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A Tale of Two Subunits: How the Neomorphic R132H IDH1 Mutation Enhances Production of αHG

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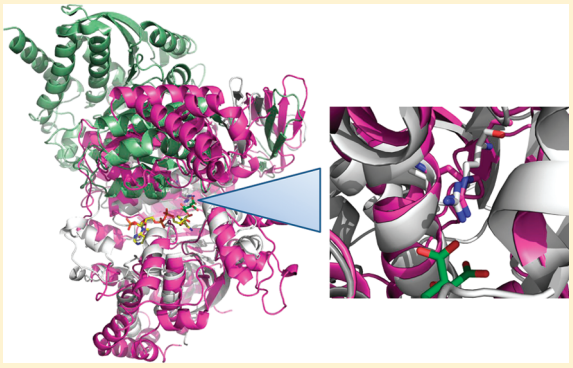
ABSTRACT: Heterozygously expressed single-point mutations in isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2, respectively) render these dimeric enzymes capable of producing the novel metabolite α-hydroxyglutarate (αHG). Accumulation of αHG is used as a biomarker for a number of cancer types, helping to identify tumors with similar IDH mutations. With IDH1, it has been shown that one role of the mutation is to increase the rate of conversion from αKG to αHG. To improve our understanding of the function of this mutation, we have detailed the kinetics of the normal (isocitrate to αKG) and neomorphic (αKG to αHG) reactions, as well as the coupled conversion of isocitrate to αHG. We find that the mutant IDH1 is very efficient in this coupled reaction, with the ability to form αHG from isocitrate and $NADP^+$. The wild type/wild type IDH1 is also able to catalyze this conversion, though it is much more sensitive to concentrations of isocitrate. This difference in behavior can be attributed to the competitive binding between isocitrate and αKG, which is made more favorable for αKG by the neomorphic mutation at arginine 132. Thus, each partial reaction in the heterodimer is functionally isolated from the other. To test whether there is a cooperative effect resulting from the two subunits being in a dimer, we selectively inactivated each subunit with a secondary mutation in the NADP/H binding site. We observed that the remaining, active subunit was unaffected in its associated activity, reinforcing the notion of each subunit being functionally independent. This was further demonstrated using a monomeric form of IDH from *Azotobacter vinelandii*, which can be shown to gain the same neomorphic reaction when a homologous mutation is introduced into that protein.



Mutations in isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2, respectively) have been identified in a number of cancers. In 2008, an exhaustive sequencing effort of Glioblastoma multiforme (GBM) tumor samples found IDH1 mutations at R132 in 12% of GBM patients.[1] Mutations of R132 to His, Ser, Cys, Gly, and Leu were all identified, though His at the highest frequency. Subsequent analyses not only confirmed the IDH1 mutations in secondary glioblastomas but also identified IDH1 and IDH2 mutations in patients with acute myeloid leukemias (AML)[2,3] and nonepithelial melanomas.[4] Though these mutant enzymes were studied and shown to have reduced enzymatic activity in vitro,[2,5] this functional analysis was at odds with the genetic signature of these mutations, which was more consistent with a gain of function phenotype. This dilemma was beautifully resolved in 2009 by Dang et al., who demonstrated that while the wild type (wt)/R132H IDH1 did indeed have impaired abilities in the normal isocitrate to αKG conversion, it was able to facilitate a new reaction producing αHG from αKG [termed normal and neomorphic reactions, respectively (Figure 1)].[6] αHG has been proposed to exert its oncogenic effect through competitive inhibition with enzymes that use αKG as a cofactor.[7] This has been shown recently in AML patients, where IDH1 and IDH2 mutations were associated with elevated levels of global 5-methylcytosine by virtue of αHG inhibition of TET2.[8] TET2 is an αKG-utilizing enzyme that hydroxylates 5-methylcytosine as a step in the demethylation of DNA.[9]

A major question about these heterodimeric IDH1 and IDH2 enzymes is the role of their associated mutations in promoting the production of αHG. In the normal reaction, R132 plays both catalytic and structural roles in promoting αKG production. From the structure of human wt/wt IDH1, R132 is proposed to contribute to substrate binding, as this residue interacts directly with the C-3 carboxylate of isocitrate.[10] This is consistent with previous kinetic studies using engineered mutations of R132.[11] In addition to this role, R132 also gates the open−closed conformational change observed in forming the active, ternary complex. Within the IDH1 dimer, R132 makes hydrogen bonds to D275 within its own chain and to D279 from the opposite chain,

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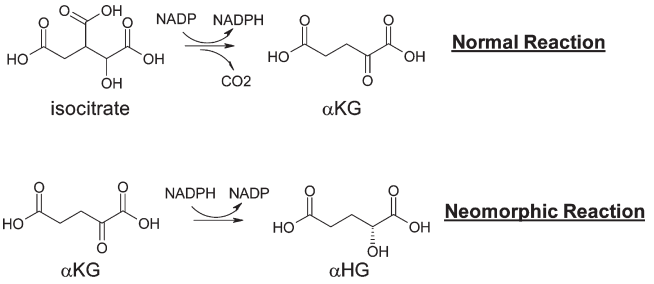

Figure 1. Normal and neomorphic reactions catalyzed by IDH1. The normal reaction converts isocitrate to αKG, while the neomorphic reaction transforms αKG to αHG.

enforcing a disordered structure to segments surrounding the active site; this results in an open conformation capable of binding the isocitrate substrate.[12] In essence, R132 acts as a doorstop, which is pulled out of the way by isocitrate upon binding to allow formation of the closed, active conformation.

