Redesigning the Substrate Specificity of an Enzyme: Isocitrate Dehydrogenase[†]

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ABSTRACT: Despite the structural similarities between isocitrate and isopropylmalate, isocitrate dehydrogenase (IDH) exhibits a strong preference for its natural substrate. Using a combination of rational and random mutagenesis, we have engineered IDH to use isopropylmalate as a substrate. Rationally designed mutations were based on comparison of IDH to a similar enzyme, isopropylmalate dehydrogenase (IPMDH). A chimeric enzyme that replaced an active site loop−helix motif with IPMDH sequences exhibited no activity toward isopropylmalate, and site-directed mutants that replaced IDH residues with their IPMDH equivalents only showed small improvements in k_{cat}. Random mutants targeted the IDH active site at positions 113 (substituted with glutamate), 115, and 116 (both randomized) and were screened for activity toward isopropylmalate. Six mutants were identified that exhibited up to an 8-fold improvement in k_{cat} and increased the apparent binding affinity by as much as a factor of 80. In addition to the S113E mutation, five other mutants contained substitutions at positions 115 and/or 116. Most small hydrophobic substitutions at position 116 improved activity, possibly by generating space to accommodate the isopropyl group of isopropylmalate; however, substitution with serine yielded the most improvement in k_{cat}. Only two substitutions were identified at position 115, which suggests a more specific role for the wild-type asparagine residue in the utilization of isopropylmalate. Since interactions between neighboring residues in this region greatly influenced the effects of each other in unexpected ways, structural solutions were best identified in combinations, as allowed by random mutagenesis.

Despite many advances in protein engineering, altering the specificity of an enzyme proves to be very difficult. When amino acids are altered to engineer a desired change, it is nearly impossible to predict all the small structural changes that occur to neighboring amino acids. Even alteration of amino acids far from the active site has been shown in numerous systems to have large effects on enzyme performance (*1*, *2*). Orbital steering, which postulates that very small changes in the orientation of active site residues and substrates play a large role in substrate binding and catalysis (*3*), can explain some of these difficulties. Because such small changes are difficult to predict and implement by rational design, random mutagenesis has become a useful complementary tool in protein engineering (*4*).

The isocitrate dehydrogenase (IDH)[1] of *Eschericia coli* [threo-Ds-isocitrate:NADP+ oxidoreductase (decarboxylating), E. C. 1.1.1.42] catalyzes the conversion of isocitrate and NADP to the products α-ketoglutarate, NADPH, and CO_2 in the presence of divalent magnesium ions. Many X-ray crystallographic and kinetic studies have identified important structural and catalytic features of IDH activity (*5*−*9*). The gamma-carboxylate group of isocitrate forms a hydrogen bond to S113 in the active site and forms a salt bridge with the charged nitrogen of the nicotinamide ring of NADP (*8*). These interactions stabilize the substrate and NADP cofactor in the active site and align the substrates and catalytic residues for efficient catalysis. IDH exhibits drastically reduced activities toward similar substrates that contain the malate moiety (alpha and beta carbon groups) if the gamma-carboxylate is not present (*7*).

Isopropylmalate dehydrogenase (IPMDH) catalyzes a similar chemical reaction (oxidative decarboxylation) on isopropylmalate, which contains the malate core of isocitrate and an isopropyl group in place of the gamma-carboxyl group. Several IPMDH enzymes have been studied kinetically and their structures determined, including those from *Thermus thermophilus* (*10*−*12*) and *Thiobacillus ferrooxidans* (*13*), which are structurally and functionally very similar. Although these IPMDH sequences share approximately 25−30% amino acid sequence identity with IDH, the structures of IDH and IPMDH have striking similarities. The residues at the active site of IPMDH involved in catalysis and the binding of the malate core of isopropylmalate are the same as those in IDH. Yet their substrate specificities are different, as IDH utilizes isocitrate preferentially and exhibits very low levels of activity toward uncharged malate-derived substrates (*7*), while IPMDH has a broad activity toward uncharged malate-derived substrates and no detectable activity toward isocitrate (*14*).

A striking structural difference between the IDH and IPMDH active sites is a loop that connects the cofactor binding region with an active site helix. The loops in the two enzymes are different in both length and sequence and adopt different conformations. The longer IPMDH loop has been proposed to be involved in a loop-closing mechanism that aids in catalysis by stabilizing and orienting the substrate for efficient catalysis, in addition to interacting with the

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[1] Abbreviations: IDH, isocitrate dehydrogenase; IPMDH, isopropylmalate dehydrogenase; NAD, β-nicotinamide adenine dinucleotide; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate.

