Second-site suppression of regulatory phosphorylation in *Escherichia coli* isocitrate dehydrogenase

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Abstract

Inactivation of *Escherichia coli* isocitrate dehydrogenase upon phosphorylation at S113 depends upon the direct electrostatic repulsion of the negatively charged γ -carboxylate of isocitrate by the negatively charged phosphoserine. The effect is mimicked by replacing S113 with aspartate or glutamate, which reduce performance ($k_{cat}/K_{i-isocitrat}/K_{m-NADP}$) by a factor of 10^7 . Here, we demonstrate that the inactivating effects of the electrostatic repulsion are completely eliminated by a second-site mutation, and provide the structural basis for this striking example of intragenic suppression. N115 is adjacent to S113 on one face of the D-helix, interacts with isocitrate and $NADP^+$, and has been postulated to serve in both substrate binding and in catalysis. The single N115L substitution reduces affinity for isocitrate by a factor of 50 and performance by a factor of 500. However, the N115L substitution completely suppresses the inactivating electrostatic effects of S113D or S113E: the performance of the double mutants is 10^5 higher than the S113D and S113E single mutants. These mutations have little effect on the kinetics of alternative substrates, which lack the charged γ -carboxylate of isocitrate. Both glutamate and aspartate at site 113 remain fully ionized in the presence of leucine. In the crystal structure of the N115L mutant, the leucine adopts a different conformer from the wild-type asparagine. Repacking around the leucine forces the amino-terminus of the D-helix away from the rest of the active site. The hydrogen bond between E113 and N115 in the S113E single mutant is broken in the S113E/N115L mutant, allowing the glutamate side chain to move away from the γ -carboxylate of isocitrate. These movements increase the distance between the carboxylates, diminish the electrostatic repulsion, and lead to the remarkably high activity of the S113E/N115L mutant.

Keywords: intragenic suppression; isocitrate dehydrogenase; regulatory phosphorylation

That inactive mutants can regain a semblance of wild-type function through second-site intragenic mutations is well documented (Benzer, 1961; Crick et al., 1961; Yanofsky, 1971). This phenomenon of pseudoreversion has recently gained popularity as a means of identifying residues important to protein function (Dunn et al., 1988; Li & Baltimore, 1990; Supek et al., 1994; Anand et al., 1995; Lazzaroni et al., 1995). Yet, the structural bases by which second-site mutations restore function remain, for the most part, mysterious. Plausible hypotheses have been proposed where wild-type or related protein structures are available (Hermes et al., 1990; Thow et al., 1994; diRago et al., 1995; Shinde & Inouye, 1995), but detailed structural analyses of pseudorevertants (Poteete et al., 1991) are rare.

In NADP-dependent isocitrate dehydrogenase of *Escherichia coli* (IDH, EC 1.1.1.42), S113 forms a hydrogen bond with the γ -carboxylate of bound isocitrate (Hurley et al., 1990a) which, in turn, forms a salt bridge to the nicotinamide ring of the coenzyme (Stoddard et al., 1993; Bolduc et al., 1995). Regulatory phosphorylation of S113 results in the direct electrostatic repulsion of the γ -carboxylate of isocitrate. This inactivates IDH in the absence of conformational changes (Hurley et al., 1990b). Complete inactivation is mimicked when S113 is replaced by the negatively charged residues, aspartate and glutamate, but not by asparagine or glutamine, which are uncharged (Thorsness & Koshland, 1987; Dean et al., 1989; Dean & Koshland, 1990).

N115 is postulated to be the second key determinant of specificity in IDH. Like S113, N115 interacts with the γ -carboxylate of bound isocitrate (Hurley et al., 1990a, 1991). The interaction has a nonideal hydrogen bond angle and is probably purely electrostatic in nature. N115 also interacts with the phosphoserine of inactivated IDH (Hurley et al., 1990b) and with the carbox-

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ylate of the inactive S113E mutant in complex with isocitrate (Hurley et al., 1990a).

Here, we demonstrate that replacing N115 by leucine reactivates the S113D and S113E mutants by completely suppressing the electrostatic repulsion, and analyze the structural basis underlying this striking example of second-site intragenic suppression.

