Kinetic Analysis of NAD$^+$–Isocitrate Dehydrogenase with Altered Isocitrate Binding Sites: Contribution of IDH1 and IDH2 Subunits to Regulation and Catalysis†

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ABSTRACT: NAD$^+$-dependent isocitrate dehydrogenase from *Saccharomyces cerevisiae* is an allosterically regulated enzyme that exists as an octamer composed of two nonidentical subunits, designated IDH1 and IDH2. To determine the contribution of each subunit to regulation and catalysis, a conserved serine residue at the proposed active site of each subunit was mutated to alanine. This mutation in IDH1 resulted in a 6-fold decrease in V_{max} and a decrease in cooperativity, but little change in $S_{0.5}$ for isocitrate. The mutant IDH2, in contrast, exhibited a 60-fold decrease in maximal velocity and a 2-fold reduction in $S_{0.5}$ for isocitrate, but the cooperativity was unaffected. Responses to the allosteric modifier AMP also differed for the two mutant enzymes. The IDH1 mutant enzyme was not activated by AMP, whereas the IDH2 mutant enzyme exhibited an increase in isocitrate affinity in the presence of AMP similar to that observed with the wild-type enzyme. On the basis of these kinetic results, a model is presented which proposes that IDH1 functions as a regulatory subunit while IDH2 functions in catalysis. To determine if IDH1 or IDH2 alone is catalytically active, we also expressed the individual subunits in yeast strains in which the gene encoding the other subunit had been disrupted. Mitochondrial extracts from strains overexpressing solely IDH1 or IDH2 contained no detectable activity in the presence or absence of AMP. Gel filtration of these extracts showed that both IDH1 and IDH2 behaved as monomers, suggesting that the major subunit interactions within the octamer are between IDH1 and IDH2.

NAD$^+$-dependent isocitrate dehydrogenase (NAD$^+$–IDH)[1] found in eukaryotes is a complex oligomeric enzyme that is subject to extensive allosteric regulation. The enzyme from *Saccharomyces cerevisiae* functions as an octomer composed of two nonidentical subunits, designated IDH1 and IDH2 (Keys & McAlister-Henn, 1990), and is responsive to cellular energy levels as a result of binding and activation by AMP and NAD$^+$ (Hathaway & Atkinson, 1963). The roles of the individual subunits in catalysis and regulation, however, are not well understood. On the basis of early kinetic studies, Atkinson and co-workers (Hathaway & Atkinson, 1963) proposed that NAD$^+$–IDH from *S. cerevisiae* contains functionally distinct isocitrate binding sites: a reaction site at which catalysis occurs and a regulatory site whose occupancy increases enzyme activity. This model was supported by equilibrium binding studies which indicated that there are twice as many binding sites for isocitrate as for NAD$^+$ and Mg^{2+} (Kuehn et al., 1971) which are required for catalysis. The role of the individual IDH1 and IDH2 subunits in catalysis and regulation, however, could not be determined by these methods.

Recently, we reported the cloning of the genes encoding each subunit (Cupp & McAlister-Henn, 1991, 1992). The amino acid sequences deduced from the genes indicate that the IDH1 and IDH2 subunits are synthesized as precursors of 360 and 369 amino acids, respectively, and are processed upon mitochondrial import to yield mature proteins of 349 (M_r 38 001) and 354 (M_r 37 755) amino acids, respectively. While similar in size, the overall sequence identity of IDH1 and IDH2 is only 42% (Cupp & McAlister-Henn, 1992). Some insights into the roles of the individual subunits of NAD$^+$–IDH in catalysis and regulation can be made by comparisons with isocitrate dehydrogenase from *Escherichia coli*. While the *E. coli* enzyme differs from the yeast enzyme in utilizing NADP$^+$ as a cofactor and existing as a dimer of identical subunits (Borthwick et al., 1986), alignment of the amino acid sequence with both IDH1 and IDH2 reveals ~32% sequence identity (Cupp & McAlister-Henn, 1991, 1992). The amino acid residues which participate in Mg^{2+}/isocitrate binding and NADP$^+$ binding in the *E. coli* enzyme have been determined by X-ray crystallography (Hurley et al., 1989, 1990, 1991), and alignment of IDH1 and IDH2 with the *E. coli* enzyme reveals that both subunits have conserved residues in the region comprising the isocitrate binding site of *E. coli* NADP$^+$–IDH. In particular, the sequence of the IDH2 subunit appears to contain many of the residues involved in substrate and cofactor binding (Cupp & McAlister-Henn, 1991, 1992). IDH1 is also similar, but has substitutions at several positions in the putative Mg^{2+}/isocitrate binding site and the cofactor binding site (Cupp & McAlister-Henn, 1992). These differences suggest the possibility that the isocitrate sites on IDH1 and IDH2 may bind isocitrate differently and may have separate functions in regulation and catalysis.