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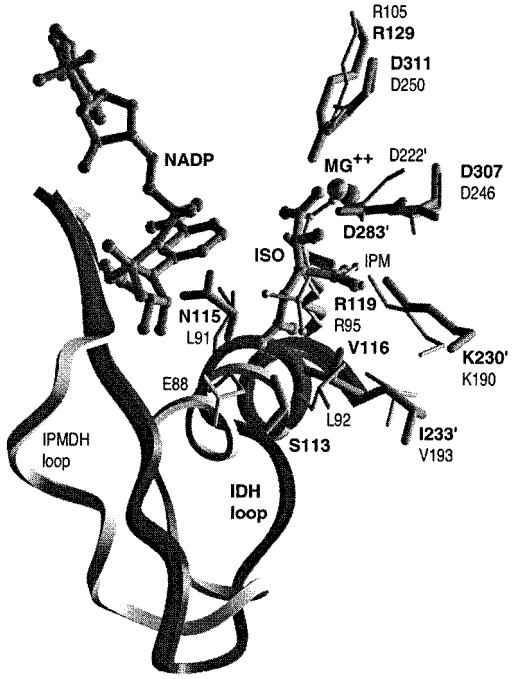

FIGURE 1: Superposition of IDH and IPMDH active sites with bound substrates. *E. coli* IDH (*5*, *19*) and *T. ferrooxidans* IPMDH (*13*) structures were superimposed using the conserved residues that are involved in binding the malate core of the substrates (R119, R129, K230, D283, D307, and D311 in IDH). The residues drawn and labeled in bold represent the IDH sequences, while IPMDH residues are drawn and labeled in plain text. Most features important for catalytic activity are nearly identical in both structures, including catalytic and malate core binding residues, as well as positioning of the substrates and Mg^{2+}. Only the IDH structure contained the cofactor, NADP. The main features that differ between the structures are the loop and helix sequences, including residues 113, 115, and 116 in IDH, which corresponds to residues 88, 91, and 92 in IPMDH.

cofactor NAD (*11*). The active site helices, which superimpose closely, contain residues known to be important for its substrate specificity: E88, L91, L92, as well as a neighboring residue, V193 (numbered as for *T. ferrooxidans* IPMDH) (*13*). These residues correspond in three-dimensional space to S113, N115, V116, and I233 of the IDH active site based on superimposed structures (Figure 1). These features create different environments for the gamma-moieties of the substrates and may hold the key to how to engineer IDH to act on alternative substrates.

We set out to engineer IDH to learn what structural components are critical for its performance with isopropylmalate as a substrate. We chose three different approaches to this aim: (i) a chimeric mutant was created that contained the loop and helix sequence of *T. ferrooxidans* IPMDH within the framework of the IDH enzyme; (ii) a series of site-directed mutants with substitutions in an active site helix and a neighboring residue was created based comparison to the structure of IPMDH; (iii) random mutagenesis of residues in the active site helix was used to test a large library of possible sequence combinations for improved activity toward isopropylmalate.

EXPERIMENTAL PROCEDURES

Mutagenesis. The loop−helix chimera, which replaced the entire loop−helix sequence of IDH with the IPMDH sequence, was created in two steps. First, the Exsite mutagenesis kit (Stratagene, La Jolla, CA) was used, following the manufacturer's protocol, on the template plasmid pTK513, which carries the IDH gene inserted into the vector pEMBL18$^-$ (*15*). The resultant plasmid contained 45 new base pairs but was missing the central 21 bp of the sequence. The final plasmid containing the entire insertion was created using PCR mutagenesis using the Quickchange Mutagenesis Kit (Stratagene, La Jolla, CA), following the manufacturer's protocol, using the plasmid generated by the first mutagenesis experiment. The sequence of the entire coding region of the final IDH mutant was confirmed by DNA sequencing (UC Berkeley DNA Sequencing Facility). This resulted in the replacement of the 16 loop and helix residues of IDH, from amino acids 102 to 117 (PLTTPVGGGIRSLNVALA) with the 22 residues of the *T. ferrooxidans* IPMDH loop−helix sequence, from residues 72 to 93 (AVGGPRWDAYPPAKRPEQGLLR).

Site-directed mutagenesis was performed using the Mutagene Phagemid In Vitro Mutagenesis Kit Version 2 (BioRad, Hercules, CA) or PCR mutagenesis. A sequence encoding six His residues was also placed on the 3′-end of the IDH coding region in pTK513 by site-directed mutagenesis. All final clones were confirmed by DNA sequencing (UC Berkeley DNA Sequencing Facility).

Random mutagenesis was performed using the Mutagene kit as described above, using an oligonucleotide that contained several site-specific mutations (to alter S113 and create a new *Bam*HI site) and two positions that were randomized (115 and 116). The oligonucleotide sequence used was 5′-GGTGGCGG**G**AT**C**CGCGAACTGNNCNNTGCCC TGCGCCAGG- 3′, with nucleotides in bold indicating substitutions created to add the *Bam*HI site and underlined nucleotides indicating substitutions that altered residues 113, 115, and 116. The codon changed to GAA substituted a glutamate residue in place of serine 113, and residues 115 and 116 were substituted with random amino acids (except Gln, Glu, Met, Lys, and Trp) by substituting the first two positions of the codons with an equal mixture of all four dNTPs (Operon Technologies, Alameda, CA).