Results

Electrophoresis

Following SDS-PAGE, staining with Coomassie blue reveals single bands in each lane, indicating that preparations of purified wild-type and mutant enzymes are substantially free of contaminating protein (data not shown). Following native PAGE, staining for NADP-dependent IDH activities reveals single bands in each lane. As expected (Dean & Koshland, 1990), the mobilities of mutants with acidic amino acids at site 113 (namely the S113E, S113D, S113E/N115L, and S113D/N115L mutants) are slightly advanced compared to wild type and other mutants with uncharged side chains at this site (namely the S113N, S113Q, S113N/N115L, and S113Q/N115L mutants) (data not shown).

Kinetics of the single mutants

Replacing S113 by negatively charged residues, aspartate and glutamate, causes a dramatic 2,000-fold reduction in k_{cat} , a 100-fold increase in $K_{m \cdot isocitrate}$, and a 20-fold increase in $K_{m \cdot NADP}$ (Table 1). Overall, performance ($k_{cat}/K_{i \cdot isocitrate}/K_{m \cdot NADP}$) is reduced by a factor of 10^7 (Fig. 1). By contrast, substituting uncharged residues, asparagine and glutamine, reduces performance by a much smaller factor of 2×10^3 . These

results confirm previous reports that negatively charged amino acids at site 113 inactivate *E. coli* IDH to a far greater degree than other substitutions at this site (Thorsness & Koshland, 1987; Dean & Koshland, 1990).

The N115L substitution, like the S113Q mutant, causes a marked increase in $K_{i \cdot isocitrate}$ and a modest increase in $K_{m \cdot isocitrate}$ (Table 1). k_{cat} is reduced by a factor of 50. Unlike all single mutants at site 113, however, the effects on $K_{i \cdot NADP}$ are less dramatic, whereas $K_{m \cdot NADP}$ is reduced, even compared to wild type. Overall, this substitution reduces performance ($k_{cat}/K_{i \cdot isocitrate}/K_{m \cdot NADP}$) by a factor of 500 (Fig. 1).

Kinetics of the double mutants

Combining the N115L mutation with the S113Q mutation causes only minor shifts in $K_{i \cdot isocitrate}$, $K_{m \cdot isocitrate}$, and k_{cat} (Table 1). More marked are the reductions in $K_{i \cdot NADP}$ and $K_{m \cdot NADP}$. A similar situation arises with the S113N/N115L double mutant, although here the introduction of the N115L mutation raises the $K_{i \cdot isocitrate}$ compared to the S113N single mutant. The performances of the double mutants are higher than that of the most active single mutant, N115L (Fig. 1). Clearly, mutations at site 113 and 115 are nonadditive.

The nonadditive effects are even more dramatic when the N115L mutation is combined with the S113D and S113E mutations: k_{cat} values increase 100-fold, $K_{m \cdot isocitrate}$ values are reduced by a factor of 100, and $K_{m \cdot NADP}$ values are reduced by a factor of 50 when the S113D/N115L and S113E/N115L double mutants are compared to the S113E and S113D single mutants (Table 1). Overall, performance is increased by a factor of 5×10^4 for glutamate and 2×10^5 for aspartate (Fig. 1) so that the double mutants are again more efficient than the most active N115L single mutant.

No contaminating activities are present

These startling results inevitably raise concerns that a contaminant might be present. The IDH deletion strain SL4 was con-

Table 1. Kinetic constants from initial rate studies^a

Enzyme	Substrate	K_i (mM)	K_m (mM)	k_{cat} (s ⁻¹)
S113/N115	Isocitrate	0.004	0.011	80.5
	NADP	0.006	0.017	
S113E/N115	Isocitrate	ND ^b	0.700	0.053
	NADP	ND	0.320	
S113D/N115	Isocitrate	ND	1.750	0.033
	NADP	ND	0.27	
S113Q/N115	Isocitrate	0.121	0.019	2.24
	NADP	0.344	0.054	
S113N/N115	Isocitrate	0.017	0.018	3.85
	NADP	0.275	0.291	
S113/N115L	Isocitrate	0.189	0.029	1.72
	NADP	0.026	0.004	
S113E/N115L	Isocitrate	0.033	0.007	3.13
	NADP	0.042	0.009	
S113D/N115L	Isocitrate	0.131	0.007	5.25
	NADP	0.052	0.003	
S113Q/N115L	Isocitrate	0.111	0.007	6.75
	NADP	0.062	0.004	
S113N/N115L	Isocitrate	0.120	0.012	4.61
	NADP	0.040	0.004	

^a $K_{i \cdot isocitrate}$ and $K_{m \cdot isocitrate}$ are for the Mg^{2+} -isocitrate complex. All standard errors are less than 15% of the estimates.

