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Evolution of a Transition State

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Abstract

An active site lysine essential to catalysis in isocitrate dehydrogenase (IDH) is absent from related enzymes. As all family members catalyze the same oxidative β -decarboxylation at the 2R-malate core common to their substrates, it seems odd that an amino acid essential to one is not found in all. Ordinarily, hydride transfer to a nicotinamide C4 neutralizes the positive charge at N1 directly. In IDH the negatively charged C4-carboxylate of isocitrate stabilizes the ground state positive charge on the adjacent nicotinamide N1, opposing hydride transfer. The critical lysine is poised to stabilize – and perhaps even protonate – an oxyanion formed on the nicotinamide 3-carboxamide, thereby enabling the hydride to be transferred while the positive charge at N1 is maintained. IDH may catalyze the same overall reaction as other family members, but dehydrogenation proceeds through a distinct, though related, transition state. Partial activation of lysine mutants by K^+ and NH_4^+ represents a throwback to the primordial state of the first substrate promiscuous family member.

Keywords

isocitrate dehydrogenase; evolution; catalysis; hydride transfer

Introduction

Isocitrate, homoisocitrate, isopropylmalate, 3-(2'-methylthio)ethylmalate and tartrate dehydrogenases belong to an ancient, ubiquitous and highly divergent family of enzymes^[1]

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Supporting information for this article is available.

that share a common distinct protein fold (Figure 1A) and, despite many insertions deletions and changes in quaternary structure, a highly conserved active site (Figure 1B). Family members employ a random steady-state kinetic mechanism^[2-5] (Scheme S1 in Supporting Information) and a three-step catalytic mechanism^[6], with C2 dehydrogenation to an oxaloacid preceding C3 decarboxylation to an enol followed by tautomerization to the ketone product (Scheme 1). In *Escherichia coli* isocitrate dehydrogenase^[7] (IDH) Lys230* initiates hydride transfer by abstracting the C2 hydroxyl proton, with the negative charge on the carbonyl stabilized by the positive charge of Mg^{2+} in the transition state. Lys230* returns the proton to reform the hydroxyl during decarboxylation at C3, again with Mg^{2+} stabilizing the negative charge on the carbonyl. Lys230* once again abstracts the hydroxyl proton during tautomerization, while the absence of the C3 carboxylate (lost as CO_2) allows Tyr160 to approach and protonate C3 to form the final ketone product. Residues that interact with the 2R-malate core common to all substrates are invariant and residues that interact with the different substrate C3-moieties differ (Table S1 in Supporting Information).

In the ternary substrate complex of IDH the coenzyme nicotinamide C4 is positioned to receive the C2-hydride of isocitrate, with its positively charged N1 3.5 Å 'above' the negatively charged substrate C4-carboxylate (Figure 1C). The ionic interaction stabilizes the positive charge at N1, and in so doing must oppose catalysis, which requires that the charge at N1 be neutralized upon hydride transfer. A catalytic Catch 22 exists in IDH: a nicotinamide positioned to receive the hydride is in no position to receive the hydride.

Model dehydrogenase reactions show that nonenzymatic reductions of N-methylacridinium by N-2'-carboxybenzylidihydronicotinamide and N-cis-2'-carboxycyclopentylidihydronicotinamide are two to three orders of magnitude faster than by uncharged N-benzylidihydronicotinamide and N-cyclopentylidihydronicotinamide derivatives.^[8] The rate accelerations are attributable to intramolecular electrostatic stabilization of the positively charged transition state by the carboxylates. If a carboxylate close to dihydronicotinamide assists carbocation formation at C4 in the transition state, then a carboxylate adjacent to nicotinamide will stabilize the positive charge at N1 in the ground state.

How can the nicotinamide C4 be activated for hydride transfer when its ground state is stabilized? Reactivity at C4 depends on the strength of the inductive effect of the group at C3;^[9] even halogens are sufficient to activate the C4 for enzymatic reduction.^[10] Lys100 is unique to, and invariant in, all IDHs (Table S1 in Supporting Information). Only 3.5 Å from the nicotinamide 3-carboxamide (Figure 1C),^[7] the side chain ϵ -amine of Lys100 is positioned to enhance the inductive effect of the carbonyl. Here, we investigate the role of Lys100 in promoting hydride transfer by IDH.