To improve our understanding of how changing these functions in the mutant IDH1 and IDH2 enzymes contributes to the elevated levels of αHG observed in patient samples, we studied the kinetics of the wt/wt and several mutant forms of IDH1, as well as a monomeric homologue of IDH1 from bacteria. From these studies, we conclude that a major role of the R132H mutation is to reduce the level of competition between isocitrate and αKG on the mutant subunit, functionally isolating the normal (isocitrate to αKG) and neomorphic (αKG to αHG) reactions to separate subunits of IDH1. As a result of this independent functioning, cancer cells harboring mutant IDH1 and IDH2 can produce αHG over a wider range of isocitrate concentrations than cells that have the wild-type enzyme.

METHODS

Protein Production. *wt/wt IDH1, R132H/wt IDH1, R132H/H315A IDH1, R132H-H315A/wt IDH1.* The human isocitrate dehydrogenase cDNA (IDH1, NM_005896) was cloned from a human brain cDNA using standard polymerase chain reaction (PCR) techniques. Then the open reading frame of human IDH1 was subcloned into vector pENTR/TEV/D-TOPO (Invitrogen K252520).

R132H and H315A mutations were introduced using the mutagenesis kit (Stratagene 200513), and the sequence was confirmed. The wild type and the three IDH1 mutants of pENTR-TEV/D-TOPO clones were then subsequently transferred to a Gateway pDEST 8 FlagMBP (N-terminally tagged) and pDEST8 H6MBP (N-terminally tagged) baculovirus expression vector by recombination, respectively.

The recombinant baculovirus was generated using the Bac-to-Bac system (Invitrogen 10359-016) for the following constructs: wt FlagMBP-IDH1, R132H FlagMBPtev-IDH1, R132H H6MBPtev-IDH1, R132H/H315A H6MBP-IDH, and H315A H6MBP-IDH1. Proteins were expressed by co-infection with rBV in Sf9 cells. The culture was harvested by centrifugation 48 h after infection.

The wt/wt protein was purified using anti-Flag resin (Sigma). The self-packed Flag column was extensively washed with storage buffer, and wt/wt IDH1 was eluted with 100 μg/mL Flag peptide in storage buffer. The R132H/R132H mutant homodimer protein was purified via Ni-NTA affinity chromatography, and the target protein was eluted with elution buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, and 200 mM imidazole]. Both wt/wt and R132H/R132H proteins were cleaved with Tev and further purified by MonoQ chromatography.

Purification of mutant IDH1 heterodimers (R132H/wt, R132H/H315A, and R132H-H315A/wt) was first performed via Ni purification as described above except that the elution fractions from the Ni-NTA affinity column were subjected to further purification using anti-Flag resin (Sigma). The self-packed Flag column was extensively washed with storage buffer, and the IDH1 heterodimer was eluted with 100 μg/mL Flag peptide in storage buffer.

The purified proteins were analyzed via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) with a purity of >95% and the expected migration. All the mutations were confirmed by peptide mapping.

Azotobacter vinelandii wt and R547H. The coding sequence of *A. vinelandii* IDH (isocitrate dehydrogenase) was PCR amplified from genomic DNA (ATCC12837 strain 3A) and TOPO cloned into the pENTR-TEV/D-TOPO vector. Primers were designed on the basis of Uniprot C1DL01 (GenBank entry NC_012560, strain DJ). A single G>A nonsynonymous point mutation (base pair 2110) was found in all clones, resulting in an Ala704Thr mutation; this appears to be a substrain variation.

The R547H mutation is analogous to the R132H mutation in humans and was introduced by site-directed mutagenesis in pENTRtevAzoIDH. The recombination reaction generated wt and R547H pDESTT7H6Sumo tevAzoIDH. Both wild-type and mutant proteins were expressed in *Escherichia coli* BL21DE3 (Invitrogen).

Transduced cells were grown in LB (50 μg/mL carbenecillin) at 37 °C with shaking until an optical density at 600 nm of 0.7 was reached. The temperature was changed to 18 °C, and protein expression was induced by adding IPTG to a final concentration of 1 mM. Cells were harvested after IPTG induction for 18 h.

Approximately 8 g of cell pellet from 1.5 L of culture was lysed in 100 mM Hepes (pH 7.4), 150 mM NaCl, and 10% glycerol lysis buffer by sonication. The supernatant was obtained via 30000*g* centrifugation at 4 °C for 30 min. The protein was first purified with 5 mL of Ni-NTA SF beads. The beads were first washed with 30 mM imidazole buffer, and the protein was then eluted with 200 mM imidazole in lysis buffer. The HisSumo tag was removed by adding TEV protease to the HisSumo-IDH1 Ni elution pool (50:1 protein:enzyme ratio). The mixture was incubated at 4 °C overnight while the protein solution was changed to lysis buffer by dialysis. The Ni beads were used to remove all the cleaved HisSumo tag, HisTev protease, and other contaminants from the Ni elution. The untagged IDH1 protein was further purified by Superdex 200 size exclusion chromatography.

The purified proteins were analyzed via SDS−PAGE and were judged to be >95% pure with the expected migration. All the mutations were confirmed by peptide mapping.

Enzyme Assays. $NADP^+$, NADPH, diaphorase, resazurin, and resorufin were purchased from Sigma-Aldrich. αKG was purchased from Fluka. Isocitrate was purchased from MP Biomedicals, LLC. αHG (disodium salt) was purchased from Toronto Research Chemicals.

The conversion of isocitrate to αKG was followed by coupling the production of NADPH to the diaphorase-catalyzed reduction of resazurin to the fluorescent product resorufin (excitation at 530 nm and emission at 561 nm). Assays were conducted in a 10 μL volume in a 384-well microplate (Greiner 784706) in

buffer consisting of 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.05% CHAPS, 0.01% BSA, 12.5 μM resazurin, 1.25 units/mL diaphorase, and 1 nM IDH1, with varying concentrations of either isocitrate or $NADP^+$. When the concentration of isocitrate was varied, the level of $NADP^+$ was held at fixed, saturating concentrations (typically 100 μM). When the concentration of $NADP^+$ was varied, the level of isocitrate was held at fixed, saturating concentrations (typically 100 μM). For IDH1 mutants with lower k_{cat} values, higher enzyme concentrations were used.