^b ND, not determined.

Fig. 1. Effects on enzyme performance ($k_{cat}/K_{i \cdot isocitrate}/K_{m \cdot NADP}$) of the various site-directed mutants. The first letter of the single-letter amino acid code refers to the amino acid at site 113 and the second to the amino acid at site 115.

Table 2. Kinetic constants from inhibition studies

Enzyme	Varied substrate	Inhibitor	Pattern ^a	K_{is} (mM)	K_{ii} (mM)	Fixed substrates	Concentration (mM)
S113/N115	Isocitrate	Tartrate ^b	NC ^c	0.435 ± 0.082	1.377 ± 0.154	NADP	0.028
	Isocitrate	Tartrate	C ^c	0.232 ± 0.011		NAD	0.250
	Isocitrate	NADPH	NC	0.015 ± 0.003	0.131 ± 0.024	NADP	0.040
	NADP	Tartrate	NC	0.862 ± 0.097	0.533 ± 0.049	Isocitrate ^c	0.027
S113N/N115	NADP	NADPH	NC	0.020 ± 0.002	0.169 ± 0.018	Isocitrate	0.027
	Isocitrate	Tartrate	C	0.171 ± 0.028		NADP	0.313
	Isocitrate	NADPH	NC	0.034 ± 0.004	0.186 ± 0.011	NADP	0.080
	NADP	Tartrate	NC	0.169 ± 0.013	0.265 ± 0.045	Isocitrate	0.019
S113E/N115L	NADP	NADPH	C	0.019 ± 0.003		Isocitrate	0.019
	Isocitrate	Tartrate	C	0.132 ± 0.010		NADP	0.016
	Isocitrate	NADPH	NC	0.019 ± 0.004	0.145 ± 0.042	NADP	0.023
	NADP	Tartrate	NC	1.574 ± 0.185	0.534 ± 0.029	Isocitrate	0.025
S113D/N115L	NADP	NADPH	C	0.019 ± 0.002		Isocitrate	0.040
	Isocitrate	Tartrate	C	0.105 ± 0.013		NADP	0.020

^a C, competitive inhibition; NC, noncompetitive inhibition.

^b Concentrations are for the Mg^{2+} -tartrate complex.

^c Concentrations are for the Mg^{2+} -isocitrate complex.

firmed as having no detectable IDH activity. Unfractionated extracts were assayed from 10 independent isolates from each of the S113E and S113D single mutants freshly transformed into strain SL4. None produced detectable IDH activities. This confirms previous results and suggests that active enzymes generated by rare mutations, recombinant events, or arising from contaminant organisms, cannot account for the results. Unfractionated extracts were assayed from 10 independent isolates from each of the S113E/N115L and S113D/N115L double mutants freshly transformed into SL4. All displayed similar values of k_{cat} and K_m -isocitrate, confirming that rare contaminants, reversions, and recombinant events are unlikely to account for these activities. Moreover, the plasmids from two of the S113E/N115L isolates assayed were again purified and the presence of the mutations again confirmed by DNA sequencing. Finally, the entire experiment, from mutagenesis through sequencing and kinetic assays, was repeated, producing precisely the same results.

Inhibition studies

The four noncompetitive inhibition patterns seen with the wild-type enzyme (Table 2) are typical of a steady-state random kinetic mechanism with two abortive complexes (Dean & Koshland, 1993). Two of the patterns become competitive when the mechanism is brought into rapid equilibrium, either by use of alternative substrates and/or coenzymes, or by mutation. For example, tartarate is a noncompetitive inhibitor with respect to isocitrate when the wild-type enzyme utilizes NADP, but becomes a competitive inhibitor when the same enzyme utilizes NAD (Table 2) because the lower k_{cat} forces the mechanism into rapid equilibrium (Dean & Koshland, 1993). The S113N mutation reduces k_{cat} by a factor of 20, also bringing the mechanism into rapid equilibrium, and again tartarate becomes a competitive inhibitor with respect to isocitrate (Table 2). A similar qualitative shift in the inhibition patterns, from noncompetitive to competitive, occurs with the double mutants S113E/

N115L and S113D/N115L (Fig. 2; Table 2), indicating that their kinetic mechanisms are in rapid equilibrium.