Results and Discussion

Lys100 is essential to catalysis

Lys100 is essential to catalysis by IDH (Table 1). Replacing Lys100 by Met (hydrophobic side chain), Gln (polar side chain) or His (ionizable side chain) obliterates activity; turnover (k_{cat}) falls 2×10^4 -fold and performance ($k_{cat}/K_{m.isocitrate}/K_{ia.NADP} = k_{cat}/K_{m.NADP}$)

$K_{ia, \text{isocitrate}}$) falls 3.5×10^6 -fold. Yet Lys100, essential to IDH activity, is irrelevant to catalysis in related enzymes.

Mutations at Lys100 reduce affinity for isocitrate (Table 1). Nevertheless, isocitrate binds in the same position in the Lys100Met mutant as in wildtype IDH^[11] (PDB 5icd), with the same hydrogen bonds and with Mg^{2+} chelated in the same fashion (Figure 2A). Even the largest displacements, all $< 0.5 \text{ \AA}$, cannot be considered significant given the resolution of the structures ($2.5 \text{ \AA}$ for PDB 5icd, and $2.0 \text{ \AA}$ for the Lys100Met mutant). The ϵ -amine of Lys100 lies $6.8 \text{ \AA}$ from isocitrate, too distant for direct physical contact. The effects of mutations at Lys100 on affinity for isocitrate are likely electrostatic.

Mutations at Lys100 also reduce affinity for NADP (Table 1). Nevertheless, structural studies provide no evidence that Lys100 plays a role in positioning the nicotinamide ring (Figure 2B). Instead, the nicotinamide 3-carboxamide hydrogen bonds with Glu336, the C2-carboxylate of bound isocitrate, the main chain N of Leu103, and the side chain amide of Asn115.^[7] The same hydrogen bonds are evident in the ternary product complex of the Lys100Met mutant. As a consequence, the nicotinamide occupies a similar position in both structures. Moreover, evidence shows that the wildtype steady-state mechanism distorts kinetic estimates of $K_{ia, \text{NADP}}$, giving the impression that IDH binds NADP tightly. Simply bringing the kinetic mechanism into rapid equilibrium, by mutation or by use of alternative substrates, lowers NADP affinities.^[3,12,13] The effects of mutations at Lys100 on affinity for NADP are also indirect.

Ammonium rescues catalysis in Lys100 mutants

Ammonium, which inhibits wildtype IDH activity, partially rescues Lys100Met activity (Figure 3A). Kinetic analysis yields a half saturation constant of 0.061 M , an activation constant of 130 and the same nonspecific inhibition exponent of 1.41 M^{-1} seen with wildtype IDH. Larger amines, methylamine, hydrazine and hydroxylamine, have no effect suggesting small size is a criterion for activation. Potassium also activates Lys100Met, although with a larger half saturation constant of 0.20 M , a smaller activation constant of 30 and a smaller inhibition exponent of 0.35 M^{-1} (Figure 3B). No inhibition of wildtype IDH by K^+ was detected. Activation by Li^+ and Rb^+ is modest while Na^+ has no effect and Cs^+ is slightly inhibitory (Table 2). Activation of the Lys100Gln mutant by NH_4^+ or K^+ is modest. Two additional IDH mutants that retain Lys100, their random kinetic mechanisms brought into rapid equilibrium by amino acid replacements outside their active sites, are not activated by NH_4^+ or alkali metal ions (Table 2). Activation of IDH by monovalent cations is specific to mutants at Lys100.

pH profiles lend further support for the role of the Lys100 ϵ -amine in catalysis (Figure 3C). Wildtype IDH has $V_{max} \text{ p}K_a$ of 6.3 and a $\text{p}K_b$ of 10.3 with optimal activity near pH 8. The V_{max} pH profile of the Lys100Met mutant lacks both limbs seen with wildtype IDH and is essentially flat with a modest peak activity near pH 6. Rises in the K_m s for isocitrate and NADP seen with wildtype IDH at high pH are absent from the Lys100Met mutant (Figure 3D). The limbs of the Lys100Met mutant V_{max} profile reappear when $0.2 \text{ M NH}_4\text{Cl}$ is present, with a $\text{p}K_a$ in the vicinity of 6.3 and a $\text{p}K_b$ in the vicinity of 9.3. The Lys100Gln mutant behaves similarly to the Lys100Met mutant (Table 3). We ascribe the wildtype IDH

pK_b of 10.3 to the Lys100 ϵ -amine, the absence of a pK_b in either mutant to the absence of this group, and its reappearance at pH 9.3 to NH_4^+ . Neither pK_b is greatly perturbed from its value in water (10.5 for a lysine ϵ -amine and 9.3 for NH_4^+).