In an effort to define the roles of the IDH1 and IDH2 subunits of NAD$^+$–IDH, we have utilized site-directed mutagenesis to introduce mutations at the proposed isocitrate binding sites. Mutant proteins were overexpressed in strains lacking NAD$^+$–IDH and were analyzed *in vitro* for their ability to convert isocitrate to α-ketoglutarate. In addition, kinetic

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[1] Abbreviations: NAD$^+$–IDH, NAD$^+$-dependent isocitrate dehydrogenase; NADP$^+$–IDH, NADP$^+$-dependent isocitrate dehydrogenase; AMP, adenosine 5′-monophosphate; kb, kilobase(s).

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studies were performed in the presence of the positive modifier AMP to analyze the relationship between isocitrate binding and activation by AMP. On the basis of these kinetic studies, we present a model involving different roles for IDH1 and IDH2 in regulation and catalysis.

MATERIALS AND METHODS

Protein Purification. Proteins were partially purified from yeast strains overexpressing wild-type or mutant NAD$^+$–IDH. Induction of NAD$^+$–IDH expression was obtained by growth on the carbon source 2% glycerol/2% lactate plus 0.5% glucose in rich YP medium. For protein purification, cells were harvested and mitochondria prepared as described by Daum et al. (1982). Briefly, pelleted cells were washed and resuspended in a buffer containing zymolase (ICN Pharmaceuticals) to produce spheroplasts. Cells were broken using a Dounce homogenizer, and mitochondria were isolated by differential centrifiguation at 10 000 rpm. Mitochondrial extracts were obtained by vortexing with glass beads. Wild-type enzyme, IDH(1:S92A), and IDH(2:S98A) were further purified by ammonium sulfate fractionation (45–60%) and blue dextran chromatography (Pharmacia LKB). Samples were stored in 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, and 50% glycerol at –20 °C. A Bio-Gel A0.5 column (1 cm × 78 cm) equilibrated in 25 mM potassium phosphate, pH 7.5, 50 mM KCl, and 0.5 mM citrate was used for native molecular weight estimation of all samples. Samples without activity were followed by immunoblot analysis. Fractions were slot-blotted onto nitrocellulose membrane, blocked with 5% bovine serum albumin, and incubated in a 1:300 dilution of anti-IDH antiserum. Detection was performed using ^{125}I-labeled protein A followed by autoradiography.

To quantitate the NAD$^+$–IDH in the purified samples, proteins were separated by 10% polyacrylamide–sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970), Coomassie Blue stained, and quantitated by densitometry. A standard curve of carbonic anhydrase and bovine serum albumin was used for protein concentration determination. Quantities reported are for total NAD$^+$–IDH (IDH1 plus IDH2).

Recombinant DNA Technology. Synthetic oligonucleotides containing double point mutations to convert serine to alanine were obtained from Operon Technologies (Alameda, CA). Oligonucleotide-directed mutagenesis was performed independently on IDH1 and IDH2 in the plasmid pBS (Stratagene) using the Transformer method (Clontech). A 2.0-kb *XbaI*/*Hind*III fragment of the *IDH2* gene and a 1.4-kb *Xba*I/*XBa*I fragment of the *IDH1* gene were subcloned into the multicopy shuttle vector YEp-352 (Hill et al., 1986). In addition to the mutated gene, the YEp-352 vector contained a wild-type copy of the other subunit gene to ensure overexpression of both subunits. Multicopy plasmids constructed included YEp352-(*IDH1*/*IDH2*), YEp352-(*IDH1S92A*/*IDH2*), and YEp352-(*IDH1*/*IDH2S98A*). All multicopy plasmids were transformed into the NAD$^+$–IDH double-disruption strain Δ(IDH1/IDH2).

Plasmids were amplified in the *E. coli* strain DH5αF′ and purified using the alkaline lysis method (Maniatis et al., 1989). Separation of plasmids and restriction fragments was achieved using 0.8% agarose gels. Plasmids were transformed into yeast using the lithium chloride protocol (Ito et al., 1983). All mutations were confirmed by dideoxynucleotide sequencing using the double-stranded method described by Wang (1988).

Activity Assays. NAD$^+$–IDH activity was measured spectrophotometrically as an increase in A_{340nm} versus time. Initial velocities were expressed as units per milligram of protein, where one unit of enzyme activity was defined as the amount of enzyme required to catalyze the production of 1 μmol of NADH/min at 24 °C. V_{max} values are reported as the maximal rate per milligram of NAD$^+$–IDH. All reagents for activity assays were purchased from Sigma. Assays contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM NAD$^+$, and DL-isocitrate. The concentration of D-isocitrate was calculated as 50% total isocitrate. AMP assays contained 100 μM adenosine 5′-monophosphate. All assays were performed at 24 °C and were initiated by the addition of enzyme.