Alternative substrates

Compounds retaining the 2R-malate core of isocitrate serve as substrates for IDH (Dean & Koshland, 1990; Dean et al., 1996). The dramatic reduction in K_m upon introducing the N115L mutation into the S113E mutant occurs only when 2R,3S-isocitrate is utilized, and is not replicated with the alternative substrates 2R-malate, 2R,3S-methylmalate, and 2R,3S-tartarate (Table 3). Overall, the substitutions at sites 113 and 115, singly or in combination, have far less effect on kinetics, with alternative substrates lacking negatively charged γ -moieties.

Fig. 2. Inhibition patterns for the wild-type enzyme (lower panels) and the S113E/N115L double mutant (upper panels). Both noncompetitive inhibition patterns generated by 2R,3S-tartrate with respect to isocitrate and NADPH for the wild-type enzyme become competitive patterns for the S113E/N115L double mutant because the mutations bring the steady-state mechanism into rapid equilibrium.

Table 3. Kinetic constants with alternative substrates

Enzyme	Isocitrate			2R-malate			2R,3S-methylmalate			2R,3S-tartrate		
	K_m^a (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m^a (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m^a (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)	K_m^a (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ mM ⁻¹)
S113/N115	0.011	80.5	7300	0.83	0.0038	0.0046	0.256	0.026	0.10	0.091	0.01	0.11
S113E/N115	0.700	0.05	0.08	1.46	0.002	0.0014	0.035	0.012	0.34	0.059	0.0005	0.01
S113/N115L	0.029	1.72	60	1.0	0.0036	0.0036	0.174	0.039	0.22	0.088	0.00076	0.01
S113E/N115L	0.007	3.13	450	2.0	0.012	0.0060	0.256	0.038	0.15	0.189	0.0023	0.01

^a K_m -isocitrate is for the Mg²⁺-isocitrate complex. All standard errors are less than 15% of the estimates.

pH profiles

The V_{max} profile of the wild-type enzyme displays an acidic limb with a limiting slope of 1 and a $pK_a = 6.3$ separated from a basic limb with a pK_b above pH 9.5 by a broad optimum around pH 8 (Fig. 3). The V_{max} profiles of the S113N single mutant and the S113D/N115L double mutant remain similar to that of wild type. This suggests that changes in the kinetic parameters, particularly k_{cat} , need not affect the profile. In contrast, the V_{max} profile of the S113D single mutant is displaced, producing an optimum at pH 6.2, and a basic limb with a slope less than 1 from pH 6 to pH 9. The latter suggests that its Michaelis complex is protonated at low pH to produce a second species with a higher k_{cat} .

Fig. 3. pH profiles: ○, wild type; □, S113N; ●, S113D; and ■, S113D/N115L mutants. Disturbance in the V_{max} profile of the S113D single mutant is absent in that of the S113D/N115L double mutant (top), whereas changes in pH do not affect affinity below pH 8.5 (bottom).

The K_m -isocitrate profile of the S113D single mutant, like those of the wild-type enzyme and the other mutants investigated, shows little change over the entire pH range. Evidently, the effects of protonation and ionization of groups on the enzyme and the substrate, if any occur within the pH range studied, have negligible effects on affinity.

Mutant structures

The overall structures of the binary complexes of the N115L and S113E/N115L mutants and the wild-type enzyme are very similar and can be superimposed with an RMS deviation of 0.3 Å for all main-chain atoms. The side chains of E113 and L115 are clearly visible in the unrefined positive difference electron density, as is Mg²⁺-isocitrate, which occupies the same position in both structures (Fig. 4).

However, a comparison of the refined N115L and wild-type structures reveals two significant differences (Fig. 5). First, the N-terminal part (113–117) of the D-helix (113–124) shifts away from the catalytic pocket by 0.5 Å. The shift is primarily by a local distortion of the helix at residues 114–116, rather than by an overall rigid-body motion. The hydrogen bond between S113 and the γ -carboxylate of isocitrate remains intact. Second, the side chain of L115 rotates away from the position occupied by N115 in the wild-type enzyme, thereby eliminating all interactions with the γ -carboxylate. The introduced leucine side-chain is in the t+ conformer, with $\chi_1, \chi_2 = (-176, 63)$. For comparison, the wild-type asparagine side chain has $\chi_1 = -73^\circ$ (the χ_2 angles are not comparable because the γ -groups of the two are nonisosteric). Instead, L115 packs against R119, a residue that interacts with the α - and β -carboxylates of the bound substrate.