Screening Assay. The screening assay used was a modification of a permeabilized cell assay for isopropylmalate dehydrogenase (*16*). IPMDH-deficient cells, strain JA221 (*17*) (CGSC#47303) containing mutant and control plasmids, were grown overnight at 37 °C in 2 mL of LB-ampicillin in 96-well blocks. One milliliter of culture from each well was transferred to a new block, and the cells were pelleted by centrifugation (4 °C, 4000 rpm for 5 min), washed by resuspension in 0.5 mL of buffer KP (KH_2PO_4, pH 7.2), followed by centrifugation at 4 °C, 4000 rpm for 10 min. The cells were resuspended in 105 μL of buffer KP, lysed by addition of 2 μL chloroform and 5 μL of 1% sodium deoxycholate, and followed by incubation on ice for 5 min. Fifty microliters of each mixture was added to two 96-well assay plates (V-bottom) and mixed with 80 μL of assay cocktail containing 3 mM isopropylmalate (plate 1) and no substrate (plate 2). The plates were incubated for 15 min at 37 °C, in a shaking incubator. Then 2,4-dinitrophenylhydrazine (62.5 μL) was added, and the plates were incubated at room temperature for 15 min on a shaker. To stop the reaction, 37.5 μL of 40% KOH was added. The plates were centrifuged at 4 °C, at 4000 rpm for 15 min to pellet the cellular debris, and 200-μL samples from each well were

Table 1: Kinetic Characteristics of Site-Directed Mutants of IDH with Isopropylmalate and Isocitrate as Substrates[a]

	IDH position				isopropylmalate (IPM)			isocitrate (ISO)			$\frac{k_{cat}/K_M \text{ IPM}}{k_{cat}/K_M \text{ ISO}}$
enzyme	113	115	116	233	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M	
W. T. IPMDH	E^{87}	L^{90}	L^{91}	V^{192}	2.4×10^{-1}[A]	0.017[A]	1.4×10^{1}	*			
					1.9×10^{1}[B]	44[B]	4.3×10^{-1}	*			
W. T. IDH	S	N	V	I	3.4×10^{-4}	200	1.7×10^{-6}	8.0×10^{1}	5	1.6×10^{1}	1.0×10^{-7}
SNLI	S	N	**L**	I	6.5×10^{-4}	321	2.0×10^{-6}	2.4×10^{-3}	8	2.3×10^{1}	0.0
SNVV	S	N	V	**V**	1.9×10^{-4}	209	9.1×10^{-7}	2.4×10^{-3}	8	5.1×10^{1}	0.0
SNLV	S	N	**L**	**V**	8.6×10^{-4}	421	2.1×10^{-6}	2.4×10^{-3}	5	4.4×10^{1}	0.0
ELLI	**E**	**L**	**L**	I	$<1.0 \times 10^{-4}$			$<1.0 \times 10^{-4}$			
loop-helix chimera (103−117)					$<1.0 \times 10^{-4}$			$<1.0 \times 10^{-4}$			

[a] *T. thermophilus* IPMDH residues listed are equivalent to the IDH positions based on structural alignment (with equivalent residue numbers in superscript). Two sets of kinetic values are shown for IPMDH, values denoted by footnote A were obtained at 21 °C (*7*) and by footnote B were obtained at 60 °C (*21*). The residues in bold and underlined are mutations in IDH that are the same as IPMDH residues in equivalent locations in the active site. The asterisk (*) indicates that activity was tested and reported as zero activity. Standard errors are less than 5% for k_{cat} values and 20% for K_M values.

transferred to a new 96-well plate (flat bottom) and the absorbance at 540 nm was read on a Molecular Devices SpectraMAX 340 Microplate reader. The difference between plates with and without substrates was calculated, and samples with greater differences in A_{540} (generally >0.04) were run through a repeat assay to identify false positives.

Protein Purification. Two methods were used for protein purification following overexpression of the mutant enzymes in an *E. coli* strain JLK-1, which has the IDH gene deleted. One method was the standard protocol for the purification of native IDH as previously described (*18*), and the second method utilizes a His tag on the C-teminal end of the IDH sequence. The His-tagged proteins were purified using Ni-NTA Agarose resin (Qiagen, Valencia, CA) following the manufacturer's protocols. Wild-type IDH and several mutant proteins were purified using both methods, characterized kinetically and compared and were found to be identical. IDH concentrations were determined at 280 nm using a molar extinction coefficient of 66 300 M^{-1} cm^{-1}.

IDH Activity Assay. IDH activity was determined by monitoring the conversion of NADP to NADPH over time by measuring the change in absorbance at 340 nm using a Hewlett-Packard model 8453 diode array spectrophotometer. The reactions were performed in 100 mM Tris-Cl (pH 7.8) containing 1 mM NADP and 10 mM $MgCl_2$, in a 1.00-cm path length cuvette that was maintained at 25 °C. The concentration of NADPH was determined using a molar extinction coefficient for NADPH of 6.22 mM^{-1} cm^{-1}. The precise concentrations of substrate and NADP used in the activity assays were determined using a spectrophotometric end point assay as described previously (*18*). The data were fit to the Michaelis−Menten equation to determine the kinetic constants using Table Curve 2D version 4.0 (SPSS Scientific, Chicago, IL).

