IDH2 may contribute to the interdependence of subunit function. These residues were targeted for alanine replacement based on previous reports that a homologous residue in the enzyme from pig heart can be specifically labeled with an adenine nucleotide analogue (17, 32). Both replacements in the yeast enzyme have significant effects, with loss of activity for the D191A replacement in IDH1 and pleiotropic effects on all kinetic parameters except isocitrate affinity for the D197A replacement in IDH2. These effects cannot simply be attributed to major alterations in structure since subunit assembly and holoenzyme purification are apparently unchanged. Assuming the location of the yeast residues to be near adenine nucleotide-binding sites, as suggested by the study of Huang and Colman (17), and that our basic model for subunit-specific functions is correct, the loss of activity obtained with a D191A replacement in the “regulatory” subunit IDH1 could be due to a critical role for this residue in NAD\(^{+}\) affinity at the active site of IDH2. Similarly, the loss of AMP activation and the dramatic reduction in cooperativity observed following a D197A replacement in IDH2, the presumed catalytic subunit, could be due to a crucial role for this residue in the regulatory regions of IDH1 or in a region essential for communication between the subunits. While this is a highly speculative argument, there is some supportive structural information: Asp-191 and Asp-197 in the IDH1 subunits correspond in aligned sequences to Glu-238 of the homodimeric E. coli NADP\(^{+}\)-specific isocitrate dehydrogenase, a residue near Lys-230 that contributes to hydrogen bonding of isocitrate in the active site formed otherwise by residues of the other subunit (10). Future tests of these ideas will involve more conservative replacements of Asp-191 and Asp-197 and replacement of lysine residues in IDH1 and IDH2 corresponding to NAD\(^{+}\)-specific isocitrate dehydrogenase Lys-230.

The metabolic contributions in vivo by mutant forms of IDH have, to date, only been assessed as a function of growth rate with acetate, a nonpermissive carbon source for yeast strains lacking IDH1 and/or IDH2. However, we have designed our expression system to obtain normal cellular levels of mutant enzymes. This establishes the basis for future work to examine effects of altered allosteric and catalytic properties on adaptation to changes in growth conditions and on respiratory mitochondrial functions.

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