The refined structure of the S113E/N115L double mutant manifests all of the features observed in the N115L mutant structure: the shift in the α -helix is again apparent and L115 remains packed against R119. The leucine side-chain is in the same conformer as in the single mutant, with $\chi_1, \chi_2 = (-174, 71)$. Although the 2R-malate moiety of isocitrate occupies the same position as for wild type, the γ -carboxylate rotates away from that occupied during hydrogen bond formation with S113 (Fig. 5). The same type of shift is seen in the binary complex of the S113E single mutant (Hurley et al., 1990a). However, the hydrogen bonds between N115 and E113 and the γ -carboxylate seen in the S113E binary complex are necessarily eliminated in the double mutant. This allows the glutamate side chain to shift 1 Å further away from the γ -carboxylate of isocitrate, pointing toward solution rather than isocitrate (Fig. 5).

Fig. 4. Initial unbiased difference electron densities from $(F_o - F_c)_{calc}$ syntheses at 2.5-Å resolution with F_c and phases calculated from wild-type apo-IDH with side chains of mutated residues, isocitrate, and all solvent molecules removed. Both the N115L (top) and the S113E/N115L (bottom) maps are contoured at ± 2.5 SD. Positive density is shown in solid lines, negative density in dashed lines (Arnez, 1994). In both cases, difference densities corresponding to Mg^{2+} -isocitrate and L115 are clearly visible. Additional difference density corresponding to E113 is present in the S113E/N115L map (bottom). The γ -carboxylate of isocitrate is less well-defined in the S113E/N115L mutant than in the N115L mutant, due to its increased mobility and lack of interactions with the enzyme. Electron density for this group appeared more strongly following refinement.

Discussion

Roles of S113 and N115 in binding

The $K_{i\text{-isocitrate}} = 17 \mu\text{M}$ of the S113N mutant is lower than the $K_{i\text{-isocitrate}} = 121 \mu\text{M}$ of the S113Q mutant. Modeling suggests a possible explanation: a hydrogen bond may form between the γ -carboxylate of bound isocitrate and the asparagine of the S113N mutant, whereas the additional methylene in the glutamine side chain may force the γ -carboxylate to adopt a conformation that prevents hydrogen bond formation. Loss of this hydrogen bond would then explain the raised $K_{i\text{-isocitrate}}$ of the S113Q mutant.

The increase in the N115L $K_{i\text{-isocitrate}}$ is consistent with the observation that N115 interacts with the γ -carboxylate (Hurley et al., 1990a, 1991). Although the $K_{i\text{-NADP}}$ of the N115L single mutant is higher than that of wild type, a case can be made that the substitution actually stabilizes the binary complex with

NADP. The $K_{i\text{-NADP}} = 6 \mu\text{M}$ of the wild-type enzyme does not reflect the true $K_{d\text{-NADP}} = 125 \mu\text{M}$ estimated by equilibrium dialysis (Dean et al., 1989) because the random kinetic mechanism is steady state (Dean & Koshland, 1993). The low k_{cat} with the alternative substrate 2R-malate brings the mechanism into rapid equilibrium and the true $K_{i\text{-NADP}} = 130 \mu\text{M}$ is recovered (Dean & Koshland, 1993). The low $k_{cat} = 1.72 \text{ s}^{-1}$ of the N115L mutant suggests that this mechanism is also in rapid equilibrium. Comparing the $K_{i\text{-NADP}} = 26 \mu\text{M}$ of the N115L mutant to the true $K_{i\text{-NADP}} = 130 \mu\text{M}$ of the wild-type enzyme suggests that the leucine substitution stabilizes the binary complex with NADP. This effect on $K_{i\text{-NADP}}$ is quite general, and occurs in all mutants where N115 is substituted by leucine (Table 2).