RESULTS

Site-Directed Mutants. Loop−Helix Chimera. Although IDH and IPMDH exhibit a high degree of structural similarity, and most of their active site residues are conserved and superimpose well, a striking difference exists in their active sites (Figure 1). In this study, we report comparisons to the structure of the *T. ferrooxidans* IPMDH, since its crystal structure is in the same conformation as IDH crystal structures, as opposed to *T. thermophilus* IPMDH crystal structures, which adopt a conformation with a more open interdomain hinge. A loop−helix motif, which forms the portion of the site that contacts the gamma moieties of the substrates, is very different in IDH and IPMDH. Yet despite this difference, isocitrate and isopropylmalate bind to the active sites in an almost identical orientation. The beginning and end of the loop−helix motifs superimpose very closely, suggesting that replacement of the entire IDH sequence with that of IPMDH might create a stable chimera with dehydrogenase activity toward isopropylmalate.

The result of the insertion of IPMDH sequences into IDH is shown in Table 1. Although the chimera was less soluble than wild-type IDH, as judged by precipitation in solution, it was sufficiently stable to perform kinetic experiments to assess its activity toward isopropylmalate and isocitrate. This mutant's activity toward each of these two substrates was below the detectable level (k_{cat} < 0.0001 s^{-1}). Therefore, no further characterization of this mutant was performed.

Site-Directed Mutants. This report focuses on four amino acid positions in IDH, 113, 115, 116, and 233. The mutant enzymes are designated by listing the residues in these positions, with mutated residues underlined. In most cases only the first three positions (113, 115, 116) are altered, and thus those mutants are designated by a three-letter name. Some mutants have a fourth letter in their name, indicating an additional mutation at position 233. The residues of the IDH active site helix that are in contact with the gamma-moieties of the substrate, residues 113, 115, 116, are not conserved with residues in the equivalent location of IPMDH (Figure 1). Site-directed mutagenesis was used to assess whether replacing these IDH residues (SNV) with those found in the equivalent positions in IPMDH (ELL) would increase the level of IDH activity toward isopropylmalate. As seen in Table 1, the triple substitution reduced the activity toward isopropylmalate to undetectable levels.

Additional rational design attempts included position 116 (contained in the mutants already described) and position 233. Position 233 is far from the active site in the tertiary structure of an IDH subunit, but it forms part of the active site (near 116) upon dimerization of IDH subunits (Figure 1). These positions were chosen because they are in close contact to the gamma-moiety of the substrate yet are distant from the cofactor NADP (unlike positions 113 and 115). Therefore, by substituting the IDH residues 116 and 233 with

Table 2: Kinetic Characteristics of Random Mutants of IDH toward Isopropylmalate and Isocitrate as Substrates[a]

	IDH position			isopropylmalate (IPM)			isocitrate (ISO)			$\frac{k_{cat}/K_M \text{ IPM}}{k_{cat}/K_M \text{ ISO}}$
enzyme	113	115	116	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M	
W. T. IPMDH	E^{87}	L^{90}	L^{91}	2.4×10^{-1}[A]	0.017[A]	1.4×10^{1}	*			
				1.9×10^{1}[B]	44[B]	4.3×10^{-1}	*			
W. T. IDH	S	N	V	3.4×10^{-4}	200	1.7×10^{-6}	8.0×10^{1}	5	1.6×10^{1}	1.0×10^{-7}
EVG	**E**	V	G	9.3×10^{-4}	84	1.1×10^{-5}	2.4×10^{-3}	228	1.1×10^{-5}	1.0
ENA	**E**	N	A	8.2×10^{-4}	54	1.5×10^{-5}	2.9×10^{-2}	4860	5.9×10^{-6}	2.5
ENS	**E**	N	S	2.7×10^{-3}	173	1.6×10^{-5}	6.4×10^{-2}	4340	1.5×10^{-5}	1.1
ENL	**E**	N	L	1.0×10^{-3}	51	2.0×10^{-5}	9.1×10^{-2}	2750	3.3×10^{-5}	0.6
ENV	**E**	N	V	5.5×10^{-4}	5.5	1.0×10^{-4}	6.1×10^{-2}	1190	5.1×10^{-5}	2.0
ETV	**E**	T	V	4.5×10^{-4}	2.5	1.8×10^{-4}	8.3×10^{-3}	212	3.9×10^{-5}	4.6

[a] *T. thermophilus* IPMDH residues listed are equivalent to the IDH positions based on structural alignment (with equivalent residue numbers in superscript). Two sets of kinetic values are shown for IPMDH, values denoted by footnote A were obtained at 21 °C (*7*), and by footnote B were obtained at 60 °C (*21*). The residues in bold are mutations in IDH that are the same as IPMDH residues in equivalent locations in the active site. Underlined residues are mutations generated from the random mutagenesis. The asterisk (*) indicates that activity was tested and reported as zero activity. Standard errors are less than 10% for k_{cat} values and 20% for K_M values.

their IPMDH counterparts, we hoped to improve isopropylmalate binding and positioning yet not disrupt interactions of the enzyme with NADP.

The results of the kinetic experiments using are shown in Table 1. K_M values for isopropylmalate remained the same or increased, indicating that the apparent affinity (as measured by $1/K_M$)[2] of isopropylmalate was either not affected or was hindered. Both mutants that showed increased K_M values toward isopropylmalate contained the V116L mutation (SNL and SNLV), whereas the corresponding enzymes with an additional mutation at position 116 [wild-type IDH (SNV) and SNVV] exhibited K_M values equivalent to wild-type IDH. Substitution of I233 with valine, the amino acid in the equivalent position in IPMDH, did not affect the apparent binding affinity of isopropylmalate; however, it lowered the k_{cat} of the reaction. The best results were seen when the two mutations at 116 and 233 were combined. This mutant (SNLV) exhibited a 2.5-fold improvement in k_{cat}, even though the single mutant V116L (SNL) increased k_{cat} by 1.9-fold and the single mutant I233V (SNVV) decreased k_{cat} by a factor of 1.6. However, the double mutant also exhibited a reduction in the apparent binding affinity of isopropylmalate by a factor of 2.1.