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                        90                  100
IDH1        P A D Q T G H G S L N V A L R K Q L K I Y
            : .     :   : : : : : : :   :   . :
E.coli (106)P V G G - G I R S L N V A L R Q E L D L Y (125)
            : . :   :   : : : : . : :       : .
IDH2        P I G K - G H R S L N L T L R K T F G L F
                            100             110
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FIGURE 1: Amino acid sequence comparison of *E. coli* NADP$^+$–IDH with IDH1 and IDH2 of NAD$^+$–IDH from *S. cerevisiae*. Identical residues are indicated by a colon (:), and similarities are shown by a period (.). Gaps (–) were introduced into IDH2 and *E. coli* NADP$^+$–IDH to optimize alignment with IDH1. The conserved serine proposed to be involved in isocitrate binding is indicated with boldface type.

Yeast Strains and Growth Conditions. The wild-type haploid yeast strain used in these studies was S173-6B (*MATa leu2-3, 112 his3-1 ura3-52 trp1-289*); the NAD$^+$–IDH disruption strains Δ*IDH1*, Δ*IDH2*, and Δ(*IDH1*/*IDH2*) were constructed using this wild-type haploid yeast strain as previously described (Cupp & McAlister-Henn, 1991, 1992). Yeast strains were grown in rich YP medium [1% yeast extract/2% Bacto-Peptone (Difco)] with the indicated carbon source added to 2%. Cell growth was measured spectrophotometrically at A_{600nm}.

RESULTS

Production of Mutant Enzymes. Figure 1 shows an alignment of *E. coli* NADP$^+$–IDH residues 106–125 with IDH1 and IDH2 [*cf.* Cupp and McAlister-Henn (1991, 1992) for complete alignment]. Both subunits exhibit substantial sequence similarity to the bacterial enzyme. Structural analysis of the *E. coli* enzyme by X-ray crystallography has established that several of these residues function in isocitrate binding (Hurley et al., 1990). In particular, Ser-113 of the *E. coli* enzyme forms a hydrogen bond with the γ-carbonyl group of isocitrate. Ser-92 of IDH1 and Ser-98 of IDH2 align with this residue and thus may participate in substrate binding in the yeast enzyme. To determine if these residues are involved in binding of isocitrate in yeast NAD$^+$–IDH, alanine residues were substituted for serine at each of these positions. The mutant NAD$^+$–IDH enzymes are designated IDH(1:S92A) for the Ser → Ala mutation in IDH1 and IDH-(2:S98A) for the Ser → Ala mutation in IDH2.

For all analyses, wild-type and mutant enzymes were partially purified from mitochondrial extracts as described under Materials and Methods. To determine whether changes in quaternary structure had occurred as a result of amino acid substitutions, gel filtration chromatography was performed. Wild-type enzyme, IDH(1:S92A), and IDH(2:S98A) each eluted with an apparent molecular weight of ~300 000 (data not shown). This result suggests that both IDH(1:S92A) and IDH(2:S98A) exist predominantly in an octameric structure similar to the wild-type enzyme.

Activity of Mutant Enzymes. To test whether substitution of alanine for serine in IDH1 or IDH2 affected catalysis, the activities of the mutant NAD$^+$–IDH enzymes were measured, and the results are plotted in Figure 2 and summarized in

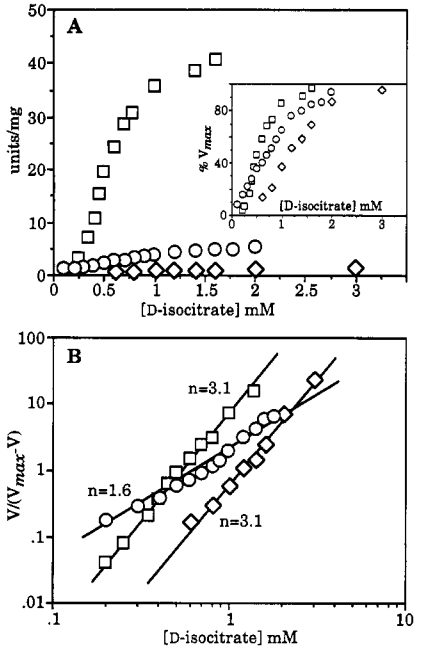

FIGURE 2: Isocitrate-dependent reduction of NAD^+ by wild-type and mutant NAD^+–IDH enzymes. Initial reaction rates were determined spectrophotometrically as an increase in A_{340nm}. Assays were carried out at 24 °C and contained 40 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM NAD^+, and the indicated amount of D-isocitrate. All assays were performed in duplicate and average values are plotted. Data are presented for wild-type enzyme (□), IDH(1:S92A) (○), and IDH(2:S98A) (◇). (Panel A) Specific activity versus D-isocitrate concentration. Inset: specific activity normalized by V_{max}. (Panel B) Hill plot of the specific activity of wild-type and mutant NAD^+–IDH enzymes. Hill coefficients (n) were calculated as a best fit using the Macintosh–Cricket graph program.

