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Basis for Half-Site Ligand Binding in Yeast NAD$^+$-Specific Isocitrate Dehydrogenase

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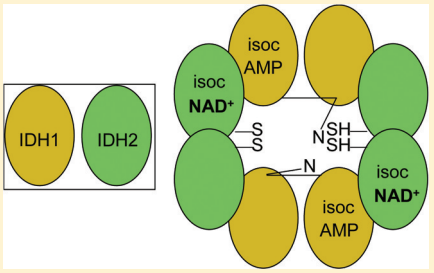
ABSTRACT: Yeast NAD$^+$-specific isocitrate dehydrogenase is an allosterically regulated octameric enzyme composed of four heterodimers of a catalytic IDH2 subunit and a regulatory IDH1 subunit. Despite structural predictions that the enzyme would contain eight isocitrate binding sites, four NAD$^+$ binding sites, and four AMP binding sites, only half of the sites for each ligand can be measured in binding assays. On the basis of a potential interaction between side chains of Cys-150 residues in IDH2 subunits in each tetramer of the enzyme, ligand binding assays of wild-type (IDH1/IDH2) and IDH1/IDH2^{C150S} octameric enzymes were conducted in the presence of dithiothreitol. These assays demonstrated the presence of eight isocitrate and four AMP binding sites for the wild-type enzyme in the presence of dithiothreitol and for the IDH1/IDH2^{C150S} enzyme in the absence or presence of this reagent, suggesting that interactions between sulfhydryl side chains of IDH2 Cys-150 residues limit access to these sites. However, only two NAD$^+$ sites could be measured for either enzyme. A tetrameric form of IDH (an IDH1^{G15D}/IDH2 mutant enzyme) demonstrated half-site binding for isocitrate (two sites) in the absence of dithiothreitol and full-site binding (four sites) in the presence of dithiothreitol. Only one NAD$^+$ site could be measured for the tetramer under both conditions. In the context of the structure of the enzyme, these results suggest that an observed asymmetry between heterotetramers in the holoenzyme contributes to interactions between IDH2 Cys-150 residues and to half-site binding of isocitrate, but that a form of negative cooperativity may limit access to apparently equivalent NAD$^+$ binding sites.



Mitochondrial NAD$^+$-specific isocitrate dehydrogenase (IDH) catalyzes a rate-limiting step in the tricarboxylic acid (TCA) cycle. The oxidative decarboxylation reaction catalyzed by IDH is essentially irreversible,[1] and the enzyme is subject to extensive allosteric regulation. IDH from *Saccharomyces cerevisiae* is allosterically activated by AMP[2] and inhibited by NADH,[1] suggesting that rates of respiratory metabolism may be controlled by this enzyme in response to cellular energy levels.[2] We have also reported that the activity of yeast IDH is subject to negative covalent control through formation of a disulfide bond that forms in the stationary phase of growth.[3]

Yeast IDH is an octamer composed of four IDH1 subunits (M_r = 38001) and four IDH2 subunits (M_r = 37755). The two types of subunits are 42% identical in primary sequence,[4,5] and disruption of the gene encoding either subunit eliminates cellular IDH activity. However, IDH2 primarily contributes to catalytic function, while IDH1 primarily contributes to allosteric properties of the enzyme. Both subunits contain isocitrate binding sites (for catalysis in IDH2 and for cooperativity in IDH1), and both subunits contain nucleotide binding sites (for NAD$^+$ in IDH2 and for AMP in IDH1).[6,7] On the basis of mutagenesis studies, two-hybrid analyses, and crystallographic structures,[8−10] the most extensive interactions within the octameric enzyme are between an IDH1 and an IDH2 subunit within a heterodimer (e.g., between E and F and between G and H, respectively, in Figure 1). Among these interactions are contributions to ligand binding sites in both subunits (IDH1 and IDH2) by residues from the other subunit

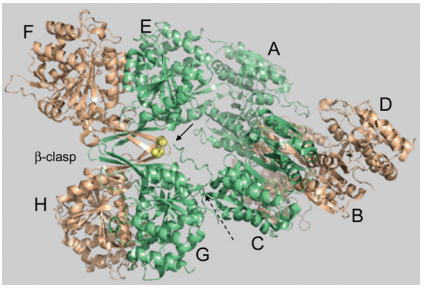


Figure 1. Structure of the yeast IDH octamer (PDB entry 3BLV). Catalytic IDH2 subunits are colored light gold, and regulatory IDH1 subunits are colored light green. Heterodimer pairs of IDH1 and IDH2 subunits are designated A and B, C and D, E and F, and G and H, respectively. One heterotetramer (A/B and C/D) is twisted in space relative to the other heterotetramer (E/F and G/H). The *β*-clasp region between the E/F and G/H heterodimers is denoted. IDH2 Cys-150 residues from subunits F and H are colored yellow. The amino terminus of IDH1 subunit C interacts with residues in the IDH1 G subunit (dashed arrow) and extends toward the Cys-150 residues (solid arrow).

(IDH2 and IDH1, respectively) that represent direct mechanisms for communication between regulatory and catalytic sites.[8,9] The organization of two heterodimers into a

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tetramer includes regions of interaction between subunits of each heterodimer (e.g., E/F and G/H in Figure 1) to form an eight-stranded β-clasp region (noted in Figure 1) that can be stabilized by a disulfide bond between Cys-150 residues from adjacent IDH2 subunits (yellow in Figure 1).[3,10]

In contrast to the extensive interactions within heterodimers and heterotetramers, the only substantial interaction between two heterotetramers to form the octamer enzyme is a protrusion (dotted arrow in Figure 1) of residues 12−16 of the amino terminus from an IDH1 subunit (e.g., C in Figure 1) from one tetramer into an IDH1 subunit (e.g., G in Figure 1) of the other tetramer. Because of a deviation from pseudo-222 symmetry, only one IDH1 subunit from each tetramer forms this interaction.[10] In support of the importance of this interaction to formation of the octamer, we have shown that a single mutation (IDH1 G15D) is sufficient to produce a stable tetrameric form of the enzyme.[11] In the ligand-bound form of the IDH structure[a] shown in Figure 1, the remainder of the IDH1 amino terminus (e.g., of subunit C in Figure 1) extends to place the first residue in the proximity of the IDH2 Cys-150 residues in the other tetramer (solid arrow in Figure 1). The proximity of the IDH1 amino terminus was proposed to help stabilize the reduced forms of the IDH2 Cys-150 residues.[10] In the ligand-free structure determined for IDH, the IDH1 amino terminus is not in the vicinity of the IDH2 Cys-150 residues and a disulfide bond can form between the side chains.[10] In support of these structural observations, we have found that the tetrameric form of IDH (the IDH1^{G15D}/IDH2 enzyme) is particularly sensitive to oxidation and formation of the IDH2 Cys-150 disulfide bond,[11] presumably because of the absence of the IDH1 amino terminus from the other tetramer.