Comparison of Activity Toward Isopropylmalate and Isocitrate. The activities of all the mutants were tested with isocitrate as a substrate to compare directly the new substrate specificities of the mutant enzymes (Tables 1 and 2). The site-directed mutants listed in Table 1 showed a significant reduction of k_{cat} values toward isocitrate while K_M values remained unchanged, except for mutant ELL and the loop−helix chimera, which displayed no detectable activity. All of the mutants that displayed activity toward isopropylmalate exhibited higher catalytic rates and higher apparent binding affinity toward the original substrate isocitrate than for isopropylmalate.

Degenerate Oligonucleotide-Directed Mutants. The random mutagenesis experiment introduced both a site-directed mutation at S113 and random mutations at N115 and V116. E88 in *T. ferrooxidans* IPMDH (E87 in *T. thermophilus* IPMDH), which occupy equivalent positions to S113 in IDH, have been implicated as a major determinant of activity (*10*, *13*), based on their negative charge and postulated interaction with the nicotinamide ribose. On the basis of the site-directed mutant ELL described above, substitution of a glutamate at position 113 (S113E) would not be expected to improve activity of IDH toward isopropylmalate. However, since it was shown that substitutions might have different effects on activity depending on the surrounding environment, we reasoned that S113E might be beneficial in conjunction with other substitutions at the neighboring positions, 115 and 116. Although substitution of N115 and V116 with the residues found in IPMDH did not lead to improvements, as described earlier, the local environments of IDH and IPMDH are not exactly the same, since other residues that comprise the active site helix are different. Therefore, we set out to determine if the combination of S113E with different substitutions at positions 115 and 116 would accommodate better isopropylmalate binding and orient the substrate with the NADP cofactor for catalysis.

Targeted randomization means that specific residues were randomized with a defined number of substitutions, based on the design of the oligonucleotide, excluding only a few possible amino acids and stop codon sequences. Sequencing of eight randomly chosen clones showed that the chosen positions were altered in an unbiased fashion, with each nucleotide position being fully randomized. Random residue substitutions at position 115 included F, I, D, Y, and at position 116 included G and D, in addition to the positive clones selected by the screening assay that contained N, V, T and S, V, A, L at positions 115 and 116, respectively. These results suggested that no major bias was present in the variant library. Since four nucleotide positions were randomized (the first and second positions of two codons) and 256 possible variants existed, approximately 3000 mutants were tested in the screening assay to increase the probability that most combinations of single and double amino acid changes were analyzed. Six random mutants with improved activity toward isopropylmalate were identified from this screen. Further kinetic analyses were performed on the purified proteins, and the results are summarized in Table 2.

The S113E Mutation. The first surprising result was that the S113E mutation alone (ENV) improved activity toward isopropylmalate. Although this mutant showed only a small increase in k_{cat} (1.6-fold), it decreased the K_M of isopropy-

[2] Previous kinetic studies of IDH have shown the K_M of isocitrate was equal to the thermodynamic dissociation constant, suggesting that $1/K_M$ may be a good measure of substrate affinity (*6*). Since we are dealing with mutant enzymes and a different substrate, thermodynamic studies would need to be performed to certify this conclusion.

lmalate by a factor of 36. Thus, the overall enzymatic performance (k_{cat}/K_M) improved 59-fold. In addition, this indicated that in the case of the ELL site-directed mutant, which had lower activity than wild-type, the beneficial changes caused by S113E were masked by the presence of N115L and V116L.

V116 Mutations. The other five random variants contain mutations at 115 and/or 116 in addition to S113E. In all four mutants containing substitution of V116 (to alanine, serine, leucine, and glycine), the k_{cat} values were significantly improved (2.4- to 7.9-fold) as compared to wild-type. Isopropylmalate apparent affinity was also improved, as indicated by K_M values that were lower than wild-type by a factor of 1.2 to 3.9. Mutants that contain a substitution at 116 showed an improvement in the k_{cat}/K_M of 6.5- to 11.8-fold over wild-type IDH with isopropylmalate as a substrate. Interestingly, only one of the identified mutants, ENL, contains substitutions that mimic the IPMDH sequence, ELL. The k_{cat} and K_M values differed greatly from both related mutants from the site-directed study, SNL and ELL, as well as the S113E mutant (ENV) from the random study.

N115 Mutations. Two of the identified mutants contain substitutions at position 115, ETV and EVG. The EVG mutant (also mentioned above) showed a 2.7-fold improvement in k_{cat} and improved the apparent affinity of isopropylmalate over wild-type by 2.3-fold. This resulted in an improvement in catalytic efficiency of 6.5-fold over the wild-type enzyme. Similar improvements in k_{cat} were seen in all mutants containing substitutions of V116, suggesting that the main component in EVG responsible for these improvements may be the V116G mutation and not N115V. The other N115 mutant (ETV) showed very different results; the k_{cat} was not significantly increased over wild-type, but the K_M for isopropylmalate decreased by a factor of 80 (to 2.5 μM).