Ammonium shifts the wildtype IDH pK_b from pH 10.3 to pH 8.9 with a non-integer limiting slope (Figure 3C). The effect of NH_4^+ on the wildtype IDH pH profile can be predicted assuming bound NH_3 inhibits, bound NH_4^+ cannot fully compensate for an unprotonated Lys100 ϵ -amine, and a protonated ϵ -amine prevents NH_4^+ binding through charge repulsion (Scheme S2 in Supporting Information). The prediction, which uses estimates from the pH profiles of wildtype IDH in the absence of NH_4Cl and the Lys100Met mutant in the presence of NH_4Cl , tracks the observed profile accurately (dashed line, Figure 3C).

Our experiments demonstrate Lys100 is essential to IDH catalysis. Too far (3.5 Å) to hydrogen bond to the 3-carboxamide carbonyl in the ground state, the Lys100 ϵ -amine is nevertheless poised to engage the carbonyl during catalysis.

The nicotinamide transition state in IDH is distinct from that in other dehydrogenases

Experiments with lactate and formate dehydrogenases support a transition state for nicotinamide with carbocation character at C4^[14-16] and a positively charged and near planar N1^[17] (**1** in Scheme 2A). Theoretical computations^[18-23] confirm nicotinamide should adopt this dipolar quasi-boat conformation in the transition state. Recent atomic resolution structures of horse liver alcohol dehydrogenase Michaelis complexes reveal puckering in the nicotinamide ring consistent with a partial transition towards the quasi-boat conformation expected of the transition state.^[24]

Ordinarily, the nicotinamide dipolar transition state dissipates upon hydride transfer, as the positive charge at N1 is neutralized and the full-boat conformation typical of dihydronicotinamide is assumed (**2** in Scheme 2A). Hydride transfer by IDH is problematic because the positive charge at N1 is stabilized by the adjacent negatively charged C4-carboxylate of bound isocitrate. Either the hydride must be returned to the substrate, or there must exist alternative means to achieve transfer.

Evidence suggests that Lys100 in IDH directly stabilizes resonance structure **3** in Scheme 2B over **2** in Scheme 2A, and may even protonate the oxyanion formed on the 3-carboxamide in the transition state (**4** and **5** in Scheme 2B). Either route enables hydride transfer to be completed while a full positive charge at N1 is retained. These transition states are unique to IDH. The reaction then continues through substrate C3-decarboxylation and tautomerization to the final product, 2-oxoglutarate, the oxyanion by then having neutralized the positive charge at N1. Our model explains why Lys100 is essential to catalysis in IDH, and yet is not found in related enzymes where hydride transfer proceeds by the standard model (Scheme 2A).

IDH belongs to an ancient enzyme family

The last universal common ancestor was undoubtedly prototrophic and capable of synthesizing amino acids glutamate, leucine and lysine. It would have needed an IDH function (for glutamate biosynthesis), an isopropylmalate dehydrogenase function (for

leucine biosynthesis), and possibly a homoisocitrate dehydrogenase function (depending on which of the two lysine biosynthetic pathways was ancestral)^[25]. Phylogenetic analysis (Figure 4) shows that modern enzymes derive from a central branch deep in the family tree on which the primordial ancestor lay (its exact position cannot be determined in the absence of a known out-group).

Modern IDHs are highly substrate specific^[12,26] and while most isopropylmalate dehydrogenases (IMDH) and tartrate dehydrogenases (TDH) retain broad substrate specificity towards isopropylmalate, 2R-tartrates and 2R-malate, they do not use isocitrate^[27-31]. *Thiobacillus thiooxidans* IMDH is exceptional in being highly specific for isopropylmalate.^[32] Homoisocitrate dehydrogenases (HIDH) vary widely in specificity^[33-38] and, though they may not form a monophyletic group, their long evolutionary branches coalesce on or near the deep central branch of the tree.