Previous analyses of ligand binding properties of yeast IDH[6,7] showed a strict order of binding: a prerequisite for NAD^+ binding is the binding of (iso)citrate and Mg^{2+} at the IDH2 catalytic site, and a prerequisite for AMP binding is the binding of (iso)citrate by the IDH1 regulatory site. In these studies, we also found a major discrepancy between the number of ligand binding sites predicted by the structure of the octameric enzyme and the number of these sites actually measured in direct ligand binding assays. With four catalytic IDH2 and four regulatory IDH1 subunits per octamer, the predicted numbers of ligand binding sites are eight for isocitrate and four each for NAD^+ and AMP. These numbers are twice those measured in ligand binding assays performed by us and others.[6,7,12] To determine the structural basis for this occlusion of half of the ligand binding sites in the octameric enzyme, we have compared the ligand binding properties of the octamer (IDH1/IDH2 enzyme) with those for a stable tetrameric form of IDH (IDH1^{G15D}/IDH2). In addition, to determine the effect of the IDH2 Cys-150 disulfide bond, we have analyzed ligand binding properties of an IDH1/IDH2^{C150S} enzyme unable to form this bond with those for an IDH1/IDH2^{C56S,C242S} enzyme that contains a single cysteine residue (Cys-150) and with those for an IDH1/IDH2^{C56S,C150S,C242S} enzyme that contains no cysteine residues.

EXPERIMENTAL PROCEDURES

Mutagenesis, Expression, and Purification. Construction of the IDH1/IDH2^{C150S} and IDH1/IDH2^{C56S,C242S} enzymes was previously described.[3] The plasmid for expression of the latter enzyme (pET-15b *IDH1*^{His}/*IDH2*^{C56S,C242S} containing codons for a histidine tag on the 3′-end of the *IDH1* gene)[3,8] was used for subsequent site-directed mutagenesis to construct a plasmid for expression of the IDH1/IDH2^{C56S,C150S,C242S} enzyme. All mutations were confirmed by DNA sequence analysis.

For expression of wild-type and mutant forms of IDH, plasmids were transformed into *Escherichia coli* BL21-Gold (DE3) competent cells (Stratagene). Induction of expression and purification using Ni^{2+}-nitrilotriacetic acid chromatography was performed as previously described[8] except that harvested cells were suspended in a buffer [50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 20 mM imidazole, and 20% glycerol] containing 2 mg/mL lysozyme and incubated on ice for 15 min prior to being lysed with glass beads. The purified enzymes were dialyzed in a buffer containing 40 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 10% glycerol, then concentrated to ∼50 mg/mL (4 mL Amicon Ultra 50K filter, Millipore), and stored at −20 °C. Concentrations of purified enzymes were determined by measuring A_{280} and using a molar extinction coefficient of 168810 M^{-1} cm^{-1}.[13]

Electrophoretic Analyses. Nonreducing gel electrophoresis was conducted using 10% polyacrylamide gels made according to the method of Laemmli[14] but using a sample loading buffer lacking β-mercaptoethanol. For reducing gel electrophoresis, the sample loading buffer contained 0.35 M β-mercaptoethanol. Following electrophoresis, IDH subunits were stained using Coomassie blue or visualized using an antiserum against the holoenzyme.[15]

Kinetic and Ligand Binding Analyses. IDH activity (units per milligram of protein; 1 unit = 1 μmol of NADH/min at 24 °C) was measured in 1 mL assays containing 40 mM Tris-HCl (pH 7.4), 1 mM D-isocitrate, 4 mM $MgCl_2$, and 0.5 mM NAD^+. $S_{0.5}$ values, Hill coefficients, and apparent V_{max} values with respect to isocitrate were determined in the absence of AMP using concentrations of D-isocitrate ranging from 0 to 10 mM, in the presence of 0.1 mM AMP using concentrations of D-isocitrate ranging from 0 to 2 mM, and in the absence or presence of 0.1 mM dithiothreitol.

Ligand binding assays using an ultrafiltration method were conducted essentially as previously described.[6,7] Samples (2−5 mg) of purified enzymes were incubated for 30 min at 24 °C in 0.5 mL of binding buffer in the absence or presence of 0.1 mM dithiothreitol. The binding buffer for all ligands contained 40 mM Tris-HCl (pH 7.4) and 4 mM $MgCl_2$. For isocitrate binding assays, the binding buffer contained no AMP with D-isocitrate concentrations ranging from 0 to 7.5 mM or contained 0.1 mM AMP with D-isocitrate concentrations ranging from 0 to 2.5 mM. For AMP binding assays, the binding buffer also contained 2.5 mM D-isocitrate and AMP concentrations ranging from 0 to 2 mM. For NAD^+ binding assays, the binding buffer also contained 20 mM citrate and NAD^+ concentrations ranging from 0 to 2 mM. Following incubation, the samples were transferred to 0.5 mL Amicon Ultra 30K filtration devices (Millipore) and centrifuged at 7000 rpm in a microcentrifuge for 1 min. The filtrates (∼150 μL) were used to measure concentrations of unbound ligand. Isocitrate concentrations in filtrates were determined by enzymatic assays using standard curves made on the same day using wild-type IDH. NAD^+ and AMP concentrations in the filtrates were measured spectrophotometrically with standard curves at A_{260}. Binding is expressed as moles of bound ligand per mole of IDH enzyme.