The conversion of αKG to αHG was monitored directly by liquid chromatography−tandem mass spectrometry (LC−MS/MS) methods or by the consumption of NADPH and its concomitant decrease in fluorescence (excitation at 340 nm and emission at 460 nm). Fluorescence assays were conducted in a 10 μL volume in a 384-well microplate (Greiner 784706) or in a 500 μL volume in a cuvette using a PC1 photon counting spectrofluorimeter (ISS) in buffer consisting of 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 0.05% CHAPS, 0.01% BSA, 50 nM IDH1, and varying concentrations of either αKG or NADPH. When the concentration of αKG was varied, the level of NADPH was held at fixed, saturating concentrations (typically 100 μM). When the concentration of NADPH was varied, the level of αKG was held at fixed, saturating concentrations (typically 5 mM). For IDH1 enzymes with lower k_{cat} values, higher enzyme concentrations were used. LC−MS/MS assays were conducted in a 50 μL volume in a 384-well microplate (Greiner 781280) in the same buffer with the reagent concentrations used in the fluorescence format. Reactions were quenched after 2 h with the addition of 50 μL of 100 mM EDTA.

The conversion of isocitrate to αHG was followed directly by LC−MS/MS methods. Assays were conducted in a 50 μL volume in a 384-well microplate (Greiner 781280) in buffer consisting of 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 50 nM IDH1, 100 μM $NADP^+$, and varying concentrations of isocitrate. Reactions were quenched after 2 h with the addition of 50 μL of 100 mM EDTA.

LC−MS/MS Methods. All analyses were performed on an API 4000 triple-quadrupole tandem mass spectrometer (Applied Biosystems). Other instrumentation consisted of a Shimadzu binary LC10ADvp HPLC system and a HTS Pal (Leap Technologies) autosampler. LC was performed on a Phenomenex Luna C18 analytical column [150 mm × 4.6 mm (inside diameter); 5 μm particle size] with a water (solution A)−acetonitrile (solution B) gradient containing 1% acetic acid as the mobile phase. Solution A at 95% was held for 1 min, before a linear gradient from 5.0 to 90% B over 1.75 min with a hold for 1.25 min was generated by Shimadzu LC 10ADvp pumps. The 1.0 mL/min flow was directed into the mass spectrometer, which operated in the negative multiple-reaction-monitoring mode. The MS/MS system was optimized by continuous infusion of each standard (10 mM) at a flow rate of 200 μL/min. αHG was monitored by the transition from m/z 146.7 to 128.9 using a DP of −35.0, a collision energy of −16.0, and a CXP of −9.0. The parameters were set as follows: temperature of the electrospray, 600 °C; collision-activated dissociation gas (nitrogen), 4; curtain gas (nitrogen) flow, 30; turbo ion gas (zero air), 50; desolvation gas (zero air), 50; ion spray voltage, −4200 V. Data were acquired and processed with Analyst for Windows (version 1.4.2).

Thermal Shift Assay (TSA). Fluorescence-based protein thermal stability analysis as a function of ligand concentration was performed using a LightScanner instrument (Idaho Technology Inc., Salt Lake City, UT). Briefly, 1 μL of 1:2 serially diluted DL-isocitrate in TSA assay buffer [25 mM cacodylic acid (pH 7.0), 2 mM $CaCl_2$, and 150 mM NaCl] with a final top concentration of 33.3 mM was mixed with 2 μL of protein dimers (4.0 μM) in TSA buffer with 21 μM dapoxyl sulfonic acid (D12800 from Invitrogen) in a 384-well PCR microtiter plate (HSP3865 from Bio-Rad). The assay solution was overlaid with 1 μL of silicone oil DC200 (85411 from Fluka/Sigma Aldrich) to prevent evaporation. The plate was analyzed immediately, and the change in dapoxyl fluorescence due to protein unfolding was measured with the excitation wavelength set to 380 nm and the emission wavelength to >435 nm, over a temperature range of 31−80 °C at a ramp rate of 1 °C/min.

K_d values at T_m were determined from nonlinear fits of T_m as a function of ligand concentration using the following equation[13] in Excel using the XLFit add-on from IDBS:

$$[L]_t = (1 - e^x)\left(\frac{1}{e^y e^x} + \frac{[P]_t}{2}\right)$$

$$x = \Delta_U H_{T_m} \frac{\left(\frac{1}{T} - \frac{1}{T_m}\right) - \Delta_U C_p\left[\frac{T_m}{T} - 1 - \ln\left(\frac{T}{T_m}\right)\right]}{R}$$

$$y = -\frac{\Delta_b G_{T_m}}{R T_m}$$

$$K_d = e^{(\Delta_b G_{T_m})/(R T_m)}$$

where $\Delta_U H_{T_m}$ is the enthalpy of unfolding for the unbound protien, $\Delta_U C_p$ is the heat capacity of unfolding for the unbound protein, $[P]_t$ is the total protein concentration, $[L]_t$ is the total ligand concentration, and $\Delta_U G_{T_m}$ is the Gibbs free energy change for ligand binding at T_m. The heat capacity (ΔC_p) and enthalpy (ΔH) for the protein were set to 2500 cal mol^{-1} K^{-1} and 110 kcal/mol, respectively.