The phylogeny is consistent with duplications of an ancestral gene encoding a substrate-promiscuous enzyme followed by divergence in substrate specificity of individual enzymes towards isopropylmalate, isocitrate and homoisocitrate. These events happened in the last universal common ancestor, before the Bacteria split from the Archaea, and long before the Eukarya first appeared. Later, the tartrate dehydrogenases appeared in the Bacteria^[39] and the 3-(2'-methylthio)ethylmalate dehydrogenases in the green plants.^[40]

The IDH mechanism diverged early in evolution

The appearance of Lys100 is contemporaneous with the origination of the IDHs. This means that the primordial ancestor was not only substrate promiscuous, but likely used monovalent cations (K^+ , Na^+ , or even NH_4^+ found in the strongly reducing environment of a primitive earth) to assist catalysis with isocitrate. Monovalent cations activate modern TDH,^[31] HIDH^[41] and some (though not all) IMDHs between 20- and 30-fold.^[42-44] The structure of IMDH with bound NADH and isopropylmalate^[45] reveals K^+ bound 3.8 Å from the 3-carboxamide (Figure S1). The structure of TDH with bound NADH and oxalate^[46] reveals electron density, plausibly NH_4^+ , bound 3.2 Å from the 3-carboxamide. Similarly, the structure of the Lys100Met product ternary complex reveals electron density, plausibly NH_4^+ , bound in plane 3.2 Å from the 3-carboxamide. Cation activations in these enzymes (and possibly HIDH) are modest, the remnants of a promiscuous past. The ability of Lys100 to stabilize dipolar dihydronicotinamide during hydride transfer is the key ancient adaptation that enabled much larger rate accelerations in specialist IDHs.

Conclusions

Sequence alignments reveal an invariant lysine in the catalytic subunits of IDH. Structural alignments reveal the lysine occupies a similar position in the active sites of IDHs from across the tree of life. Site directed mutagenesis with *Escherichia coli* IDH shows the lysine is essential to catalysis – even though it is not found in highly conserved active sites of related enzymes catalyzing homologous reactions. Ammonium rescues activity. X-ray crystallography shows replacing the lysine with methionine does not affect the position of isocitrate bound in binary and ternary complexes. X-ray crystallography also shows the negatively charged C4-carboxylate of isocitrate adjacent to the positively charged N1 of the

nicotinamide ring. The ensuing ionic interaction must stabilize the nicotinamide ground state and oppose the hydride transfer that would ordinarily neutralize the charge at N1. We suggest that the ϵ -amine of the lysine stabilizes – and perhaps even protonates – the oxyanion formed on the nicotinamide 3-carboxamide in the transition state. The result is that hydride transfer proceeds to completion even as the full positive charge at N1 is maintained. IDH catalyzes the same overall reaction as other family members, but dehydrogenation proceeds through a distinct, though related, transition state.

Experimental Section

Media

E. coli strains were grown in Luria broth (LB)^[47] and minimal Davis salts media (MD)^[48]. The minimal media contained 2g/L glucose supplemented with 100 mg/L glutamic acid when needed. For enzyme purification, the strains were grown in Terrific Broth (TB)^[49] supplemented with 15 mg/L tetracycline, 50 mg/L kanamycin or 100 mg/L ampicillin as necessary. Agar plates included 15 g/L Bactoagar.

Strain Construction

Isolation of the mutant IDH proteins required construction of the *E. coli* strain ST *icd* (*icd::kan^r, his4, lacBK1, rpsL, sfiB, thi1*)^[7] which has a complete deletion of the chromosomal *icd* gene, encoding IDH, in order to eliminate any possibility of recombination repair. The gene was deleted using the method of Datsenko and Wanner.^[50] Kan^r transformants were selected on LB/kanamycin plates at 37 °C and screened on MD/kanamycin (with and without glutamate) to confirm the glutamate auxotrophy indicative of a *icd::kan^r* genotype. The deletion was also confirmed by genomic PCR analysis. The *icd::kan^r* construct was then moved into strain ST2010 by generalized transduction using bacteriophage P1(cml, clr100).^[47]

Site-Directed Mutagenesis

The QuikChange™ Site-Directed Mutagenesis method (Stratagene) was used to mutate Lys100 of wildtype *E. coli* IDH. The plasmids pTK509, containing a *tet^r* gene (pSML1), or pTK513^[51] were used as the template for mutagenesis. These plasmids contain the entire *E. coli icd* gene with expression driven from the native promoter. A pair of mutagenic primers (Integrated DNA Technologies) was used for construction of each mutation. The primers were complementary to each other and contained the Lys100 mutation with 20 nucleotides of *icd* sequence on both sides of the mutation. For screening purposes, the primers also contained a silent mutation in an adjacent codon that introduces a new restriction site. The primers for the Lys100 to methionine (AAA → ATG) and glutamine (AAA → CAG) mutations contained a silent mutation in Gly101 (GGG → GGC) which introduces a new *ApaI* site (GGGCCC). The Lys100 to histidine (AAA → CAT) mutant primers contain an Ile99 silent mutation (ATT → ATC) that introduces an *NcoI* site (GGTACC). The primers were extended in a QuikChange™ PCR reaction containing 40 ng plasmid under conditions recommended by Stratagene. The reactions were then treated with 10 units of DpnI (Stratagene) at 37 °C for 3 hrs in order to digest the parental plasmid. The resulting DNA was used to transform *E. coli* ST *icd* via the RbCl/CaCl₂ method.^[52] Transformants were