Table I: Kinetic Parameters of Wild-Type and Mutant NAD^+–IDH Enzymes[a]

	$S_{0.5}$ (mM)	V_{max} (units/mg)	Hill coefficient
–AMP			
wild type	0.56	42	3.1
IDH(1:S92A)	0.61	5.5	1.6
IDH(2:S98A)	1.26	0.7	3.1
+AMP			
wild type	0.10	44	2.5
IDH(1:S92A)	0.71	7.0	1.2
IDH(2:S98A)	0.15	0.85	2.8

[a] Parameters were calculated from the data presented in Figures 2 and 3.

Table I. V_{max} values are expressed as a maximal rate per milligram NAD^+–IDH. The kinetic parameters of wild-type NAD^+–IDH are similar to those from previously published studies (Hathaway & Atkinson, 1963; Gabriel & Plaut, 1990, 1991); the enzyme exhibited a maximal rate of 42 μmol of NADH produced min^{-1} mg^{-1} and an isocitrate $S_{0.5}$ = 0.56 mM. In addition, the wild-type enzyme had a Hill coefficient of 3.1 for isocitrate, indicative of the high cooperativity of substrate binding.

Mutations in IDH1 and IDH2 had differing effects on the kinetics with respect to isocitrate. IDH(1:S92A) exhibited a reduced maximal rate, with the V_{max} decreasing 6-fold compared to wild type. In addition, IDH(1:S92A) exhibited a slight decrease in isocitrate affinity, $S_{0.5}$ = 0.61 mM. Despite this small effect on apparent isocitrate binding affinity, the mutation in IDH1 resulted in substantially reduced cooperativity with a Hill coefficient of 1.6.

The mutation in IDH2 produced larger effects on the maximal rate of catalysis and isocitrate binding affinity. The V_{max} for IDH(2:S98A) decreased 60-fold compared to wild type, and the isocitrate binding affinity decreased 2-fold relative to wild type, giving $S_{0.5}$ = 1.25 mM. Alteration of isocitrate binding to the IDH2 subunit, however, did not affect cooperativity. IDH(2:S98A) exhibited a Hill coefficient equal to that of wild type, n = 3.1.

Taken together, these results suggest that both IDH1 and IDH2 participate in isocitrate binding. The effects of the Ser → Ala mutation, however, differ for the two subunits. The change in IDH1 results in decreased cooperativity whereas the change in IDH2 results in decreased isocitrate binding affinity. These contrasting effects suggest that IDH1 and IDH2 play different roles in catalysis and regulation.

Activation by AMP. To test whether mutations in NAD^+–IDH isocitrate binding sites affect activation by AMP, we performed kinetic assays in the presence of saturating AMP. Previous studies have shown that AMP results in a 5-fold decrease in isocitrate $S_{0.5}$ with no effect on V_{max} (Hathaway & Atkinson, 1963; Gabriel & Plaut, 1990). Furthermore, binding studies using yeast NAD^+–IDH have shown that the binding of AMP is dependent on the presence of isocitrate (Kuehn et al., 1971). Activities obtained in the presence of 100 μM AMP are plotted in Figure 3 and are summarized in Table I. The kinetic parameters obtained with wild-type enzyme are in agreement with previously published studies (Hathaway & Atkinson, 1963; Gabriel & Plaut, 1990). As shown in Figure 3A, the isocitrate binding affinity increased approximately 5-fold in the presence of AMP. Activation by AMP resulted in a slight decrease in cooperativity, the Hill coefficient decreasing from 3.1 to 2.5.

The mutant IDH(1:S92A) exhibited a small decrease in affinity for isocitrate; $S_{0.5}$ increases to 0.7 mM in the presence of AMP compared to 0.61 mM in its absence (Figure 3B). There was also a small decrease in cooperativity, n = 1.2 in the presence of AMP, but the Hill coefficient remained half that of the wild-type enzyme.

IDH(2:S98A), in contrast, was activated by AMP in a manner similar to the wild-type enzyme (Figure 3C). The binding affinity of IDH(2:S98A) for isocitrate increased 8.4-fold; $S_{0.5}$ shifted from 1.26 to 0.15 mM, a value close to that of the wild-type enzyme. In addition, a slight decrease in cooperativity, the Hill coefficient decreasing from 3.1 to 2.8, was observed; a similar decrease was seen with the wild-type enzyme.

Taken together, these results indicate that mutations in IDH1 and IDH2 have different affects on AMP activation. IDH(1:S92A) exhibited a diminished responsive to the positive modifier, while IDH(2:S98A) was activated by AMP in a manner similar to wild-type NAD^+–IDH. In addition, AMP did not restore the cooperativity lost in the IDH(1:S92A) mutant.

Activity of Individual Subunits. To determine whether the individual IDH1 or IDH2 subunits are catalytically competent, we assayed each in a background void of the other subunit. IDH1 was overexpressed using the multicopy vector YEp352-*IDH1* in the Δ*IDH2* strain. Similarly, IDH2 was overexpressed using the multicopy vector YEp352-*IDH2* in the Δ*IDH1* strain. No NAD^+–IDH activity could be detected in mitochondrial extracts from either of these strains in the presence or absence of AMP. These results suggest that both subunits are required for a functional enzyme.