RESULTS AND DISCUSSION

IDH1 and IDH2 contain heterozygously expressed mutations that allow the enzyme to produce elevated levels of the metabolite αHG in tumor samples. Though the increased level of production of αHG is a clear and consistent result both in vitro and in vivo with IDH1 and IDH2 mutations, it is less clear at a molecular level how these mutations enable the enzyme to perform its neomorphic activity. In the first reported structure of the mutant IDH1, it was suggested that the effect of the R132H substitution is to favor the closed conformation of the enzyme, which would enable the neomorphic reaction through tighter binding of the NADPH substrate.[6] In contrast, a more recent structure found that the mutant IDH1 was less capable of forming the closed, active conformation needed for catalysis.[12] Instead, it was suggested that the reorganization of the IDH1 active site introduced by the R132H mutation resulted in other residues of the protein being brought into the proximity of the active site to assist in catalysis, such as Y139. There have been no biochemical analyses of the mutant protein to help address these and other possibilities.

To improve our understanding of the role of the R132H mutation in IDH1, we began by studying the kinetics of the wt/wt, R132H/wt, and R132H/R132H enzymes. As expected, the wt/wt enzyme displayed robust turnover in the normal reaction, whereas the R132H/wt and R132H/R132H enzymes

Table 1. Kinetic Constants for IDH Enzymes in the Normal and Neomorphic Reactions

			Isocitrate to αKG		
construct	k_{cat} (min^{-1})	$K_{isocitrate}$ (μM)	$k_{cat}/K_{isocitrate}$ ($min^{-1}\,\mu M^{-1}$)	K_{NADP^+} (μM)	k_{cat}/K_{NADP^+} ($min^{-1}\,\mu M^{-1}$)
wt/wt	749	7	115	4.7	160
R132H/wt	121	33.4	3.6	5	15.2
R132H-H315A/wt	3.3	34	0.1	4.4	0.75
R132H/H315A	43.2	106	0.41	4.4	9.8
azo wt	9213	17.4	530	ND[a]	ND[a]
azo R547H	99	1280	0.08	ND[a]	ND[a]
			αKG to αHG		
construct	k_{cat} (min^{-1})	$K_{\alpha KG}$ (μM)	$k_{cat}/K_{\alpha KG}$ ($min^{-1}\,\mu M^{-1}$)	K_{NADPH^+} (μM)	k_{cat}/K_{NADPH^+} ($min^{-1}\,\mu M^{-1}$)
wt/wt	0.067	43	0.0015	ND[a]	ND[a]
R132H/wt	11.2	700	0.0025	<0.4	>28
R132H-H315A/wt	6.6	970	0.007	68.2	0.1
R132H/H315A	13.4	250	0.054	<0.4	>33
azo wt	0.22	175	0.0012	ND[a]	ND[a]
azo R547H	40.7	6600	0.0062	ND[a]	ND[a]

[a] Not determined.

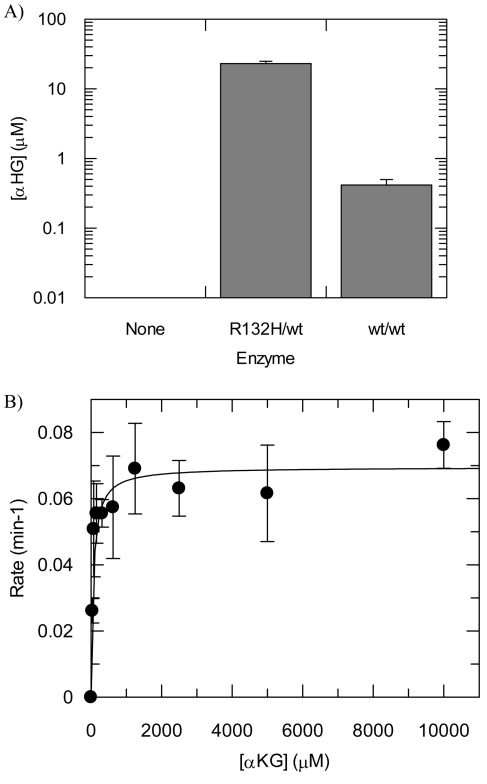

Figure 2. (A) Wild-type IDH1 can perform the neomorphic conversion of αKG to αHG. Reactions with 1 mM αKG and 100 μM NADPH were conducted for 2 h with either buffer, 50 nM R132H/wt IDH1, or 50 nM wt/wt IDH1. The amount of αHG formed was determined by LC−MS analysis. (B) Kinetics of wt/wt IDH1 in the neomorphic reaction. The substrate αKG was diluted serially from 10 mM to 39 μM, and the amount of αHG formed after 2 h was determined by LC−MS analysis. Fitting the data yielded a k_{cat} of 0.07 min^{-1} and a K_m(αKG) of 43 μM.

displayed the highest activity in the neomorphic reaction (Table 1). However, we surprisingly found that the wt/wt enzyme had measurable activity for the neomorphic reaction, producing 2% αHG compared to the mutant enzyme (Figure 2A). The k_{cat} value for wild-type IDH1 was 0.07 min^{-1}, and k_{cat}/K_m was equal to 0.0015 μM^{-1} min^{-1} (Figure 2B), representing 1 and 6%, respectively, of those of the R132H/wt IDH1 reaction. This finding is in contrast to the initial report about IDH1 neomorphic activity, where the authors were not able to measure the ability of the wt/wt enzyme to catalyze the reduction of αKG.[6] This may be due to the fact that in that study the neomorphic activity was measured spectrophotometrically by the consumption of NADPH, which is less sensitive than measuring the production of αHG by LC−MS, which we used to determine these enzyme kinetics.