To assess the effects of diamide, we incubated 2 mg samples of the IDH1/IDH2^{C56S,C242S} enzyme with concentrations of diamide ranging from 0 to 10 mM for 30 min at 24 °C prior to

isocitrate binding assays. Samples (1.2 μg) of each incubation mix were also used for IDH enzyme assays, and samples (4.5 μg) were used for nondenaturing gel electrophoresis. To assess the effect of dithiothreitol on the reversing effects of diamide treatment, 2 mg samples of the IDH1/IDH2^{C56S,C242S} enzyme preincubated with 10 mM diamide were subsequently incubated with concentrations of dithiothreitol ranging from 0 to 30 mM for 30 min at 24 °C prior to the use of aliquots as described above for isocitrate binding assays, enzyme assays, and nondenaturing gel electrophoresis. Note that isocitrate concentrations and standard curves in this case were determined enzymatically using the IDH1/IDH2^{C150S} enzyme that is refractory to inhibition by diamide.[3]

Analysis of Disulfide Bonds during Catalysis. To analyze disulfide bonds in IDH during the process of catalysis, five 1 mL enzymatic assays were set up using an assay mix as described above but containing 10 μg/mL bovine serum albumin to serve as a protein carrier for precipitation of small amounts of IDH. One assay mix was used to spectrophotometrically follow the course of the reaction. The others were used for TCA precipitation of IDH from the assay mixes at various times: (a) prior to the addition of substrate, (b) immediately following the addition of substrate, (c) during the linear phase of catalysis, and (d) 3 min after the end of the catalytic assay. The protein precipitates were dissolved in a urea buffer[3] and used for nondenaturing gel electrophoresis as described above.

RESULTS

Effects of Dithiothreitol on IDH Kinetics and Ligand Binding. The wild-type yeast IDH enzyme (IDH1/IDH2) was expressed in *E. coli* and affinity purified as described in Experimental Procedures. The purities of this enzyme and other enzymes used in this study are illustrated in Figure 2.

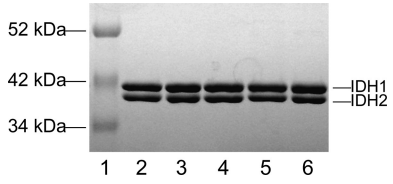

Figure 2. Purified wild-type and mutant forms of IDH. Denaturing gel electrophoresis was conducted using 2.5 μg samples of affinity-purified wild-type (IDH1/IDH2, lane 2) and mutant (IDH1^{G15D}/IDH2, lane 3; IDH1/IDH2^{C150S}, lane 4; IDH1/IDH2^{C56S,C242S}, lane 5; and IDH1/IDH2^{C56S,C150S,C242S}, lane 6) enzymes. Protein standards were run in lane 1, and proteins were stained with Coomassie blue.

Kinetic and ligand binding analyses were performed using the wild-type octameric enzyme as previously described,[6,7] except that the purified enzyme was dialyzed to remove imidazole prior to being stored. Data listed in Table 1 related to the kinetic properties with respect to isocitrate determined in the absence of dithiothreitol were similar to those previously described for the wild-type yeast enzyme.[6] For example, $S_{0.5}$ values with respect to isocitrate were reduced ~6-fold by the presence of 100 μM AMP, indicative of positive allosteric regulation. Hill coefficients of 3–4 also indicated substantial cooperativity.

Isocitrate binding analyses conducted with wild-type IDH in the absence of dithiothreitol (Table 1) indicated the presence of four isocitrate binding sites, a positive AMP effect of ~2-fold on isocitrate binding, and substantial cooperativity with Hill coefficients of ~4. Other ligand binding assays suggested there are two ligand binding sites each per octamer for AMP and NAD^+. Thus, as mentioned above, the total number of binding sites measured for isocitrate, AMP, and NAD^+ is half the number predicted by the subunit composition and structure of octameric IDH. These data are similar to those previously reported,[6] except our studies indicate some cooperativity in the binding of both AMP and NAD^+, with Hill coefficients of ~2, whereas the previous study produced values of 1.4 for these ligands.

To determine potential effects of disulfide-bond formation (e.g., between IDH2 Cys-150 residues) on the activity and ligand binding properties of the wild-type enzyme, kinetic and ligand binding properties were evaluated using assays containing 0.1 mM dithiothreitol. As shown in Table 1, the presence of dithiothreitol primarily affected the apparent V_{max} (V_{max}^{app}) of IDH, with an increase of ~17%, but had little effect on kinetic activation by AMP or on cooperativity with respect to isocitrate. In contrast, the presence of dithiothreitol had a dramatic effect on the number of ligand binding sites for isocitrate (eight) and AMP (four), but no effect on the number of NAD^+ (two) binding sites. K_D values for ligands and Hill coefficients determined in the presence of dithiothreitol were not substantially different from those determined in the absence of dithiothreitol.

These data suggest that formation of a disulfide bond, presumably between the IDH2 Cys-150 residues, or some interaction between cysteine side chains may normally limit the availability of isocitrate binding sites and subsequently of AMP binding sites, because isocitrate binding is essential for binding of AMP to IDH1 subunits.[7] The data also imply that sulfhydryl side chain interactions have no impact on binding of NAD^+, suggesting that there may be only two functional NAD^+ binding sites in the wild-type enzyme. Given that the IDH octamer exhibited half-site ligand binding in the absence of dithiothreitol, and because previously published data suggested that the IDH1 and IDH2 subunits within a heterodimer act in concert,[8,9] one important question about ligand binding sites and allosteric interactions in the wild-type enzyme is whether the "active" heterodimers are located in opposite tetramers or in the same tetramer. On the basis of the model for IDH shown in Figure 3A, these respective possibilities are shown schematically in Figure 3B. Because, in the presence of dithiothreitol, all ligand binding sites except for two potential NAD^+ sites can be occupied, a corollary of this question is whether, in the presence of dithiothreitol, the two active NAD^+ binding sites are located in opposite tetramers or in the same tetramer (shown in Figure 3C). To answer these questions, we conducted kinetic and ligand binding analyses of a stable tetrameric form of IDH.

Kinetic and Ligand Binding Properties (with or without dithiothreitol) of a Tetrameric Form of IDH. We previously constructed and analyzed three stable tetrameric forms of yeast IDH[11] and determined that a single residue change (G15D) in IDH1 was alone sufficient to produce a tetramer. As previously reported[11] and as shown in Table 2, the IDH1^{G15D}/IDH2 enzyme, relative to the wild-type octamer, exhibited ~50% reductions in V_{max}^{app} values and Hill coefficients with respect to those of isocitrate but retained kinetic allosteric activation by AMP. The tetrameric enzyme was previously shown to be much more sensitive to oxidation of the Cys-150 disulfide bond,[11] presumably because of the

Table 1. Kinetic and Ligand Binding Analyses of the Wild-Type IDH Octamer

	Kinetic Properties with Respect to Isocitrate (−AMP/+AMP)					
	−DTT			+DTT		
	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient
	36/36	0.56/0.09	3.0/3.7	42/42	0.51/0.10	3.8/3.8
	Ligand Binding Properties					
	−DTT			+DTT		
ligand	no. of binding sites	K_D (mM)	Hill coefficient	no. of binding sites	K_D (mM)	Hill coefficient
isocitrate (−AMP/+AMP)	3.9/4.1	0.58/0.27	3.6/4.3	8.0/8.4	0.64/0.31	4.4/4.0
AMP	2.2	0.04	2.2	3.9	0.05	1.6
NAD^+	2.0	0.20	2.3	2.1	0.20	1.6

absence of the amino terminus of an IDH1 subunit from another tetramer.[10]

We analyzed the ligand binding properties of the $IDH1^{G15D}$/IDH2 enzyme and determined there are two isocitrate binding sites, two AMP binding sites, and one NAD^+ binding site per tetramer (Table 2). K_D values for isocitrate and AMP were ∼2-fold higher than those for the octamer, but similar K_D values for NAD^+ were observed for the octameric and tetrameric enzymes. Interestingly, the tetramer retained Hill coefficients of >3 with respect to isocitrate binding, no apparent cooperativity for AMP binding, and a Hill coefficient of ∼2 for NAD^+ binding.