selected at 37 °C for plasmid-based antibiotic resistance on LB plates. Plasmid DNA was isolated from antibiotic-resistant transformants grown overnight at 37 °C in liquid LB/antibiotic medium using the GeneJET™ Plasmid Miniprep kit (Fermentas). The mutant plasmids were identified by restriction digest analysis. The mutations were then confirmed by sequencing the entire *icd* gene at the University of Minnesota Advanced Genetic Analysis Center. The resulting plasmids were designated pIDH[K100M], pIDH[K100H] and pIDH[K100Q].

Enzyme Purification

E. coli strain ST *icd* was transformed with each of the plasmids. Cells harboring plasmids pSML1 (wildtype *E. coli* IDH) or pIDH[K100M] were grown at 37 °C to full density in 5 liters of TB medium containing 15 mg/L tetracycline using a 6 L NBS BioFlow 3000 fermentor (University of Minnesota Biotechnology Resource Center). Cells harboring plasmids pIDH[K100Q] or pIDH[K100H] were grown at 37 °C in 1 liter batch cultures of TB medium containing 100 mg/L ampicillin. Cells were harvested by centrifugation and then flash frozen before being stored at -80 °C.

50 g of cell paste was added to 150 mL of extract buffer (10 mM KH₂PO₄, 0.5 M NaCl, 2 mM MgCl₂, and 2-mercaptoethanol, pH 7.7) and the suspension stirred for 1 hr at room temperature to allow IDH to become fully dephosphorylated.^[13] The suspension was then chilled to 4 °C, sonicated, and the cell debris removed by centrifugation. Purification of the enzymes, using the procedure of Garnak and Reeves^[53] as modified by Chen *et al.*^[54], involved ammonium sulfate precipitation, DEAE (Pharmacia) anion-exchange chromatography and finally affinity chromatography using Affi-Gel Blue (BioRad). Protein concentrations were determined using the Bio-Rad Protein Assay dye reagent and IgG as the standard. All preparations are >98% free of contaminating protein as judged by Coomassie Blue staining following SDS-PAGE.

X-ray crystallography

Crystallization and Soaks: Crystallization of the Lys100Met mutant followed standard procedures established for wildtype *E. coli* IDH.^[7, 55] Briefly, a solution of the Lys100Met mutant (20 mg/mL protein in 0.9 mM citric acid, 3.5 mM Na₂HPO₄ pH 6.0, 100 mM NaCl, 0.02% NaN₃ and 2 mM DTT) was mixed 1:1 with a crystallization solution (1.85 M NH₄SO₄, 50 mM citric acid/Na₂HPO₄, 0.1 M NaCl and 0.2 M DTT at pH 5.8) and 2 drops equilibrated against 500 µL of the crystallization solution. Tetragonal bipyramidal crystals, 300 µm x 150 µm x 75 µm, developed within 5 days. Crystals were first transferred from their mother liquor to a soaking solution (1.58M NH₄SO₄, 156 mM NaHEPES/MES pH 7.5, 52 mM MgCl₂, 300 mM 2R,3S-isocitrate at 293 K) and then to an artificial mother liquor of the same composition as the soaking solution supplemented with 25% glycerol before being flash-cooled in liquid nitrogen.

Data Collection and Processing: Data were collected at 100 K from one crystal of the IDH[K100M]:ICT:Mg²⁺ complex using Cu Kα X-rays (1.5418 Å) on a Bruker-AXS Proteum Pt135 CCD detector system coupled to a Bruker-AXS Microstar-I rotating anode X-ray generator with Montel mirrors. Data were integrated with SAINT and scaled with

SADABS as part of the Bruker-AXS Proteum Software Suite. Diffraction statistics were obtained with XPREP (Bruker-AXS) and are listed in Table S2 in Supporting Information. Data were further processed with the CCP4 Program Suite.^[56] The crystal structure was solved by Molecular Replacement with PHASER^[57] using the coordinates of PDB entry 1ai2^[58] as the search model. As in the structure reported by Hurley *et al.*^[59], only one monomer was present in the asymmetric unit of the crystal structure, corresponding to V_m values and estimated solvent contents of ca. $4.5 \text{ \AA}^3 \text{Da}^{-1}$ and 72.4 % respectively.