The observation of measurable neomorphic activity for wt/wt IDH1 makes sense from the perspective of enzyme evolution. In the theory of divergent evolution, a given enzyme may be optimized for a particular reaction but at the same time may be capable of catalyzing several other, related transformations less efficiently but still at significant levels.[14,15] When a new activity is required due to evolutionary pressures, nature tends to take advantage of these secondary reactions as advanced starting points rather than designing an enzyme de novo. It is less understood if these secondary reactions have purposeful or unintended relevance in vivo as well.[16]

Having studied the normal and neomorphic reactions in isolation, we next wanted to understand if IDH1 can link the two partial reactions to convert isocitrate to αHG. Though it had been previously shown from isotope labeling experiments in cells that αHG can be derived from glutamine,[6] it is also possible that a portion of the αKG utilized for the neomorphic reaction is made by the wild-type subunit of IDH1 itself. Within the R132H/wt dimer, this would create an efficient strategy for the generation and utilization of the αKG intermediate, which is often seen in cells as a strategy for colocalizing enzymes that catalyze consecutive reactions along a metabolic pathway.[17]

When the fully coupled reaction was conducted with high concentrations of isocitrate (1 mM), αHG was found to be robustly produced enzymatically starting from just isocitrate and $NADP^+$; no added NADPH was needed. Thus, it appears that

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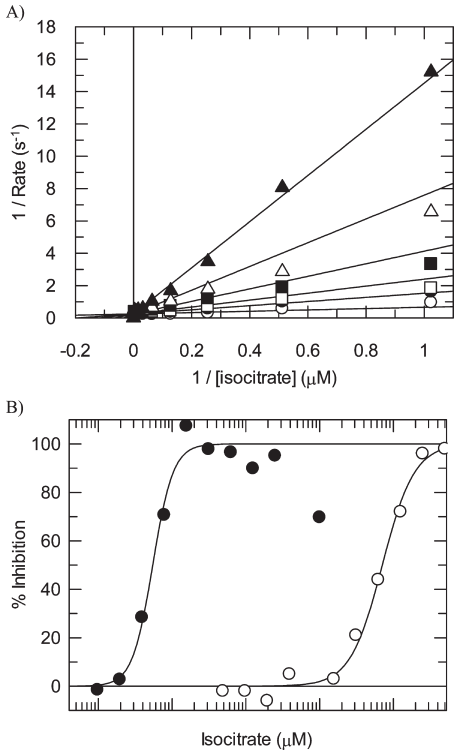

Figure 3. (A) Mechanism of product inhibition for αKG with wt/wt IDH1. Isocitrate was titrated as a substrate in the normal reaction catalyzed by wt/wt IDH1 in the presence of αKG at 0 (○), 39 (●), 78 (□), 156 (■), 312 (△), and 625 μM (▲). The data were best fit to a model of competitive inhibition. (B) Isocitrate as an inhibitor of the neomorphic reaction. The conversion of αKG to αHG was monitored in the presence of varying isocitrate concentrations, using either R132H/R132H (○) or wt/wt (●) IDH1.

the mutant IDH1 can efficiently use both products of the normal reaction as substrates for the subsequent, neomorphic conversion. We also tested wt/wt IDH1 in this coupled reaction but did not observe any production of αHG. This was unexpected, on the basis of the kinetics of the individual normal and neomorphic reactions catalyzed by the wt/wt enzyme. When we modeled the coupled isocitrate to αHG reaction as two independently functioning reactions[18] in the wild-type enzyme, we predicted the production of ~0.6 μM αHG during the 4 h time course of our experiments. Our empirical result implied that the production of αHG from isocitrate was somehow being repressed in wt/wt IDH1.

In characterizing the kinetics of the normal reaction catalyzed by wt/wt IDH1, we found that isocitrate is competitively inhibited by αKG (Figure 3A), similar to previous reports of IDH from porcine heart.[19] Given the relatively similar values for the αKG K_i (18 μM) and isocitrate K_m (7 μM), we wondered whether isocitrate could inhibit αKG binding and conversion to αHG in an analogous fashion. Studying the neomorphic partial reaction catalyzed by wt/wt IDH1, we found isocitrate to be a relatively potent inhibitor, with an IC_{50} of 8 μM (Figure 3B). Thus, under the conditions of our original experiment with 1 mM isocitrate, any αKG formed from the normal reaction would easily be outcompeted for rebinding. To test this hypothesis, we repeated the original experiment at varying concentrations of isocitrate, especially at lower ranges. As the isocitrate concentration is lowered from 1 mM, the amount of αHG produced

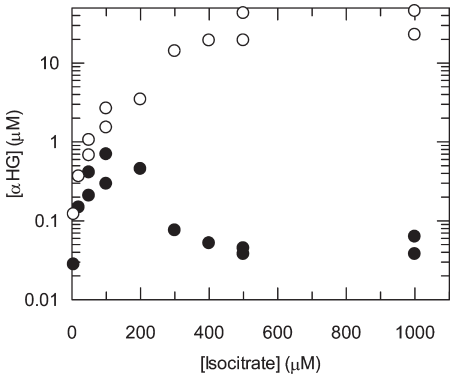

Figure 4. Dependence of the isocitrate concentration on the formation of αHG. Reactions with wt/wt IDH1 (●) or R132H/wt IDH1 (○) were initiated with NADP and varying isocitrate concentrations (5 μM to 1 mM) and analyzed for αHG production.

increases, coming to a maximum at 100−200 μM (Figure 4). Below this value, there is a diminution of αHG production, presumably because of a decreasing level of isocitrate to below its K_m value of 33 μM (Table 1). Under the conditions with a lower isocitrate concentration, wt/wt IDH1 could be seen to produce the predicted amount of αHG, forming 0.5 μM αHG after 4 h. Thus, it appears that a major limitation to producing αHG in wt/wt IDH1 is the inability of αKG to compete against isocitrate as a substrate.