Kinetic and ligand binding analyses of the $IDH1^{G15D}$/IDH2 enzyme were also conducted using assays containing 0.1 mM dithiothreitol. As shown in Table 2, with respect to isocitrate, the V_{max}^{app} for the tetramer increased ∼26% in the presence versus the absence of dithiothreitol, but kinetic $S_{0.5}$ and Hill coefficient values were largely unchanged. The major effect of dithiothreitol on ligand binding properties was an increase to four binding sites for isocitrate versus two measured in the absence of dithiothreitol.

As illustrated in Figure 4, these data suggest that, in the absence of dithiothreitol, two of the isocitrate binding sites in the tetramer are not accessible, there are two fully accessible AMP binding sites, and there is a single functional NAD^+ binding site. The latter finding suggests that the first models in panels B and C of Figure 3 may be more representative of ligand binding in the octameric enzyme, i.e., that the two active NAD^+ binding sites are located in opposite tetramers rather than within the same tetramer. The data also suggest that an interaction between IDH2 Cys-150 side chains may normally limit the number of isocitrate binding sites and activity in the tetramer. One unexpected finding was that the Hill coefficient for NAD^+ binding by the tetramer measured in the absence or presence of dithiothreitol was ∼2 despite there being a single binding site for this ligand. Another unexpected result was the presence in the untreated tetramer of two isocitrate binding sites and three sites for other ligands (two for AMP and one for NAD^+). Because (iso)citrate binding is necessary for binding both AMP in IDH1 and NAD^+ in IDH2 subunits,[7] the substantial cooperativity observed for binding of isocitrate by the tetramer (Hill coefficients of >3) may indicate some interactions among (iso)citrate binding sites to facilitate binding of the other ligands.

Kinetic and Ligand Binding Properties (with or without dithiothreitol) of an $IDH1/IDH2^{C150S}$ Enzyme. To more directly assess the role of the IDH2 Cys-150 disulfide bond or interaction, we analyzed the kinetic and ligand binding properties of the $IDH1/IDH2^{C150S}$ mutant enzyme. We previously found that this enzyme is refractory to inhibition or disulfide-bond formation in the presence of diamide in vitro and that the mutant enzyme fails to form the disulfide bond in vivo in yeast cells grown to the stationary phase.[3] As shown in Table 3, the kinetic properties of the $IDH1/IDH2^{C150S}$ enzyme were similar to those of the wild-type enzyme except that the V_{max} value for the $IDH1/IDH2^{C150S}$ enzyme was ∼17% higher than that of the wild-type enzyme and was unchanged by the presence of dithiothreitol. This result suggests that some interaction between IDH2 Cys-150 residues in the wild-type enzyme curtails catalytic activity.

The ligand binding properties of the $IDH1/IDH2^{C150S}$ enzyme were found to be very similar whether they were measured in the absence or presence of dithiothreitol (Table 3). The enzyme has eight isocitrate, four AMP, and two NAD^+ binding sites, similar to the number of binding sites measured for the wild-type enzyme in the presence of dithiothreitol. K_D values for isocitrate were ∼2-fold higher for the $IDH1/IDH2^{C150S}$ enzyme than for the wild-type enzyme, suggesting that the Cys-150 disulfide bond may facilitate binding of the substrate.

Kinetic and Ligand Binding Properties (with or without dithiothreitol) of $IDH1/IDH2^{C56S,C242S}$ and $IDH1/IDH2^{C56S,C150S,C242S}$ Enzymes. In addition to IDH2 Cys-150, there are only two other cysteine residues in IDH, IDH2 Cys-56 and IDH2 Cys-242. We previously constructed and analyzed an $IDH1/IDH2^{C56S,C242S}$ mutant enzyme to isolate effects due to IDH2 Cys-150,[3] and we constructed an IDH mutant enzyme ($IDH1/IDH2^{C56S,C150S,C242S}$) containing serine replacements for all three cysteine residues in IDH for this study.

Analysis of the kinetic properties of the $IDH1/IDH2^{C56S,C242S}$ mutant enzyme that contains only Cys-150 (Table 4) showed a reduction in V_{max} relative to that of the wild-type enzyme of ∼50%, although some activity (∼30%) was restored in the presence of dithiothreitol. $S_{0.5}$ values for isocitrate were 2−3-fold higher than wild-type values under both conditions, and Hill coefficients were reduced to ∼2. These results suggest that IDH2 Cys-56 and Cys-242 residues are important for maximal activity, apparent affinity for isocitrate, and cooperativity.

The $IDH1/IDH2^{C56S,C242S}$ enzyme displayed four binding sites for isocitrate and two binding sites for AMP in the absence of dithiothreitol, and these numbers increased to eight and four in the presence of dithiothreitol, respectively, as was observed for the wild-type enzyme. This suggests it is the remaining IDH2 Cys-150 residue that controls access to isocitrate binding sites. K_D values for isocitrate measured in the absence or presence of dithiothreitol were increased 2−3-fold and Hill