Crystallographic Refinement: The structure was refined using the amplitude-based Maximum-Likelihood target function with automatic weight optimization in PHENIX v. 1.8.2.^[60] Each refinement run consisted of three macrocycles. After an initial refinement of positional and isotropic atomic displacement parameters, the ICT and Mg^{2+} ligands were included in the model with Coot.^[61] Following convergence, Translational-Librational-Screw (TLS) rigid bodies were determined with PHENIX and the final refinement runs consisted of three macrocycles with refinement of positional, TLS anisotropic displacement, residual isotropic and occupation parameters. The occupation factor refinement was done for 10 residues with alternate side-chain conformations and the side chains of a few more residues with poorly defined side-chain electron density without clear indication of alternate conformations. During refinement, the model was periodically inspected and corrected with Coot against σ_A -weighted $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ electron density maps. A total of 527 solvent molecules were included in the refined model by inspection of the electron density maps in Coot or automatically with PHENIX. The stereochemical quality of each model was assessed with MolProbity.^[62] A summary of the refinement statistics, model composition and stereochemical quality is presented in Table S3 in Supporting Information. Coordinates and structure factors were deposited in the Protein Data Bank in Europe^[63] with accession code 4bnp.

Kinetic Analyses

Kinetic assays were conducted at 37 °C in 25 mM MOPS pH 7.5, 100 mM NaCl, 1 mM DTT, and 5 mM free Mg^{2+} .^[3] The rates of oxidative decarboxylation of 2R,3S-isocitrate were determined spectrophotometrically by monitoring the production of NADPH at 340 nm using a molar extinction coefficient of $6220 \text{ }_{340\text{nm}}/\text{cm}$. All of the data were collected on a Cary 300 spectrophotometer operating in the kinetics mode. For the initial velocity studies, the concentration of one substrate was varied at several fixed concentrations of the second substrate. The effect of salts on their activities were determined with both substrates – Mg^{2+} -2R,3S-isocitrate (ICT) and NADP – at saturating concentrations (i.e. $20 \times K_m$). Non-linear least squares Gauss-Newton regressions in JMP (SAS Institute, Inc.) were used to determine the fit of the data to the following kinetic models:

Michaelis-Menten—

$$v = k_{cat} \frac{S}{K_{m,s} + S} \quad (1)$$

where S is either isocitrate (at saturating NADP) or NADP (at saturating isocitrate).

Irreversible rapid equilibrium random—

$$v = k_{cat} \frac{\frac{ICT}{K_{ia,ICT}} \frac{NADP}{K_{m,NADP}}}{1 + \frac{ICT}{K_{ia,ICT}} + \frac{NADP}{K_{ia,NADP}} + \frac{ICT}{K_{ia,ICT}} \frac{NADP}{K_{m,NADP}}} \quad (2)$$

where v is the rate, k_{cat} is the maximum rate of turnover when the concentrations of isocitrate (ICT) and coenzyme (NADP) are in excess, the K_{ia} s are the dissociation constants of the binary complexes, the K_m s are the Michaelis constants and $K_{ia,NADP}K_{m,ICT} = K_{m,NADP}K_{ia,ICT}$.

Activation by potassium—

$$rv = \frac{K_{d,Me} + MRA \cdot Me}{K_{d,Me} + Me} \quad (3)$$

where rv is the rate relative to that in the absence of potassium, Me is the concentration of potassium, $K_{d,Me}$ is the dissociation constant of potassium from the Michaelis complex and $MRA = V_{max,Me}/V_{max}$ is the maximum relative activation.

Inhibition of wildtype IDH by ammonium—

$$rv = e^{-aNH_4^+} \quad (4)$$

where the constant a represents nonspecific inhibition with increasing ammonium (NH_4^+).

Activation and Inhibition of Lys100Met IDH by ammonium—

$$rv = \frac{K_{d,Me} + MRA \cdot NH_4^+}{K_{d,Me} NH_4^+} e^{-aNH_4^+} \quad (5)$$

which simply combines equations 3 and 4.