As introduced above, one major function of R132 in wt/wt IDH1 is to bind the C-3 carboxylate of isocitrate. Consistent with this idea, we found that the K_m for isocitrate increased nearly 500-fold from the wt/wt to R132H/R132H IDH1 enzymes (Table 1). On this basis, we wondered whether a functional consequence of the R132H mutation was to reduce the level of competition between isocitrate and αKG, such that the latter could more effectively compete for binding and turnover in the mutant subunit. When we examined the isocitrate to αHG conversion by the R132H/wt enzyme, we observed that increasing isocitrate concentrations led to higher levels of αHG production, in contrast to what was observed with the wt/wt enzyme (Figure 4). We further confirmed this by testing isocitrate as a direct inhibitor of the neomorphic reaction catalyzed by R132H/R132H IDH1. The IC_{50} for the mutant homodimer was 7 mM (Figure 3B), a 1000-fold increase from the value found with the wt/wt enzyme. The change in binding affinity for isocitrate was also confirmed by thermal shift analysis,[20] a direct binding method. For wt/wt IDH1, the K_d at T_m for isocitrate was estimated to be 10 μM, whereas for R132H/R132H, it was >1 mM (Figure 5A). The affinities of NADP and NADPH were unchanged between the wild-type and mutant subunits. Interestingly, the K_d for isocitrate with each subunit of the R132H/wt heterodimer could be determined by this method, because the T_m values were sufficiently distinct for the wild-type (53.0 ± 0.3 °C) and mutant (47.2 ± 0.1 °C) subunits. In the context of the heterodimer, the isocitrate K_d for the wild-type subunit was found to be 53 μM, whereas it was >1 mM in the mutant half of the enzyme (Figure 5B). Thus, the R132H mutation has functionally segregated the two partial reactions to different subunits of the heterodimer, to allow them to proceed efficiently in parallel without cross competition.

In glioblastomas, arginine 132 has been found to be mutated to histidine, cysteine, leucine, serine, and glycine, though the

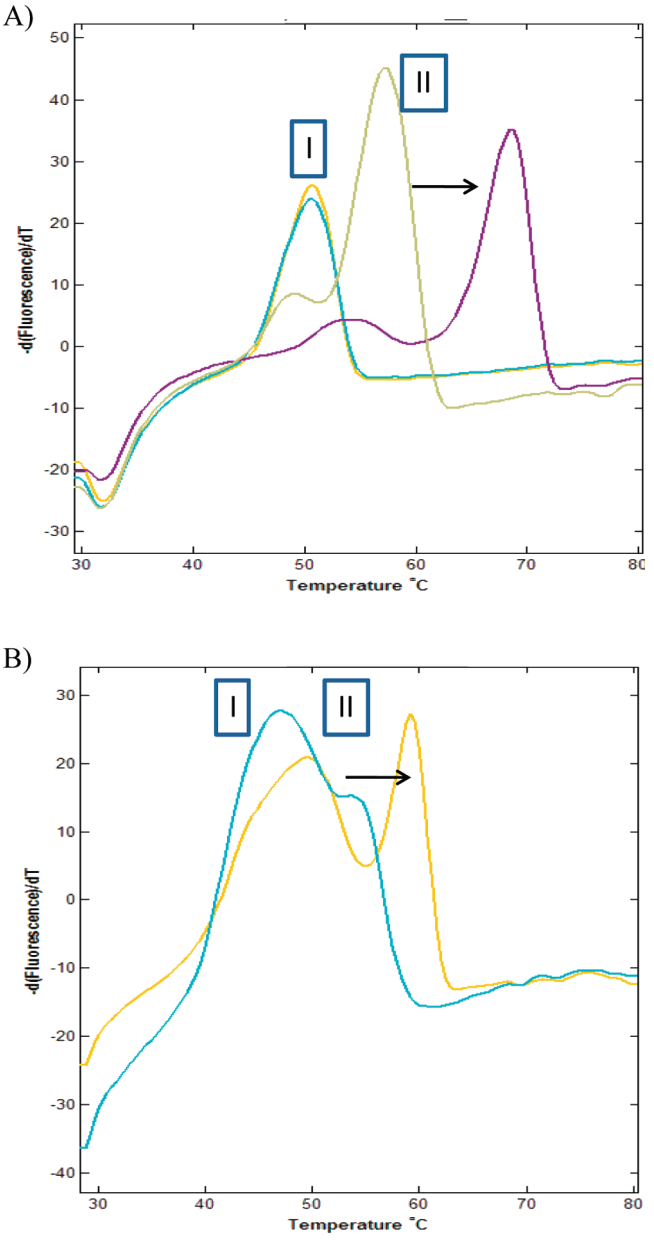

Figure 5. Titration of isocitrate in a thermoshift assay for IDH1. (A) Melting peaks of R132H/R132H and wt/wt homodimers in the presence (gold trace for the mutant and purple trace for the wild type) or absence (blue trace for the mutant and green trace for the wild type) of 4.2 mM isocitrate. A significant T_m shift was observed for wt/wt IDH1 (II) in the presence of isocitrate but not for the R132H/R132H enzyme peak (I). (B) Melting peaks of R132H/wt heterodimers in the presence (gold) or absence (blue) of 4.2 mM isocitrate. A significant T_m shift was observed for the wild-type subunit (II) in the presence of isocitrate but not for the R132H subunit (I).

R132H mutation is the most frequent.[2] An interesting aspect of this group of mutations is the lack of a consistent functional theme. While histidine, serine, and cysteine are hydrophilic and can traditionally participate in acid/base or nucleophilic reactions, leucine and glycine are hydrophobic and incapable of contributing to these types of conversions. Instead, what these mutations share in common is the fact that they are all accessible as one nucleotide changes from the codon used for arginine in

Nt Sequence	Residue 132	change
CGT	WT (Arg)	
AGT	Ser	C to A
TGT	Cys	C to T
GGT	Gly	C to G
CAT	His	G to A
CTT	Leu	G to T
CCT	Pro	G to C

Figure 6. DNA codons for amino acids accessible from one-nucleotide changes to Arg132. All five glioma-associated mutations in IDH1 (Arg132Ser, -Cys, -Gly, -His, and -Leu) can be obtained from single-nucleotide changes. The only residue possible but not found in tumors is Pro.