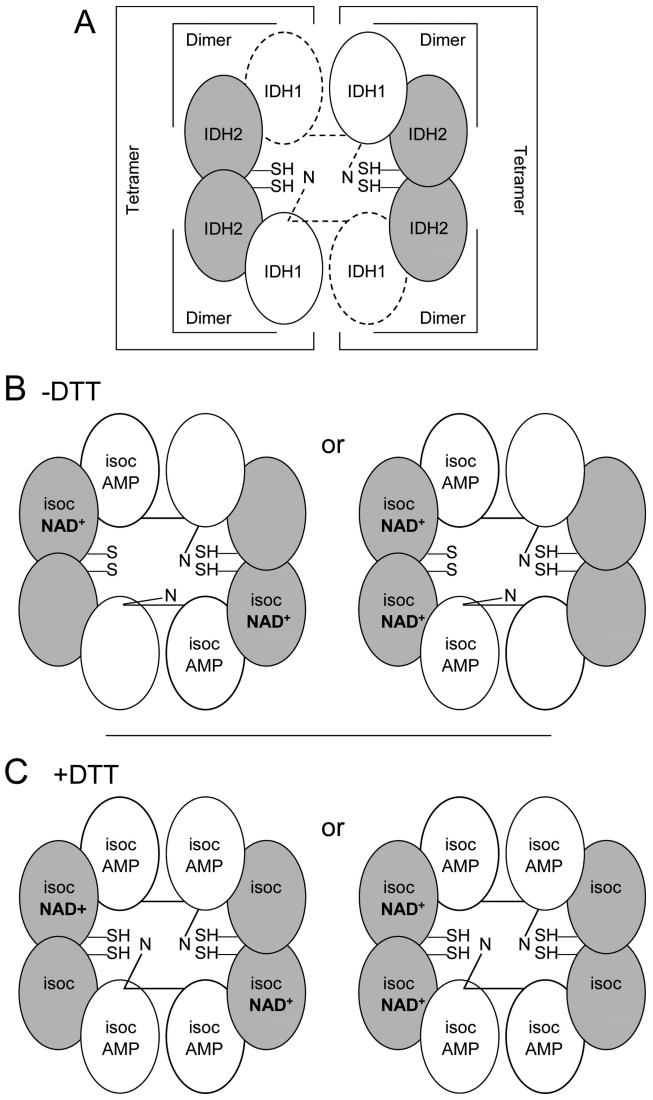

Figure 3. Models for ligand binding in octameric IDH. (A) Schematic representation of the wild-type IDH enzyme. The amino termini of two IDH1 subunits (dashed ovals) from each tetramer are shown extending toward sulfhydryl side chains of Cys-150 residues of the other tetramer. The deviation from symmetry between the tetramers has been removed for the sake of clarity. (B) Alternative possible arrangements of ligand binding sites in active dimers in opposite tetramers or in the same tetramer of the wild-type octameric enzyme based on ligand binding analyses. On the basis of the half-site binding properties of the enzyme, it is assumed that only one of the IDH1 amino termini from one tetramer would extend toward the IDH2 Cys-150 residues in the other tetramer. The side chains of these Cys-150 residues are shown as sulfhydryl groups. The oxidative state(s) of the other IDH2 Cys-150 side chains is unknown (see Discussion). (C) Alternative possible arrangements of NAD^+ binding sites in the IDH octamer treated with dithiothreitol (DTT). On the basis of the full-site binding properties of isocitrate and AMP observed with dithiothreitol, it is assumed that IDH2 Cys-150 residues in both tetramers are reduced. The alternative models show that active NAD^+ binding sites may be in opposite tetramers or in the same tetramer.

coefficients reduced to 2−2.5 relative to those of the wild-type enzyme, again suggesting that the IDH2 Cys-56 and Cys-242 residues are important for optimal and cooperative binding of this substrate.

The kinetic properties of the IDH1/IDH2^{C56S,C150S,C242S} mutant enzyme (Table 5) were intermediate between those of IDH1/IDH2^{C150S} and IDH1/IDH2^{C56S,C242S} in terms of V_{max}^{app} values, $S_{0.5}$ values, and Hill coefficients with respect to isocitrate. The V_{max} values measured for the IDH1/IDH2^{C56S,C150S,C242S} enzyme in the absence or presence of dithiothreitol were similar, as observed for the IDH1/IDH2^{C150S} enzyme, again suggesting that the IDH2 Cys-150 residue may normally limit catalytic activity. The numbers of ligand binding sites for the IDH1/IDH2^{C56S,C150S,C242S} enzyme measured in the absence or presence of dithiothreitol were similar to the numbers determined for the IDH1/IDH2^{C150S} enzyme, again suggesting that the Cys-150 residue regulates access to isocitrate and AMP binding sites. However, apparent K_D values and Hill coefficients for isocitrate for the IDH1/IDH2^{C56S,C150S,C242S} enzyme were more similar to those measured for the IDH1/IDH2^{C56S,C242S} enzyme, also suggesting that the IDH2 Cys-56 and Cys-242 residues contribute to substrate affinity and cooperativity.

Effects of Diamide and Dithiothreitol on the Number of Isocitrate Binding Sites. Data presented above suggest that formation of the IDH2 Cys-150 disulfide bond or some interaction between these residues limits access to isocitrate binding sites and subsequently curtails maximal activity in the IDH holoenzyme. To address this possibility, we compared concomitant effects of increasing concentrations of diamide (a sulfhydryl oxidant that we previously used in analyses of IDH)[3] on formation of the IDH2 Cys-150 disulfide bond, on activity, and on the number of isocitrate binding sites. For these experiments, we used the IDH1/IDH2^{C56S,C242S} mutant enzyme to limit effects to those due to the remaining IDH2 Cys-150 residue.

As shown in Figure 5A, nondenaturing gel electrophoresis of samples of the IDH1/IDH2^{C56S,C242S} enzyme following 30 min incubations with increasing concentrations of diamide indicated that levels of the free form of IDH2 decreased as levels of the disulfide-bond form of IDH2 increased. (Note that the disulfide-bond form of IDH2 was previously chemically characterized.[3]) Concomitant with an increased level of formation of the disulfide bond, IDH catalytic activity was reduced from 100% in the absence of diamide to <10% in the presence of 10 mM diamide (graph 1 in Figure 5A). Also concomitant with formation of the disulfide bond, the number of isocitrate binding sites decreased from approximately four in the absence of diamide to approximately one in the presence of 10 mM diamide (graph 2 in Figure 5A). It is interesting to note that the concentration of diamide (~0.5 mM) that produced approximately equal amounts of the free and disulfide-bond form of IDH2 also resulted in ~50% reductions in IDH activity and in the number of isocitrate binding sites (i.e., from 4 to 2.5).