To determine whether the individual subunits are capable of forming oligomeric complexes, mitichondrial extracts from the strains overexpressing the individual subunits were applied to a Bio-Gel A0.5 gel filtration column and elution patterns monitored with anti-IDH antiserum as described under Materials and Methods. Both IDH1 and IDH2 eluted as

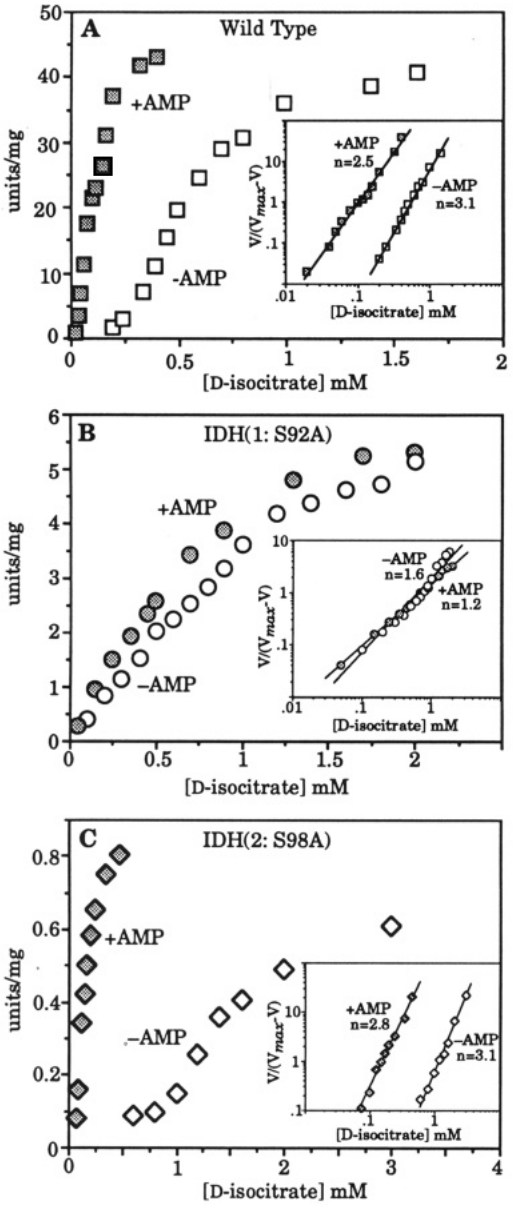

FIGURE 3: Effect of 5′-AMP on the specific activity of wild-type and mutant NAD$^+$–IDH enzymes. Activity measured in the presence of 100 μM AMP is reported in shaded symbols while open symbols indicate activity without AMP. (Panel A) Specific activity of wild-type NAD$^+$–IDH in the presence (shaded squares) and absence (open squares) of AMP. Inset: Hill plot of specific activity. (Panel B) Specific activity of IDH(1:S92A) in the presence (shaded circles) and absence (open circles) of AMP. Inset: Hill plot of specific activity. (Panel C) Specific activity of IDH(2:S98A) in the presence (shaded diamonds) and absence (open diamonds) of AMP. Inset: Hill plot of specific activity.

monomers with an apparent molecular weight of ~40 000. This result suggests that the individual subunits do not form oligomeric complexes and that interactions stabilizing the native octamer probably occur between the different subunit types.

DISCUSSION

An Allosteric Model for NAD$^+$–IDH. Early binding studies with yeast NAD$^+$–IDH indicated that the number of binding sites for isocitrate was twice that of the cofactor NAD$^+$ and the allosteric regulator AMP (Kuehn et al., 1971). On the basis of these observations, Atkinson and co-workers proposed that half of the isocitrate sites are regulatory and associated with AMP sites and half of the isocitrate sites are catalytic and associated with the NAD$^+$ sites. The role of the IDH1

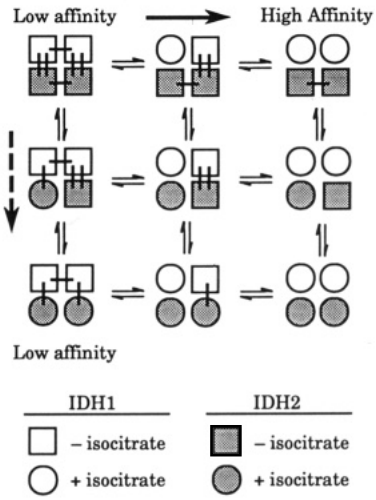

FIGURE 4: Model for the allosteric states of yeast NAD$^+$–IDH. IDH1 is proposed to be a regulatory subunit, and IDH2 is proposed to be the catalytic subunit. In this model, regulation of the catalytic activity of IDH2 is achieved primarily by interactions with IDH1. These interactions (shown by boldface lines) function to stabilize a low-affinity, low-efficiency, *tense* state. In addition, IDH1 in the *tense* reduces the apparent isocitrate affinity of IDH2 to less than that of IDH1. For the wild-type enzyme, the reaction equilibrium would favor isocitrate binding to IDH1 initially, driving the enzyme from a low-affinity to a high-affinity state (reaction pathway indicated by the boldface arrow). For simplicity, the enzyme is represented as a tetramer in this model.

and IDH2 subunits in catalysis and regulation, however, could not be determined, and the mechanism of regulation remained unclear. Using molecular biology techniques, we have been able to further examine the contribution of each subunit to regulation and catalysis, and we present here a model for the functions and interactions of IDH1 and IDH2.