Comparison of Random Mutants with S113E (ENV). Since the S113E background mutation was present in all random mutants, we compared the mutants to the S113E parent and assessed how the additional mutations affected changes caused by S113E alone. All mutants with a substitution of valine 116 showed improvement in binding isopropylmalate as compared to wild-type, as seen by the decrease in K_M values (all are below 200 μM). However, these values also represented a decrease in the apparent affinity for isopropylmalate by a factor of 10 to 31, as compared to the S113E mutation alone (ENV), which has a K_M of 5 μM (Figure 2, panel B). Thus in S113E mutants, substitution of V116 reduced isopropylmalate binding affinity. More importantly, however, the k_{cat} values of V116 mutants were 1.4−5-fold improved over the S113E value (Figure 2, panel A), showing that these mutations do further improve catalysis.

The two mutants with substitutions at position 115 did not fit into a pattern. EVG also contained a substitution at position 116 and followed the same trend as the other mutants with substitutions of V116 as described above. ETV, however, had the opposite effect, as shown in Figure 2, exhibiting decreased catalysis (panel A) and improved the apparent binding affinity (panel B), as compared to S113E.

Comparison of Activity Toward Isopropylmalate and Isocitrate. All random mutants were also tested for activity toward isocitrate (Table 2). Although k_{cat} values for isocitrate were reduced approximately by a factor of 1000−10000 as compared to wild-type IDH, they were still approximately 10−100-fold higher than values for isopropylmalate. However, the apparent binding affinities for the new substrate, isopropylmalate, were significantly improved (2.5 to 173 μM) as compared to those for isocitrate (212 to 4860 μM). This resulted in a change in substrate specificity (k_{cat}/K_M) of all the random mutants, with the exception of ENL, favoring isopropylmalate over the original substrate, isocitrate, by up to 5-fold.

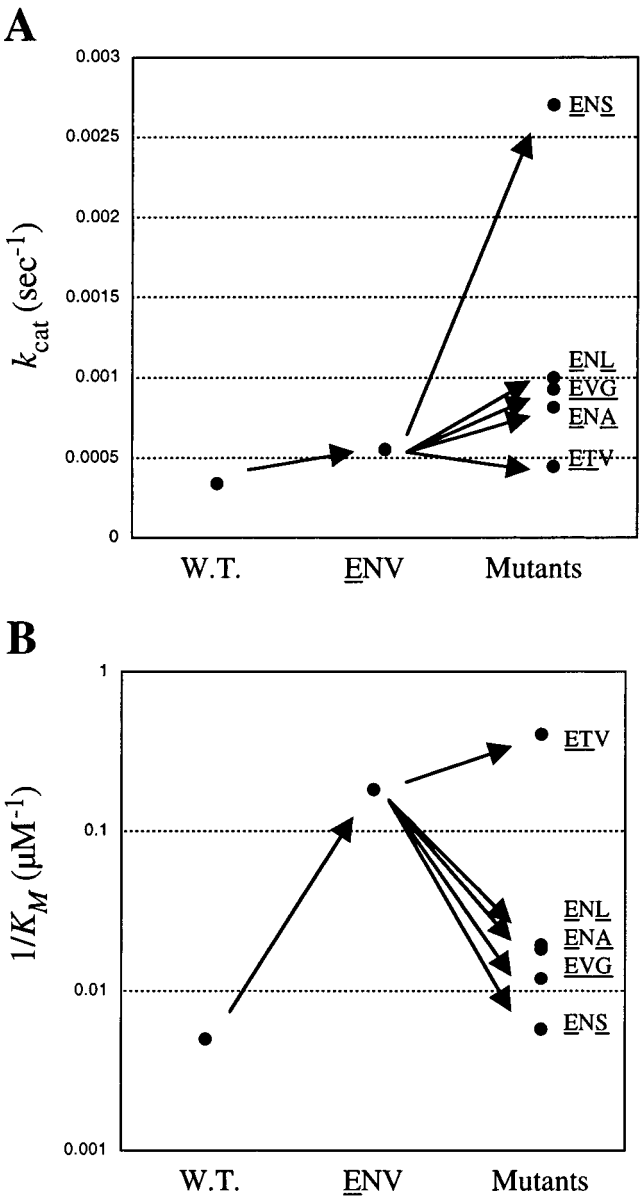

FIGURE 2: Activity of IDH random mutants toward isopropylmalate. Panel A: k_{cat} values; panel B: K_M values, plotted as $1/K_M$.

DISCUSSION

In this study, we used site-directed and random mutagenesis to attempt to engineer IDH to bind and utilize isopropylmalate as a substrate. Both methods produced mutants with improved activity toward isopropylmalate, although the random mutagenesis method gave better results.

Site-Directed Mutants. The most drastic alteration in the IDH active site was the chimeric mutant that contained the entire active site loop and helix sequence of *T. ferrooxidans*

IPMDH in place of the shorter IDH sequence. Since the precise features of the loop and helix sequence important for substrate binding and catalysis in IPMDH are not known, it seemed important to attempt to add in the complete loop−helix module of IPMDH to the IDH active site.