To determine if the diamide-induced changes in the IDH1/IDH2^{C56S,C242S} enzyme could be reversed by addition of dithiothreitol to reduce the IDH2 Cys-150 disulfide bond, samples of the enzyme preincubated with 10 mM diamide were subsequently incubated with increasing concentrations of dithiothreitol. As shown in Figure 5B, nondenaturing gel electrophoresis demonstrated that levels of the disulfide-bond form of IDH2 were reduced while levels of the free IDH2 subunit were increased by the presence of 10−30 mM dithiothreitol. Concomitantly, the activity of IDH was restored to >90% of the original activity with 15 mM dithiothreitol and to 120% of the original activity of the IDH1/IDH2^{C56S,C242S} enzyme with 20 and 30 mM dithiothreitol (graph 1 in Figure 5B). In addition, the number of isocitrate binding sites

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Table 2. Kinetic and Ligand Binding Analyses of the IDH1^{G15D}/IDH2 Tetramer

	Kinetic Properties with Respect to Isocitrate (−AMP/+AMP)					
	−DTT			+DTT		
	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient
	16/18	0.57/0.15	1.9/1.7	21/22	0.68/0.12	1.6/2.0
	Ligand Binding Properties					
	−DTT			+DTT		
ligand	no. of binding sites	K_D (mM)	Hill coefficient	no. of binding sites	K_D (mM)	Hill coefficient
isocitrate (−AMP/+AMP)	1.8/2.2	1.01/0.50	3.1/3.6	3.9/3.9	1.19/0.48	2.1/3.2
AMP	2.2	0.10	1.1	2.2	0.10	1.4
NAD^+	1.0	0.21	1.9	1.0	0.19	2.2

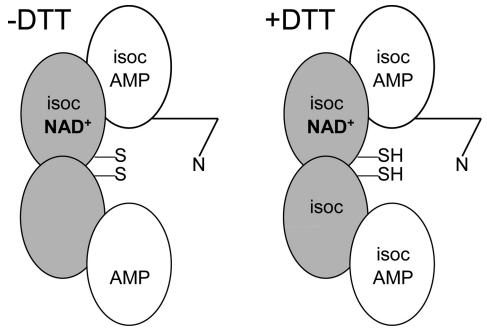

Figure 4. Models for ligand binding in tetrameric IDH. On the basis of ligand binding analyses, the IDH tetramer (IDH1/IDH2^{G15D} enzyme) exhibits half-site binding for isocitrate in the absence of dithiothreitol (DTT) but full-site binding in the presence of dithiothreitol. Full-site binding for AMP is observed under both conditions, and half-site binding for NAD^+ is observed under both conditions.

increased to eight at the higher concentrations of dithiothreitol (graph 2 in Figure 5B). This is similar to the number of sites previously measured for the enzyme in the presence of dithiothreitol (Table 4) in contrast to the four sites measured in the absence of dithiothreitol (Table 4 and the initial lane of Figure 5A). Similar to the observation described above, the concentrations of dithiothreitol (7−10 mM) producing approximately equivalent levels of free and disulfide-bond forms of the IDH2 subunit correlated with the concentrations of dithiothreitol that restored ~50% of the enzyme activity and isocitrate binding sites. Collectively, these data indicate that the formation of the IDH2 Cys-150 disulfide bond can limit both IDH enzymatic activity and the number of accessible isocitrate binding sites.

Disulfide-Bond Formation during Catalysis. Because IDH2 Cys-150 residues seem to be important for limiting IDH catalytic activity, we investigated if disulfide-bond formation occurs during the catalytic process. For this, we again used the IDH1/IDH2^{C56S,C242S} enzyme to directly assess the effects of the residual Cys-150 residues. The enzyme was precipitated as described in Experimental Procedures from an assay mix prior to addition of substrate, immediately following addition of substrate to an assay mix, during the linear phase of catalysis, and several minutes after the assay was terminated. The precipitated enzyme samples were electrophoresed using a nondenaturing gel. As shown in Figure 6, there was a minor amount of the disulfide-bond form of IDH2 present in the IDH1/IDH2^{C56S,C242S} enzyme prior to an assay and immediately following addition of substrate (lanes 1 and 2, respectively), and this amount was ~2-fold higher in samples of the enzyme taken during the linear phase of catalysis (lane 3) or after completion of the assay (lane 4). However, overexposure of the immunoblot shown in Figure 6 was necessary to visualize the disulfide-bond form of IDH2, and there was no apparent decrease in the levels of the free form of IDH2 using lower exposures (data not shown). Thus, there was little evidence of the presence of or formation of a Cys-150 disulfide bond during catalysis, suggesting that any interactions between Cys-150 residues during catalysis are likely to be transient.

DISCUSSION

The comparisons of kinetic and ligand binding properties presented here suggest that wild-type yeast octameric IDH normally exhibits less than optimal activity and that only half of the possible isocitrate and AMP binding sites are accessible because of the presence of the IDH2 Cys-150 residues. Increased activity is observed, and all binding sites for isocitrate and AMP are accessible in the octameric IDH1/IDH2^{C150S} enzyme. However, the activity of the IDH1/IDH2^{C150S} enzyme is only 17% higher than that of the wild-type enzyme perhaps because of the presence of only two active NAD^+ binding sites. The finding that only two NAD^+ sites are accessible in the octameric enzyme was unexpected because the structure suggests there are four apparently equivalent NAD^+ binding sites in IDH2 subunits.[10]

The crystal structures determined for IDH included a ligand-free structure, a citrate-bound structure, and a citrate- and AMP-bound structure.[10] The structure in the presence of NAD^+ was not determined. However, because crystallography selects for symmetry, four AMP binding sites were occupied in the citrate- and AMP-bound structure whereas only two should have been accessible in the octameric wild-type enzyme. Thus, it is likely that the structure determined in the presence of NAD^+ would show similar occupancy of four sites. We examined the general location of potential NAD^+ binding sites in the yeast octamer structure. In Figure 7, which shows a side-on view of the IDH octamer, residues in IDH2 subunits (Asp-286, Ile-287, and His-281) known by mutagenesis to be critical for NAD^+ binding[7] are highlighted. The NAD^+ binding sites appear to be easily accessible and are sufficiently distant from each other that any negative regulation of NAD^+ binding would likely be due to allosteric mechanisms. In any event, while the half-site binding properties for isocitrate and AMP exhibited by wild-type IDH are clearly due to the presence of

Table 3. Kinetic and Ligand Binding Analyses of the IDH1/IDH2^{C150S} Octamer

	Kinetic Properties with Respect to Isocitrate (−AMP/+AMP)					
	−DTT			+DTT		
	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient
	42/43	0.53/0.10	3.7/3.2	42/42	0.54/0.10	3.7/3.1
	Ligand Binding Properties					
	−DTT			+DTT		
ligand	no. of binding sites	K_D (mM)	Hill coefficient	no. of binding sites	K_D (mM)	Hill coefficient
isocitrate (−AMP/+AMP)	8.0/8.2	1.32/0.32	4.0/4.1	7.8/8.3	1.28/0.31	3.9/3.5
AMP	4.1	0.06	1.9	4.1	0.06	1.9
NAD^+	2.0	0.24	2.0	2.1	0.22	2.1