Our findings with the IDH1 and IDH2 mutant enzymes support the proposal that the different subunits have functionally distinct isocitrate binding sites. For the purpose of discussion, we have formulated a simplified allosteric model based on these observations in which the IDH1 subunit regulates the activity of the IDH2 subunit (Figure 4). In this model, we consider that the enzyme exists in a low-affinity, low-efficiency, *tense* state in equilibrium with a higher affinity, catalytically more efficient, *relaxed* state [*cf.* references in Monod et al. (1971) and Koshland et al. (1966) for development of the two-state allosteric model]. In the absence of isocitrate, IDH1 acts to stabilize the *tense* state of the enzyme; this stabilization may be achieved via interactions between IDH1 and IDH2, and such interactions are shown schematically in the model as lines between the subunits. We propose that these interactions favor the *tense* state of the enzyme and reduce the affinity of IDH2 for isocitrate such that it is less than that of IDH1. Because the affinity of IDH1 for isocitrate is greater than IDH2 in the *tense* state, the wild-type enzyme will initially bind isocitrate to IDH1, and the reaction would proceed across the upper horizontal pathway indicated by the solid arrow in Figure 4; the vertical reaction pathway is not initially populated by the wild-type enzyme because the isocitrate affinity of IDH2 is less than IDH1 while in the *tense* state. Binding of isocitrate to IDH1 releases stabilizing interactions between IDH1 and IDH2, allowing the isocitrate affinity of IDH2 to increase. This leads to binding of isocitrate to IDH2 and subsequent catalysis.

The kinetic behavior of the IDH1 and IDH2 mutant enzymes is consistent with this model. The IDH1 mutant exhibited a 2-fold decrease in cooperativity, consistent with the role of IDH1 as a regulatory subunit. The loss in

cooperativity arises because the Ser → Ala mutation decreases the affinity of IDH1 for isocitrate so that it is less than that of IDH2 in the *tense* state. Thus, isocitrate will bind to IDH2, and catalysis will occur prior to isocitrate binding to the IDH1 subunit. The reaction pathway of this mutant would travel along the left vertical pathway as indicated by the dashed arrow in Figure 4. Under these conditions, IDH2 remains in a low-affinity, low-efficiency, *tense* state because of decreased isocitrate binding to IDH1. Cooperativity is reduced in the IDH1 mutant because catalysis occurs at IDH2 prior to isocitrate binding to IDH1, thereby effectively reducing by half the number of functional isocitrate sites. The small residual cooperativity may arise from interactions between the IDH2 subunits which stabilize the *tense* state and are lost upon isocitrate binding. The decreased V_{max} observed for the IDH1 mutant could occur as a result of interactions between IDH1 and IDH2 which are not released in the IDH1 mutant due to decreased isocitrate binding to IDH1. Thus, in the IDH1 mutant enzyme, IDH2 remains in a low-efficiency, *tense* state with its active site in a conformation which is not optimal for catalysis.

In contrast to the IDH1 mutation which affected cooperativity, mutation of the IDH2 subunit affected primarily isocitrate binding affinity and V_{max}. The 2-fold change in isocitrate affinity in the IDH2 mutant enzyme can be attributed to the loss of the hydrogen bond with the γ-carboxyl group of isocitrate; a similar decrease in isocitrate binding affinity has been reported for the substitution of Ser-113 with alanine in *E. coli* NADP+–IDH (Thorsness & Koshland, 1987). The large decrease in V_{max} could result from an alteration in the positioning of isocitrate in the active site due to loss of the hydrogen bond; a 12-fold decrease in V_{max} was reported for substitution of alanine for serine at position 113 of *E. coli* NADP+–IDH (Thorsness & Koshland, 1987). In the model presented, no change in cooperativity is observed in the IDH2 mutant because the isocitrate affinity of IDH1 remains greater than IDH2. Thus, IDH1 binds isocitrate as in the wild-type enzyme, releasing interactions stabilizing the *tense* state and thereby allowing IDH2 to bind isocitrate.

AMP Activation. AMP activates yeast NAD+–IDH by reducing the amount of isocitrate required for half-saturation (Hathaway et al., 1963). Presumably, AMP functions by shifting the *tense* ⇌ *relaxed* equilibrium as a result of binding to and stabilization of the *relaxed* state. Our results are consistent with this model of AMP activation and, in addition, support a model in which two types of isocitrate binding sites are present.