The N115L substitution also reduces the Michaelis constants of isocitrate and NADP. The mechanism by which this occurs is not known. The reductions may simply reflect tighter binding to the Michaelis complexes. Alternatively, a step in catalysis, or even product release, might be rate limiting. More detailed

Fig. 5. Stereo views (Kraulis, 1991) of the superimposed binary complexes of (top) the wild type (open bonds and atoms) and the N115L mutant (solid bonds and shaded atoms), and (bottom) the S113E mutant (open bonds and atoms) and the S113E/N115L double mutant (solid bonds and shaded atoms). Rotation of L115 away from the position occupied by N115, and the shift in the D-helix are clearly evident in both structures. Rotation in the γ -carboxylate of isocitrate is present in both the S113E and the S113E/N115L mutants (bottom). Loss of a hydrogen bond to N115 in the double mutant (bottom) allows E113 to move away from the γ -carboxylate of isocitrate.

kinetic studies are necessary to determine which is the case for the mutants studied here.

Roles of S113 and N115 in catalysis

S113 is not in a position to play a direct role in promoting hydride transfer, which occurs between the α -carbon of 2R-malate and the C4 of the coenzyme nicotinamide. Substitutions at site 113 must therefore affect k_{cat} indirectly. X-ray structures of IDH ternary complexes (Stoddard et al., 1993; Bolduc et al., 1995) reveal that the nicotinamide ring is stabilized by a salt bridge to the γ -carboxylate of bound isocitrate. One possibility, then, is that substitutions at site 113 disrupt the γ -carboxylate, which in turn destabilizes the nicotinamide ring, so reducing k_{cat} .

The proximity of N115 to the nicotinamide ring in the Michaelis complex of the Y160F mutant suggested a potential role in hydride transfer (Bolduc et al., 1995). Yet the closest equivalent of N115 in the mechanistically similar NAD-dependent isopropylmalate dehydrogenase (IMDH, EC 1.1.1.85) of *Thermus thermophilus* is L90 (Imada et al., 1991; Hurley & Dean, 1994). The hydrocarbon side chain of leucine is unlikely to be catalytic.

In fact, the modest 50-fold reduction in k_{cat} of the N115L substitution is no greater than that generated by several mutations at S113. Moreover, the S113D and S113E mutants are reactivated when N115 is replaced by leucine: k_{cat} values increase 100-fold, $K_{m\text{-isocitrate}}$ values decrease 200-fold, and $K_{m\text{-NADP}}$ values decrease 30-fold. The overall increases in performance ($k_{cat}/K_{i\text{-isocitrate}}/K_{m\text{-NADP}}$) upon substituting leucine are factors of 5×10^4 for glutamate and 2×10^5 for aspartate (Fig. 1). These results cannot be attributed to contaminating enzymes (see the Results). Hence, N115 is not essential for catalysis, which explains why this residue is replaced by leucine in IMDH sequences. Rather, the primary role of N115 appears to be in binding isocitrate. Whether there is a secondary role for N115 in promoting catalysis, and what the nature of such a role might be, remains to be determined.

Effects of N115L on active site electrostatic interactions

The strength of electrostatic interactions is often sensitive to the surrounding environment, which can influence the pK_a s of the ionizable groups involved (Honig & Nicholls, 1995). For example, the close proximity of a second carboxylate group may raise

the pK_a of the first, whereas the presence of a strong hydrogen bond to a neighboring amide may counteract the effect. Native PAGE gels stained for IDH activity reveal that the mobilities of mutant enzymes with acidic residues at site 113 (namely the S113E, S113D, S113E/N115L, and S113D/N115L mutants) are slightly advanced compared to wild type and other mutants with uncharged side chains (namely S113, S113N, S113Q, S113N/N115L, S113Q/N115L). Evidently, the loss of a hydrogen bond between acidic residues at site 113 and N115, and the increased hydrophobicity of leucine, do not markedly affect ionization of aspartate and glutamate in the double mutants at pH 8.

The evident disturbance in the V_{max} profile of the S113D single mutant suggests that its Michaelis complex becomes protonated at low pH to produce a second species with a higher k_{cat} . The close proximity of D113 to the γ -moiety of bound isocitrate may raise the pK_a s of the two carboxylates to within the pH range studied, and thereby account for observed shift in the profile. However, protonation has little effect—the optimum V_{max} at pH 6 is only twofold higher than that at pH 8, whereas $K_{m \cdot \text{isocitrate}}$ remains high throughout the range.

Both V_{max} and $K_{m \cdot \text{isocitrate}}$ profiles of the S113D/N115L double mutant are similar to those of the wild-type enzyme, the gently sloping basic limb of the S113D mutant being entirely absent. Therefore, protonation of D113 or the γ -carboxylate of isocitrate, if it occurs, has no effect on the kinetics of the S113D/N115L double mutant. These results suggest that the dramatic reduction in $K_{m \cdot \text{isocitrate}}$ and the marked increase in k_{cat} of the S113D/N115L and S113E/N115L double mutants are not due to a loss of the electrostatic repulsion through protonation of carboxylates with raised pK_a s.