Table 4. Kinetic and Ligand Binding Analyses of the IDH1/IDH2^{C56S,C242S} Octamer

	Kinetic Properties with Respect to Isocitrate (−AMP/+AMP)					
	−DTT			+DTT		
	$V^{max^{app}}$ (units/mg)	$S_{0.5}$ (mM)	Hill coefficient	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient
	23/26	1.6/0.32	2.1/1.8	32/32	1.3/0.2	1.9/2.2
	Ligand Binding Properties					
	−DTT			+DTT		
ligand	no. of binding sites	K_D (mM)	Hill coefficient	no. of binding sites	K_D (mM)	Hill coefficient
isocitrate (−AMP/+AMP)	4.1/4.2	1.41/0.39	2.1/2.5	8.2/8.4	1.53/0.43	2.5/2.3
AMP	2.0	0.10	2.2	4.0	0.19	1.3
NAD^+	1.9	0.21	2.4	2.0	0.24	2.3

Table 5. Kinetic and Ligand Binding Analyses of the IDH1/IDH2^{C56S,C150S,C242S} Octamer

	Kinetic Properties with Respect to Isocitrate (−AMP/+AMP)					
	−DTT			+DTT		
	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient	V_{max}^{app} (units/mg)	$S_{0.5}$ (mM)	Hill coefficient
	31/31	1.03/0.17	3.0/3.4	32/32	1.03.19	2.9/3.0
	Ligand Binding Properties					
	−DTT			+DTT		
ligand	no. of binding sites	K_D (mM)	Hill coefficient	no. of binding sites	K_D (mM)	Hill coefficient
isocitrate (−AMP/+AMP)	8.2/8.1	1.45/0.40	2.1/2.5	8.2/8.4	1.56/0.45	2.5/2.3
AMP	4.1	0.13	1.3	4.2	0.14	1.2
NAD^+	2.1	0.15	2.1	2.3	0.22	1.5

IDH2 Cys-150 residues, the basis for half-site binding properties for the cofactor remains unresolved.

Data presented for the tetrameric IDH1^{G15D}/IDH2 enzyme also suggest that the half-site binding of isocitrate is due to sulfhydryl interactions, because there were two measurable sites in the untreated enzyme but four measurable sites in the presence of dithiothreitol. Two binding sites were measurable for AMP under both conditions, suggesting that the octameric structure may contribute to masking AMP binding sites in the holoenzyme. The tetrameric enzyme also had a single NAD^+ binding site, suggesting that only one of the two apparent NAD^+ binding sites in the tetramer is functional. Thus, any negative regulation of NAD^+ binding at one site may occur at the level of the tetramer. Along these lines, it is interesting to note that allosteric activation by AMP of activity and isocitrate binding is preserved in the tetramer, suggesting that this property is controlled at the level of the tetramer or perhaps at the level of component heterodimers.

In vitro data with diamide (Figure 5) clearly indicate that formation of an IDH2 Cys-150 disulfide bond can limit IDH activity and the number of isocitrate binding sites. However, under conditions used for kinetic and ligand binding analyses of the wild-type enzyme, there is little evidence that the disulfide bond is present, despite the half-site occupancy of isocitrate and AMP binding sites. That full-site occupancy is obtained for the wild-type enzyme in the presence of dithiothreitol suggests that some interaction between the sulfhydryl side chains of adjacent IDH2 Cys-150 residues is the key to half-site occupancy for these ligands. Thus, one possibility is that formation of a disulfide bond is so transient that the bond cannot be detected in the native holenzyme during catalysis. The IDH2 Cys-150 residues are characteristic of redox active cysteines[16] in that they are relatively exposed (see Figure 1), and when the amino

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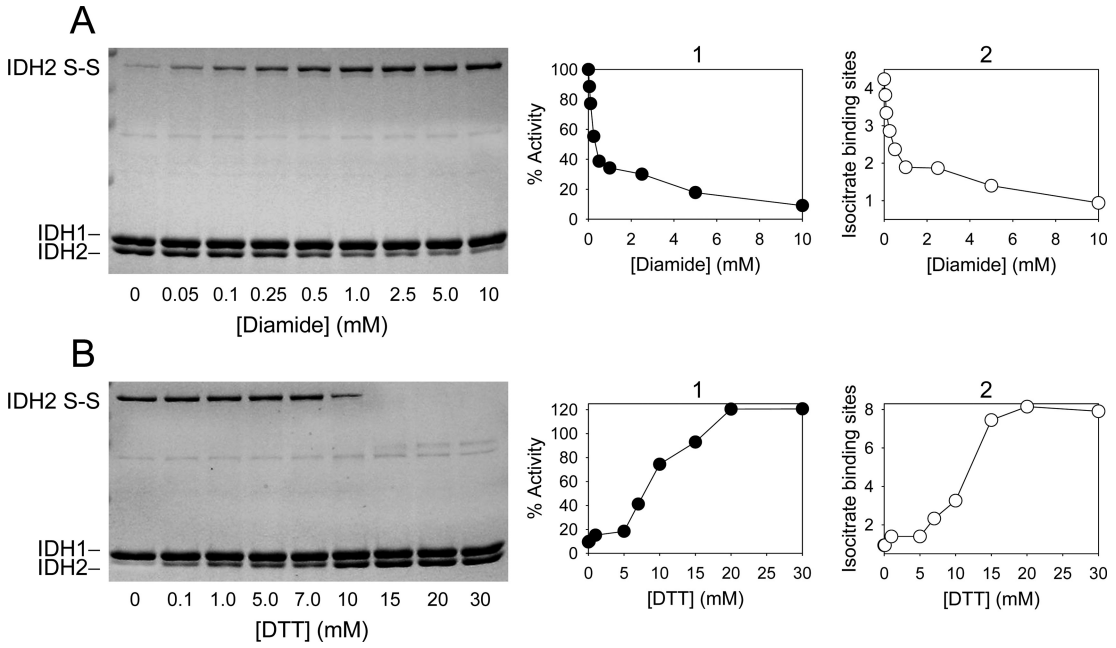

Figure 5. Effects of diamide and dithiothreitol. (A) Samples of the IDH1/IDH2^{C56S,C242S} enzyme were incubated with the indicated concentrations of diamide as described in Experimental Procedures. Aliquots of these samples were used for nondenaturing gel electrophoresis (and stained with Coomassie blue), for enzyme assays (graph 1), and for isocitrate binding assays (graph 2). (B) Samples of the IDH1/IDH2^{C56S,C242S} enzyme pretreated with 10 mM diamide were incubated with the indicated concentrations of dithiothreitol (DTT) as described in Experimental Procedures. Aliquots of these samples were used for nondenaturing gel electrophoresis (and stained with Coomassie blue), for enzyme assays (graph 1), and for isocitrate binding assays (graph 2).