IDH1 (Figure 6). In light of our biochemical results, these genetic mutations make sense if their purpose is to remove the binding interaction between isocitrate and IDH1 and thus make αKG binding more competitively favorable. Then, mutation of arginine to any of the other residues found in tumor samples would accomplish the same end. In this strategy, R132 is an obvious choice for mutation in IDH1, as the C-3 carboxylate of isocitrate is the major distinguishing feature between it and αKG, and R132 specifically interacts with this group. In an analogous fashion, both R140 and R172 in IDH2 bind the C-3 carboxylate of isocitrate, and several varied αHG-associated mutations have been found in those enzymes, as well.[21]

As a side note, it is interesting that the CGT codon used for R132 in IDH1 could conceivably be mutated to six other amino acids with one nucleotide substitutions, but only five have been found in tumor samples. The sixth is proline, which is known to disrupt protein structures. Thus, in evolving this neomorphic activity, cancer cells appear to have selected for any readily accessible genetic mutation to remove R132 but then counter-selected against mutations that could not be functionally tolerated. It will be interesting to see if similar guiding principles will apply to other enzymes that evolve these types of neomorphic activities, such as the recently reported mutations in EZH2[22,23] and SPT.[24]

Given that the R132H mutation appears to functionally isolate the normal and neomorphic partial reactions to separate halves of the enzyme, we wondered whether these reactions were regulated through cooperative, intersubunit effects. Cooperativity between subunits has been postulated on the basis of previous structural studies[12] but has not been tested functionally. To address this idea, we selectively inactivated each subunit of the IDH1 dimer through introduction of a second mutation at histidine 315. From the published structures of IDH1, H315 plays a key role in binding the phosphate portion of NADP/H (Figure 7A). We reasoned that mutation of this residue to alanine would significantly impact the binding and utilization of the NADP/H cofactor in each of the two partial reactions catalyzed by R132H/wt IDH1. By placing the H315A mutation in the context of either the wt subunit (R132H/H315A) or the R132H subunit (R132H-H315A/wt), we would create an enzyme capable of conducting only the neomorphic or normal partial reaction, respectively. Of note, H315 is not proximal to either R132 or isocitrate and therefore is not expected to have similar effects on the catalytic function as the R132 mutations.

Examining first the conversion of isocitrate to αKG, we found that R132H/H315A IDH1 displayed a significant reduction in the rate of turnover compared with that of the "parent" R132H/

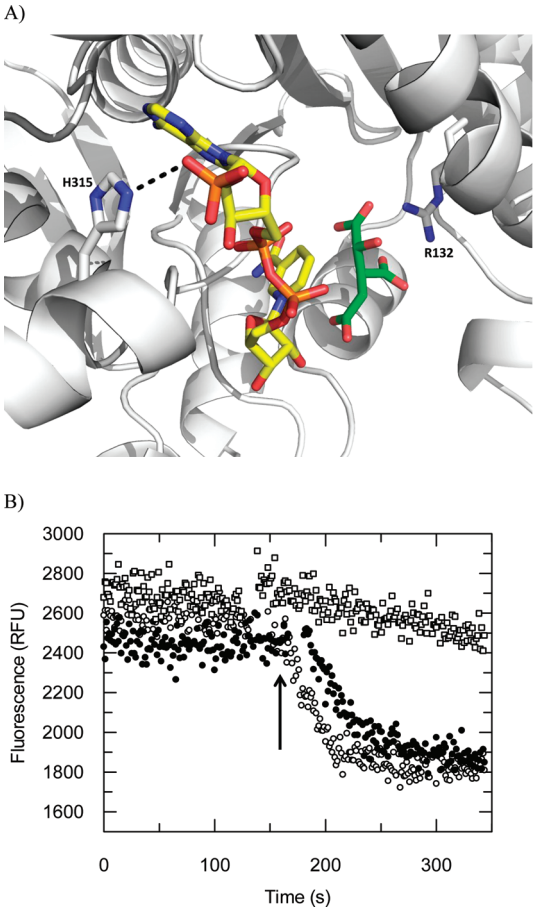

Figure 7. Kinetics of H315A IDH1 mutants. (A) Structure of IDH1 (white) showing the relative positions of R132, H315, isocitrate (green), and NADP (yellow). The dashed line indicates the interaction between H315 and the phosphate group of NADP. (B) Enzyme activity at 400 nM NADPH, 5 mM αKG, and 50 nM R132H/wt (○), R132H/H315A (●), and R132H-H315A/wt (□) IDH1. The arrow indicates when the enzyme was added to the reaction mixture.

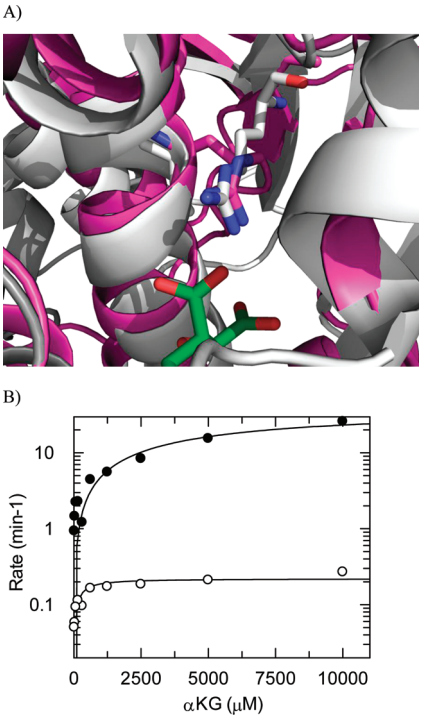

Figure 8. (A) Overlay of human IDH1 and *A. vinelandii* IDH structures. The human protein is colored white, with R132 highlighted, while the bacterial protein is colored fuschia, with R547 highlighted. Isocitrate (from the human structure) is colored green. (B) Activity of wild-type and mutant *A. vinelandii* IDH. The kinetics of the αKG to αHG reaction were studied with the wild-type (○) and R547H (●) bacterial IDH. Catalysis in the neomorphic reaction was significantly enhanced with the introduction of the mutation.