Kinetic analysis of the chimera indicates that this drastic change is not sufficient to promote the catalysis of isopropylmalate by IDH. The additional residues of the loop may have disrupted the structure of the enzyme and altered its ability to function as a dehydrogenase, due to large, global structural changes in the protein. The stability of the chimera was reduced somewhat; however, it was expressed in soluble form at a high level and could be purified, which suggests that it folded properly. The chimera also did not show activity toward isocitrate, although this is not surprising due to the repulsion of isocitrate caused by addition of the negative charge of the active site glutamate residue.

The use of site-directed mutagenesis provided modest improvements in activity toward isopropylmalate. In these experiments, in which specific residues in IDH were replaced with those present in equivalent positions in IPMDH, small increases in activity were seen, although the apparent binding affinity was decreased. The kinetic effects of combinations of substitutions at positions V116 and I233 were not additive, and the combination ELL, which contained the most IPMDH residues, showed no detectable activity. Therefore, the simple replacement of IDH active site residues with those of IPMDH does not promote isopropylmalate binding and catalysis.

Random Mutants. Significantly better results were obtained from the random mutagenesis study, which identified six mutants from a screen of approximately 3000 mutants. Although these mutants were not as active as the wild-type IPMDH enzyme, 5 out of 6 mutants identified exhibited preferential substrate specificity (k_{cat}/K_M) for isopropylmalate over the original substrate, isocitrate. Mutants were identified that contained two substitutions at position 115 and four substitutions at position 116, in addition to the site-directed substitution at position 113. Many possible substitutions of positions 115 and 116 may have been predicted to be beneficial or neutral, since these positions are mainly attributed to forming a nonspecific hydrophobic pocket. The broad substrate specificity of IPMDH, which includes substrates such as malate, methylmalate, ethylmalate, propylmalate, and isopropylmalate, suggests that the hydrophobic pocket does not need to bind its substrates in a rigidly prescribed manner. However, in this study only five mutants with new sequences at these positions exhibited improved activity even though the S113E mutation alone produced increased activity detectable in the screening assay. This indicates that to the contrary, most of the sequence combinations are detrimental to the utilization of isopropylmalate and that a high degree of specificity is required at positions 115 and 116 for improved activity.

Residue Size and Hydrophobicity at Position 116. Closer examination of the substitutions that were present and absent from the random mutants provides us with insights into the requirements for improved activity toward isopropylmalate. At position 116, size and hydrophobicity appear to play important roles. All the amino acid substitutions found at 116 were small, with residue masses ranging from 57.0 Da for glycine to 113.2 Da for leucine. The only other small residues not identified in this position were proline (which would disrupt the active site helix), isoleucine, which has the same residue mass as leucine but has a more elongated structure that could interfere with subunit interactions, and cysteine and threonine. All the hydrophobic residues were identified as beneficial at position 116 except proline, phenylalanine (which would take up too much space in the active site), and methionine and tryptophan, which were not possible substitutions based on the mutagenic oligonucleotide design. However, the substitution that provided the greatest increase in the catalytic rate was serine, which is not hydrophobic. Further studies will need to be performed to determine the role of this residue in the catalysis of isopropylmalate. The V116L substitution of ENL, which replaced a shorter, branched, nonpolar residue (valine) with a larger residue, may create a larger cavity in the area of the isopropyl group due to its shape, as compared to the wild-type residue valine. However, alanine and glycine (in combination with N115V) displayed lower k_{cat} values toward isopropylmalate. While creating more space and maintaining a hydrophobic environment generally leads to improved catalysis of isopropylmalate, other factors may be necessary for larger improvements in activity.

A New Role for N115. Only two mutants were identified with substitutions of the wild-type residue, N115. In addition, the mutants that exhibited the greatest increases in k_{cat} contained the wild-type residue. This position, which forms part of the hydrophobic substrate binding pocket, did not tolerate the addition of small, hydrophobic residues, as did position 116. These results indicate that position 115 may play a more specific role in substrate binding and catalysis, demanding more precision for exact size and the ability to interact with neighbors in a prescribed manner. This is perhaps due to its location in the active site, neighboring both the substrate and cofactor. It has been proposed that N115 forms favorable interactions with both the NADP and the gamma-carboxyl group of the natural substrate, isocitrate (*19*). Our data suggests that even in the absence of the interacting carboxyl group of the substrate, N115 appears to play a beneficial role in catalysis. In our random mutants, which contain the nearby E113 in addition to the loss of a potential interaction with the substrate, N115 may in fact play a new role in catalysis of the alternative substrate, isopropylmalate, due to the formation of new interactions. Threonine may have been tolerated at position 115 because it can form interactions similar to the wild-type asparagine that positively affect isopropylmalate binding, such as interactions with E113 or NADP. However, these interactions are not essential if other changes are made to nearby active site residues, since the EVG mutant improved activity toward isopropylmalate. In this case, where valine would not form interactions with neighboring residues or substrates, the enhanced performance may be due to the opening up of the active site while maintaining its hydrophobic nature, allowing the substrates flexibility to adopt optimal positioning for catalysis. This is consistent with the hypothesis that while N115 can be beneficial for catalysis, it is not always essential, since its substitution only moderately reduces activity toward isocitrate (*20*).