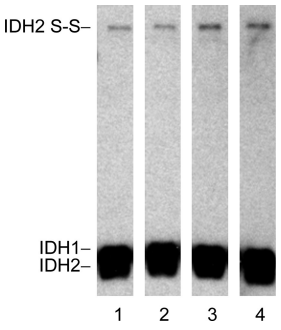

Figure 6. Analysis of disulfide-bond formation during catalysis. Samples of the IDH1/IDH2^{C56S,C242S} enzyme were precipitated from an enzyme assay mix prior to addition of substrate (lane 1), immediately after addition of substrate (lane 2), during the linear phase of the activity assay (lane 3), or 3 min after completion of the assay (lane 4). The samples were electrophoresed on a nondenaturing gel and analyzed by immunoblots using an antiserum that recognizes both IDH1 and IDH2 subunits.[15]

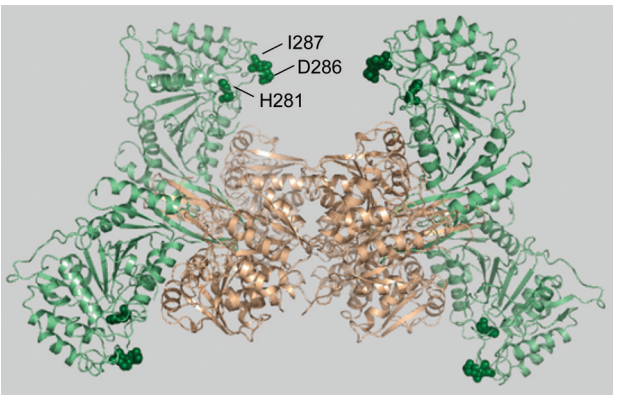

Figure 7. Location of residues in the NAD$^+$ binding sites of IDH2 subunits in the IDH holoenzyme. To visualize locations of NAD$^+$ binding sites in octameric IDH, critical residues for binding cofactor (IDH2 Asp-286, IDH2 Ile-287, and IDH2 His-281)[7] are highlighted as dark green spheres.

terminus of the IDH1 subunit from the opposite tetramer is in the vicinity of the Cys-150 residues (as is the case for ligand-bound structures for IDH), the NH_3^+ group is within ~4 Å of one of the side chains.[10] This likely stabilizes the thiolate and disfavors the disulfide bond. If the amino terminus of the IDH1 subunit is not in the vicinity of the Cys-150 bonds, as would be predicted for one tetramer in the octamer models for half-site ligand binding shown in Figure 3, the otherwise polar environment around the Cys-150 residues[10] may help stabilize possible interactions between sulfhydryl side chains.[17]

We note that in vivo analyses for yeast cells grown to the stationary phase[3] indicate that only ~25% of the IDH2 subunit is in the disulfide-bond form, and this correlates with an ~50% reduction in cellular IDH activity. Thus, the effect of IDH2 Cys-150 disulfide-bond formation in vivo is to reduce but not eliminate IDH activity during this stage of growth. This is quite similar to the regulation of isocitrate dehydrogenase (ICD) in *E. coli*. In bacterial cells shifted to acetate as the carbon source, the enzyme is phosphorylated on the Ser-113 residue in the catalytic isocitrate binding site, which eliminates catalytic activity.[18,19] However, only ~80% of the ICD molecules in the cell are phosphorylated to ensure that, while some of the isocitrate is diverted into the biosynthetic glyoxylate pathway, isocitrate can still also be utilized by the TCA cycle for energy production.[20,21] Similar metabolic changes and requirements likely apply to the stationary phase of growth of yeast cells.

Other allosteric enzymes have been reported to exhibit half-site reactivity with respect to ligand binding or interaction with

a covalent modifier.[22] In some cases, it was suggested that this phenomenon is an extreme case of negative cooperativity, so that reaction or binding in one unit of the enzyme induces a structural change in another unit of the enzyme and reduces the reactivity or affinity of the second unit to zero. However, another possibility is that half-site reactivity could result from an inherent asymmetry in the enzyme.[22] Because of the deviation from pseudo-222 symmetry in assembly of heterotetramers to form the octameric form of IDH, the latter explanation seems more relevant to IDH. However, because we also observed half-site binding for isocitrate in the tetrameric form of IDH, negative cooperativity between heterodimers within a tetramer is also a possibility. Negative cooperativity may also explain the presence of only two functional NAD^+ sites in the IDH octamer and of only one such site in the tetramer.

Mammalian IDH is also an allosterically regulated octameric enzyme.[23−25] However, the mammalian enzyme contains four catalytic α subunits and two regulatory β and γ subunits each.[25−27] The subunits of the yeast and human enzymes are 40−50% identical in sequence.[27−29] Ehrlich and Colman[30] reported that the porcine enzyme contains four binding sites per eight subunits for isocitrate, NAD^+, and the allosteric activator ADP. If we assume similar roles for catalytic and regulatory subunits in the mammalian enzyme as observed for yeast enzyme subunits, these data suggest half-site binding for isocitrate and full-site binding for NAD^+ and ADP. The mammalian catalytic α subunit lacks a residue homologous to yeast IDH2 Cys-150. However, there are numerous cysteine residues in the mammalian enzyme that may convey some property of redox reactivity. We are investigating these possibilities using the affinity-purified human enzyme.

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ABBREVIATIONS

IDH, NAD^+-specific isocitrate dehydrogenase; ICD, *E. coli* $NADP^+$-specific isocitrate dehydrogenase; DTT, dithiothreitol; PDB, Protein Data Bank.

ADDITIONAL NOTE

[a]IDH structures were obtained in the absence of ligands [Protein Data Bank (PDB) entry 3BLX], in the presence of the substrate analogue citrate (PDB entry 3BLV), or in the presence of citrate and AMP (PDB entry 3BLW). The latter two structures were similar, and the citrate-bound structure was used for most comparisons with the ligand-free structure.

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