The IDH1 mutant enzyme exhibited reduced activation by AMP. According to the model, the loss of activation by AMP arises because the IDH1 subunit is trapped in the *tense* state and AMP will not bind and activate the enzyme. This suggests that AMP activation is mediated, at least in part, by the IDH1 subunit. These results are in agreement with previous binding studies indicating that isocitrate is required for AMP binding (Kuehn et al., 1971).

The IDH2 mutant enzyme, on the other hand, was activated by AMP in a manner similar to wild type. The affinity for isocitrate is dramatically increased in the presence of AMP ($S_{0.5}$ decreases from 1.26 to 0.15 mM). On the basis of this model, this occurs because this mutant enzyme can adopt a *relaxed* state; because IDH1 is unaltered, AMP can bind and stabilize the relaxed conformation of the enzyme to increase isocitrate affinity. The $S_{0.5}$ (0.15 mM) of the IDH2 mutant is only slightly reduced compared to wild-type enzyme (0.1 mM) in the presence of AMP. Thus, in the *relaxed* enzyme, hydrogen bonds with the hydroxyl group of Ser-98 of IDH2 contribute only slightly to the overall binding energy.

Whereas the affinity of isocitrate in the IDH2 mutant enzyme in the presence of AMP is similar to that in the wild-type enzyme, the V_{max} remains only 2% that of the wild-type enzyme. This suggests that while hydrogen bonds with the hydroxyl group of Ser-98 of IDH2 contribute only a small amount to the free energy of isocitrate binding, these interactions may be critical for the precise orientation of the substrate in the active site required for high rates of catalysis.

Activity and Interactions of Subunits. Previously we have reported that extracts from the Δ*IDH1* and Δ*IDH2* stains exhibited no in vitro activity (Cupp & McAlister-Henn, 1992). However, on the basis of our kinetic analyses of the IDH1 and IDH2 mutants, we suspected that our assay conditions may not have been sensitive enough to detect small amounts of activity by the individual subunits. To address these concerns, subunits were overexpressed in the appropriate disruption strain and assayed in the presence of higher concentrations of NAD+, and in the presence of AMP. Despite these changes, we were still unable to detect activity for the individual subunits. This result indicates that both subunits are required for a functional enzyme.

Further evidence of the requirement of both subunits is that IDH1 and IDH2 are monomeric in the absence of the other subunit. This suggests that the individual subunits do not have strong interactions. Moreover, interactions stabilizing the native octamer probably occur between different types of subunits. This is consistent with a model in which interactions between IDH1 and IDH2 are most important in stabilizing the *tense* state.

Models for the Isocitrate Binding Sites of IDH1 and IDH2. Amino acid sequence alignment of IDH1 and IDH2 with *E. coli* NADP+–IDH reveals ~32% sequence identity (Cupp & McAlister-Henn, 1991, 1992). While the *E. coli* enzyme functions as a noncooperative homodimer, it catalyzes a similar reaction to NAD+–IDH, and the overall sequence similarity with IDH1 and IDH2 suggests that both subunits may have similar overall structure to the bacterial protein. In the following discussion we assume that the structures of IDH1 and IDH2 are similar to that of the *E. coli* protein. We then discuss how sequence-specific differences could give rise to differential isocitrate binding affinities and catalytic activities of the two subunits.

A schematic diagram of the Mg^{2+}/isocitrate binding site of *E. coli* NADP+–IDH as determined by Hurley et al. is shown in Figure 5A (Hurley et al., 1989, 1990, 1991). Several residues, including Ser-113, are involved in hydrogen-bonding interactions with isocitrate. In addition, a Mg^{2+} ion is held in place by aspartic acid residues 283 and 307. This combination of bonding interactions provides a high affinity for isocitrate, $K_m \sim 5$ μM (Thorsness et al., 1987). The Mg^{2+} interacts with the α-carbonyl of isocitrate and is required for activity.

The proposed structure for the Mg^{2+}/isocitrate binding site of IDH1 based on sequence alignment with the *E. coli* enzyme (Cupp & McAlister-Henn, 1992) is shown in Figure 5B. Several differences from the *E. coli* isocitrate binding site are notable. Due to replacement of aspartic acid residues (Asp-283 and Asp-307 in *E. coli* NADP+–IDH) with asparagine (Asn-245) and threonine (Thr-241), IDH1 is predicted to lack a Mg^{2+} binding site. In addition, as shown in Figure 5B, the isocitrate binding site structure predicted by this alignment suggests that IDH1 lacks three hydrogen-bonding interactions with isocitrate due to replacement of an arginine with alanine (Ala-108) and a tyrosine with phenylalanine (Phe 136). The loss of these stabilizing interactions with the