Kinetic pH profiles were conducted at 37 °C in 25 mM MES, 13 mM Tris, 13 mM ethanolamine, 100 mM NaCl, 1 mM dithiothreitol, and 5 mM free Mg^{2+} to maintain a constant ionic strength.^[54] The rates were determined spectrophotometrically at each pH using a fixed saturating concentration ($20 \times K_m$) of one substrate and varying the other. For wild-type IDH, the fixed saturating concentrations were 200 μM Mg^{2+} -2R,3S-isocitrate and 500 μM NADP. For the Lys100Met mutant, 2000 μM Mg^{2+} -2R,3S-isocitrate and 6000 μM NADP were the fixed saturating concentrations. The kinetic parameters were calculated by a nonlinear fitting of the rates to the Michaelis-Menton equation using the statistics program JMP v 2.0.5 (SAS Institute, Inc.).

The V_{max} profiles of wildtype IDH and the Lys100Met and Lys100Gln mutants in the presence of ammonia were fitted to:

$$v = \frac{V_{opt}}{(1 + 10^{pK_a - pH}) (1 + 10^{pH - pK_b})} \quad (6)$$

where V_{opt} is the apparent maximum rate at optimal pH when both substrates are in excess (at least $8 \times K_m$'s), and K_a and K_b are the acid dissociation constants associated with the acidic and basic limbs of the profile. The Michaelis constants were fitted to:

$$K_{m.apparent} = K_m \left(1 + 10^{pK_c - pH} \right) \quad (7)$$

where K_c is the acid dissociation constant.

The model for the V_{max} profile of wildtype IDH in the presence of ammonia and ammonium (Scheme S2 in Supporting Information) was:

$$V_{max.apparent} = \frac{V_{max}}{(1 + 10^{pK_a - pH}) \left(1 + 10^{pH - pK_b} + \frac{1 + 10^{pK_A - pK_b} + 10^{pH - pK_b}}{1 + 10^{pK_A - pH}} \frac{A}{K_{m.A}} \right)} \quad (8)$$

where $A = NH_3 + NH_4^+$ is the total concentration of ammonia and ammonium.

Structural Analysis: Fifteen protein structures were visualized using VMD.^[64] The C α backbone traces of the large and small domains of each protein were independently superimposed by least squares on the corresponding domain of *E. coli* IDH using DeepView/Swiss PDB-Viewer.^[65] Various loops with low sequence identity and obvious extensive differences in secondary and tertiary structures were omitted from the alignments. Structure coordinates can be obtained from the RCSB Protein Data Bank using the PDB accessions listed in Table S1 in Supporting Information.

Alignments: Amino acid sequence alignments obtained with Muscle^[66] in SeaView^[67] were adjusted using superpositioned protein structures (Figure 1A) and conserved active site residues as guides (Figure 1B) allowing gaps and insertions to be introduced into surface loops rather than the hydrophobic cores of the domains. Monomeric IDHs^[68] (formed by duplication of the small domain), 4-hydroxythreonine-4-phosphate dehydrogenases^[69] (involved in pyridoxyl phosphate biosynthesis) were excluded from the analysis because their sequences are far too divergent. The complete alignment of 2484 sequences is available upon request from the authors.

Phylogenetic Analyses: Bootstrapped maximum likelihood trees of the 2R-hydroxyacid 3S-decarboxylating dehydrogenases were obtained using ML + Bootstrap with GAMMAPROT and an LG matrix^[70] in RAxML.^[71] Similar trees were obtained using FastML with GAMMAPROT and a JTT matrix^[72] in RAxML (with SH support^[73]) and bootstrapped neighbor joining^[74] with a Poisson correction in Seaview.^[67] Phylogenetic trees are available upon request from the authors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Catalytic mechanism of IDH. Lys230* acts as the base during dehydrogenation and tautomerization and as an acid during decarboxylation. Tyr160 moves into position to protonate C3 once CO₂ has evolved. Mg²⁺ chelates the C2-hydroxyl and carboxylate and acts as a Lewis acid. The C3-carboxylate is bound anti to the transferred hydride allowing decarboxylation at the second step.

Figure 1.