The S113E Single Substitution. It was unexpected to find that the S113E mutation alone (ENV) tested positive in the screening assay and significantly improved performance, based on the results of the ELL mutant in the site-directed

study. Previous attempts to engineer IDH to utilize isopropylmalate that added a glutamate to the IDH active site at position 112 (alone and in combination of other helix mutations) did not improve performance (M. Brubaker, unpublished results). However, since structural superpositions indicate that IDH residue S113E is closer to IPMDH residue E88 than is IDH R112, due to the extra turn of the helix in IPMDH, the S113E mutation was tested. E88 in IPMDH was proposed to have a dual role, contributing to the hydrophobic pocket of the active site that binds the gamma-moiety of isopropylmalate (also formed by L91, L92, and V193), while its negatively charged carboxylate interacts with the nicotinamide ribose, stabilizing the NAD cofactor (*11, 13*). Our results suggest that this may occur in our engineered ENV mutant. However, this is in contrast to data provided by Dean et al. (*7*), which showed that S113E did not improve the binding of NADP in the presence of a similar substrate malate and only marginally improved the binding of other substrates similar to isopropylmalate. This suggests that E113 may interact favorably with NADP only when it is oriented in a specific way by interaction with isopropylmalate and not by the other similar substrates tested. Indeed, the large increase in the apparent affinity seen with isopropylmalate (37-fold) as opposed to the substrates studied by Dean et al. (*7*) (2−3-fold) indicates that large differences in the effect of the S113E substitution exist depending on the substrate present.

The ENL Mutant. Only one of the mutants, ENL, contained a substitution that mimics the IPMDH sequence, as discussed above. On the basis of the negative results of ELL, which mimics the IPMDH sequence in three positions, it was unexpected that ENL showed improved activity. The only structural change in IDH caused by a N115L substitution (alone and in combination of the S113E mutation) is a 0.5-Å shift of the N-terminal portion of the helix (113−117) away from the active site, caused by a local distortion of residues 114−116 due to the altered orientation of L115 (*20*). Therefore, the N115L mutation might be expected to improve the utilization of isopropylmalate by IDH by creating more space in the active site for the bulky isopropyl group. However, since ELL showed no activity while ENL was improved clearly this is not the case. Perhaps, the N115L substitution interferes with interactions of the beneficial substitutions S113E and V116L, resulting in an enzyme with lower activity toward isopropylmalate than wild-type IDH.

Effects on Apparent Affinity and Catalysis. A trend in the effects of binding versus catalysis was seen with the set of random mutants obtained in this study; an inverse relationship existed between the level of improvement in k_{cat} and K_M values. Mutants that show the most improved k_{cat} values (ENS and ENL) show the smallest improvement in apparent affinity toward isopropylmalate as compared to the wild-type enzyme and show significantly lower affinities than the S113E mutant. The increased activity may be achieved by altering the normal positioning of substrate or cofactor, which hinders the binding efficiency of the substrates while improving binding of the transition state. Conversely, when binding is significantly improved, as in the case of ENV and ETV, the catalytic rate is only moderately improved. Perhaps in creating a better active site to accommodate isopropylmalate, the substrate sits in the active site in a slightly different position or angle, lowering the efficiency of the reaction and increasing the free-energy difference of the substrates and transition state. Thus, engineering an enzyme solely for tighter binding may hinder the chance for progress for catalytic rate enhancement due to increased binding in a less productive or nonproductive orientation. However, not all improvements in substrate binding from wild-type levels are necessarily deleterious to improved catalysis, since all random mutants exhibited improvement in both K_M and k_{cat} values toward isopropylmalate as compared to wild-type IDH. Whether further improvements of the ENV enzyme in k_{cat} are possible by additional substitutions without significantly decreasing the binding strength of isopropylmalate remains to be seen. The correct balance needed between efficient substrate binding and the flexibility required for the precise orientation of substrates is hard to predict and no doubt is one of the main hurdles to overcome in protein engineering.

Conclusion. Comparison of IDH and IPMDH substrate-bound active sites suggests that the nonnatural substrate, isopropylmalate, binds the IDH active site in a very similar orientation as isocitrate. Therefore, the challenge in engineering an alteration in substrate specificity of IDH becomes one of identifying the consequences of structural changes that occur during the reaction trajectory. Since the gamma isopropyl group of isopropylmalate is bulkier than the carboxylate of isocitrate and occupies space in the active site closer to the NADP ribose and nicotinamide ring, it is possible that the isopropyl group interferes with conformational changes that occur during the reaction. While logical amino acid substitutions increased activity toward isopropylmalate somewhat, they do not provide as much improvement as the random combinations of substitutions. The substitution of glutamate for serine 113 clearly makes a major contribution toward binding isopropymalate and its favorable catalytic alignment. Randomization of adjacent residues, in which optimal combinations are sought without preconceived ideas of what type of changes are necessary, further improves the catalytic rate. The ways in which the random mutants enhanced activity toward isopropylmalate is not obvious or predictable, indicating that they provided structural solutions that tap features of the determinants of specificity that are just beginning to be defined and understood.

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