wt enzyme, as evidenced by a 20-fold reduction in k_{cat} (Table 1). In contrast, the R132H-H315A/wt enzyme exhibited a minimal change in activity, confirming that the wild-type subunit can catalyze the normal partial reaction when it is the only functional active site. We then undertook the same analysis with the neomorphic conversion of αKG to αHG. This was complicated by the high affinity of the mutant IDH1 for NADPH, which was reported to have a substrate K_m of 440 nM.[6] We attempted to repeat this analysis, but even using methods to maximize sensitivity (measuring changes in NADPH fluorescence in a cuvette), we were able to determine only that K_m < 400 nM. At NADPH concentrations lower than this, the reaction time was inadequate for obtaining rates of sufficient quality. However, measuring the rate of reaction at 400 nM NADPH, we were able to show that R132H/H315A IDH1 (10.6 min^{-1}) turns over substrate at an observed rate comparable to that of the R132H/wt enzyme (12.2 min^{-1}). By contrast, the R132H-H315A/wt enzyme loses the majority of its ability to reduce αKG to αHG under these conditions (0.2 min^{-1}) (Figure 7B). Thus, we conclude for the neomorphic partial reaction that the mutant subunit can function in a manner independent of activity in the wild-type half of the dimer.

Given these results, it is unclear if the dimer structure is necessary at all to allow the conversion of αKG to αHG. To address this, we studied the neomorphic reaction in a monomeric, bacterial IDH. IDH1 orthologs are all dimers in eukaryotic species but exist both as monomers and dimers in bacteria.[25,26] The structure of IDH1 is such that the active site is comprised of residues from both subunits; in the case of monomeric IDH enzymes, the portions of the second subunit needed to complete the active site of the first are all contained within one, larger gene product. An example is IDH from *A. vinelandii*,[25] which overlays structurally with portions of both subunits of the human IDH1. To assess the importance of the dimer to the neomorphic IDH1 activity, we made both a wt and mutant form of the monomeric bacterial IDH. The mutant form had an engineered R547H change in analogy to the R132H mutation in human IDH1 (Figure 8A). As expected, the wt *Azotobacter* IDH displayed rapid turnover when the isocitrate to αKG reaction was assessed, while the mutant exhibited very low activity (Table 1). The k_{cat} value for the normal reaction with wt protein was 9200 min^{-1}, comparable to the published report of 5300 min^{-1} for the specific activity of this enzyme.[27] However, when the αKG to αHG reaction was assessed, wild-type IDH displayed low activity while the R547H enzyme displayed significantly enhanced catalysis (Figure 8B). The k_{cat} value for the mutant enzyme for this reaction was found to be 40 min^{-1}, representing a 200-fold increase over that of the wt enzyme (Table 1). The ability to transfer the neomorphic reaction to the monomeric bacterial IDH strengthens the notion that in human IDH1 the

normal and neomorphic activities act in an independent fashion on their respective subunits.

CONCLUSIONS

The sum of the biochemical evidence coupled with the genetic disposition of the tumor mutations supports the idea that a major function of the R132 mutations in IDH1 is to isolate the normal and neomorphic reactions onto separate subunits of the dimer, to reduce the level of competitive binding of isocitrate and to allow αKG to be more effectively converted to αHG. This idea was further confirmed using engineered forms of IDH1, in which inactivation of either the wild-type or mutant subunit had no effect on the activity of the other, and with the monomeric IDH from *A. vinelandii*, which could be shown to gain the same neomorphic function with an analogous mutation. As a result of this independent functioning, the cancer-associated R132H/wt IDH1 can robustly produce αHG over a greater range of isocitrate concentrations than the wt/wt enzyme. This can offer a significant advantage, as the mean in vivo concentrations of isocitrate can vary from low micromolar to millimolar levels depending on location.[28,29]

The ability of wt/wt IDH1 to produce αHG calls into question whether it has some relevance in vivo. Though the levels of αHG are found to be elevated in patients bearing IDH1 or IDH2 mutations, this metabolite still accumulates to measurable levels in patients without mutations of these enzymes.[6] Metabolic acidurias have been associated with abnormally high levels of the L-αHG or D-αHG stereoisomer, previously ascribed to mutations in their respective dehydrogenases.[30,31] Natural levels of D-αHG have been proposed to originate from L-hydroxylysine metabolism via 2-keto-5-hydroxyglutarate,[32] though our results with IDH1 suggest that the wild-type forms of this enzyme and IDH2 may be additional sources of this metabolite in vivo. More recently, D-αHG aciduria has also been found to be associated with mutations in IDH2 in a separate cohort of patients who do not have alterations in the D-αHG dehydrogenase,[33] strengthening this proposal.

Beyond the effects on isocitrate binding outlined here, the R132H mutation likely plays additional roles in allowing the production of αHG, as evidenced by the increased kinetic parameters in the neomorphic partial reaction catalyzed by the mutant IDH1 enzymes. This may involve subtle changes to the active site as described in the available mutant structures and will be aided when a costructure with αKG can be obtained. Regardless, the finding that the two active sites of the R132H/wt form can be distinguished in small molecule binding offers the possibility of discovering a selective inhibitor. Specifically targeting the neomorphic, cancer-associated reaction of IDH1 offers the possibility of developing a drug with an excellent safety profile. We are hopeful other neomorphic enzyme mutations will be found from current sequencing efforts that will afford additional opportunities in drug discovery.

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