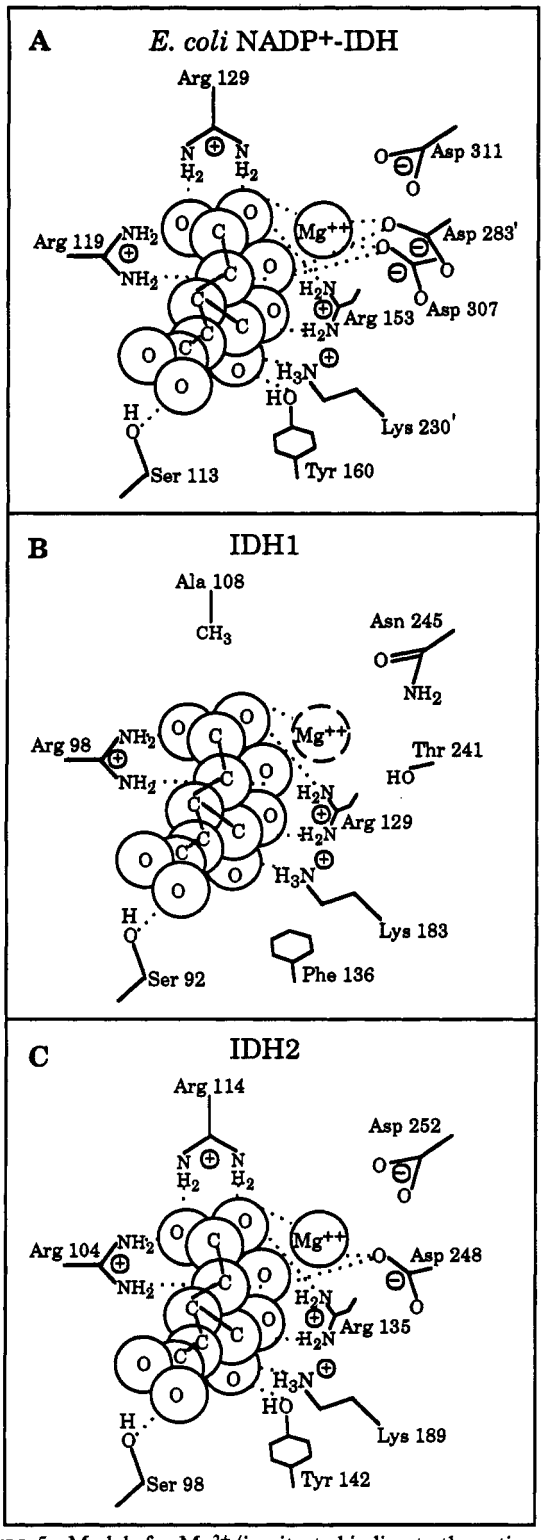

FIGURE 5: Models for Mg^{2+}/isocitrate binding to the active site of isocitrate dehydrogenase. The dashed lines indicate probable hydrogen bonds between amino acid side chains and Mg^{2+}/isocitrate. (Panel A) Isocitrate binding site of *E. coli* $NADP^+$–IDH determined by X-ray crystallography [adapted from Hurley et al. (1990)]; residues of the second subunit of the *E. coli* $NADP^+$–IDH dimer are indicated by a prime. (Panels B and C) Proposed isocitrate binding sites of IDH1 and IDH2, respectively, based on sequence alignment with the *E. coli* enzyme.

substrate would be expected to result in a decreased affinity for isocitrate compared to the *E. coli* enzyme. Sequence alignment of IDH1 and *E. coli* $NADP^+$–IDH also suggests that IDH1 lacks several residues proposed to be involved in NAD^+ binding (Cupp & McAlister-Henn, 1992). The apparent lack of a Mg^{2+} binding site and a cofactor site is consistent with a model in which IDH1 functions in a regulatory, but not catalytic, capacity.

Alignment of IDH2, in contrast, suggests that it contains each of the key residues involved in isocitrate binding in the *E. coli* enzyme (Figure 5C) (Cupp & McAlister-Henn, 1991). In addition, Asp-248 aligns with Asp-283 of the *E. coli* enzyme and may function in Mg^{2+} binding. Sequence alignment of IDH2 with *E. coli* $NADP^+$–IDH showed that several residues involved in cofactor binding also appear to be conserved (Cupp & McAlister-Henn, 1992). The presence of these features suggests that it is the IDH2 subunit which participates in catalysis.

In summary, both structural alignments of IDH1 and IDH2 with the *E. coli* enzyme and kinetic analyses of mutations in IDH1 and IDH2 support a model for yeast NAD^+–IDH in which IDH1 functions to regulate isocitrate binding to the IDH2 subunit. Regulation by the substrate at a noncatalytic site may be essential for NAD^+–IDH due to the presence of multiple isozymes in the same cellular compartment. If the NAD^+ and $NADP^+$ isozymes compete for the same isocitrate pool, the regulation of NAD^+–IDH by IDH1 would allow for maximal activation of the enzyme within a narrow concentration range of substrate while preventing shuttling of isocitrate through the NAD^+–IDH isozyme at lower isocitrate concentrations. This mechanism of regulation may provide a means of controlling the carbon flux through the citric acid cycle.

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