Cross-eyed stereo views of superpositions of 10 IDH family members. A) The IDH family fold consists of a large α + β domain (left) and a small α / β domain (right) built onto a large parallel and antiparallel β -sheet. Two vertical helices of the small domain pack against those of a second subunit (rotated 180° about the z-axis) to form a four helix bundle at the dimer interface. NADP and isocitrate in *Escherichia coli* IDH locate the active site between the large and small domains of one subunit and a small domain of the second subunit (not shown in the monomer). B) Residues (clockwise from Glu366 to Lys230*; *E. coli* IDH numbering) surrounding the 2R-malate core common to all substrates are invariant in the IDH family (the six IDHs from Bacteria, Archaea and Eukarya are red, bacterial monomeric IDH is yellow, the two bacterial IMDHs are purple and bacterial TDH is green). Residues (counterclockwise from Lys100 to Asn232*) close to substrate moieties attached at the 3S position vary. C) The ternary substrate complex of wildtype *E. coli* IDH (PDB 4aj3) showing the nicotinamide ring 'above' the isocitrate C3-carboxymethyl.

Figure 2.

Cross-eyed stereo views of superpositioned wildtype IDH (PDB 5icd) (dark colors) and the Lys100Met mutant (light colors) complexes. A) Isocitrate binary complexes showing that the Lys100Met does not affect the position of bound isocitrate.

B) Ternary complexes showing that Lys100 does not hydrogen bond to the nicotinamide C3-carboxamide, and that the Lys100Met does not affect the position of the ring.

Figure 3.

A) NH_4Cl activates Lys100Met and inhibits wildtype IDH. B) KCl activates Lys100Met with no effect on wildtype. C) Effect of 0.2 M NH_4Cl (+ NH_3) on V_{max} pH profiles. The dashed curve for wildtype with NH_4Cl is a prediction, not a fit. (D) K_m pH profiles for isocitrate and NADP of both enzymes in the absence of NH_4Cl .

Figure 4. Maximum likelihood phylogeny of the IDH family with bootstrap support for major clades. IDHs (all with Lys100) are red, IMDHs are blue, HIDHs are orange, TDHs are green and unassigned enzyme functions are grey. NADP users are darker and NAD users are lighter in color. The origin of the family lies on the central black branch. Unlabeled clades are Bacteria.

Table 1

Kinetic parameters of IDH mutants at Lys100.

Enzyme	$K_{i,ICT}^{[a]}$ [μM]	$K_{m,ICT}$ [μM]	$K_{i,NADP}$ [μM]	$K_{m,NADP}$ [μM]	k_{cat} [sec^{-1}]
wildtype	11	6	21	12	196
Lys100Met	96	110	200	240	0.0095
Lys100Gln	ND ^[b]	104 ^[c]	ND	306 ^[d]	0.0087
Lys100His	ND	108 ^[c]	ND	245 ^[d]	0.0198

^[a] ICT is 2R,3S isocitrate.^[b] Not Determined.^[c] Determined at 5 mM NADP.^[d] Determined at 2 mM ICT.

Table 2Effect of mono- and divalent cations on IDH activities^[a].

02 M Salt	Wildtype	Lys100Met	Lys100Gln	EIV ^[b]	DDIAKS ^[c]
None	1	1	1	1	1
NH ₄ Cl	1.30	80.6	2.4	1.03	0.72
LiCl	1.06	2.77	0.46		
NaCl	1.10	1.04	0.52	0.84	0.66
KCl	1.15	24.5	1.29	0.91	0.69
RbCl	1.3	7.75	0.46	0.96	0.70
CsCl	1.2	0.73	0.51		
CaCl ₂	<0.001	<0.001	<0.001		
MgCl ₂	0.75	0.02	0.02		
CuCl ₂	<0.001	<0.001	<0.001		
HgCl ₂	<0.001	<0.001	<0.001		

^[a]Normalized activities.^[b]mutant Lys344Glu, Tyr345Ile, Ala351Val.^[c]mutant Arg292Asp, Lys344Asp, Tyr345Ile, Val351Ala, Tyr391Lys, Arg395Ser.

Table 3pH profile pK_a s and pK_b s.

Enzyme	pK_a	pK_b	0.2 M NH_4Cl added	
			pK_a	pK_b
wildtype	6.32 ± 0.04	10.36 ± 0.12	6.31 ± 0.13	$(8.89 \pm 0.21)^{[a]}$
Lys100Met			6.21 ± 0.06	9.31 ± 0.06
Lys100Gln			6.05 ± 0.12	9.30 ± 0.07

^[a]Values in parentheses are unreliable.