The close proximity of the L115 side chain to R119 might enhance the electrostatic interactions with α - and β -carboxylates of isocitrate, thereby compensating for the repulsion of the γ -carboxylate. Inhibition studies show that the N115L substitution does not reduce the K_i of 2R,3S-tartarate (Table 3), a compound that retains the 2R-malate core of isocitrate but lacks its negatively charged γ -carboxylate. Moreover, the Michaelis constants and k_{cat} values with 2R-malate, 2R,3S-methylmalate, and 2R,3S-tartarate are not greatly disturbed by substitutions at sites 113 and 115, singly or in combination. Hence, the N115L substitution does not enhance electrostatic interactions with the α - and β -carboxylates of isocitrate. These data also demonstrate that reactivation by the N115L mutation depends on the presence of the γ -carboxylate of isocitrate.

Effects of N115L on active site structure

Leucine adopts a different conformer from N115, packing instead against R119 in a manner similar to that seen between the homologous L90 and R94 in *T. thermophilus* IMDH (Imada et al., 1991; Hurley & Dean 1994). This local repacking acts as a lever, driving the end of the D-helix 0.5 Å away from the active site. The γ -carboxylate of isocitrate adjusts to follow the helix shift, preserving the hydrogen bond to S113 in the single mutant. The shift of the end of the D helix away from the active site might weaken the electrostatic repulsion, whereas loss of the N115 interaction allows the γ -carboxylate greater freedom to adopt a position favorable to catalysis. In addition, loss of the E113–N115 hydrogen bond seen in the S113E single mutant frees the glutamate, which moves away from the γ -carboxylate of bound isocitrate. In the double mutant, the

E113 side chain points toward solvent rather than directly at the isocitrate γ -carboxylate. The separation between the carboxylates of isocitrate and E113 increases by 1 Å. This probably explains why the $K_{i \cdot \text{isocitrate}}$ value is reduced by a factor of 20 in the double mutants compared to the S113E and S113D single mutants.

Conclusions

The N115L substitution in IDH completely eliminates the inactivating effects of the electrostatic repulsion established between negatively charged residues at site 113 and the γ -carboxylate of isocitrate. The substitution does not perturb active site electrostatics by altering the pK_a s of the neighboring charges. Instead, reactivation occurs as a result of the leucine packing against R119, driving the N-terminal half of the D-helix away from the active site, whereas the loss of a hydrogen bond allows the negatively charged side chains at 113 to shift further from the γ -carboxylate of isocitrate and become solvent exposed. This striking example of intragenic suppression also demonstrates that N115 is not critical to catalysis.

Materials and methods

Materials

Strains, growth conditions, site-specific mutagenesis, enzyme purifications, and protein determinations were as described previously (Dean et al., 1989; Dean & Koshland, 1990). Briefly, site-directed mutagenesis of the IDH gene, cloned into a high copy number pEMBL 18 vector (Thorsness & Koshland, 1987), was conducted using the uridine-labeled template method of Kunkel (1985). Mutants were identified using the dideoxy DNA sequencing method of Sanger et al. (1977). The mutant proteins were expressed in strain SL4 (JLK1), which harbors a chromosomal deletion of the terminal two thirds of the IDH gene (Dean et al., 1989). Proteins were purified from sonicated cells using ammonium sulfate precipitation, DEAE anion exchange column chromatography, and Affi-Gel blue affinity chromatography (Dean & Koshland, 1990). Only fresh DEAE sephacel and Affi-Gel blue were used to purify the S113D, S113E, S113D/N115L, S113E/N115L enzymes to eliminate concerns regarding possible contamination from previous use.

Electrophoresis

SDS-PAGE followed by staining with Coomassie blue was used to determine the purity of the enzyme preparations. Following native PAGE of purified mutant enzymes (Dean & Koshland, 1990), gels were stained for dehydrogenase activity by coupling the production of NADPH to the reduction of nitro blue tetrazolium and phenazine methosulfate (0.1 M Tris-HCl, pH 7.6, with 0.25 mM nitro blue tetrazolium and 0.078 mM phenazine methosulfate, 2 mM NADP, and 1 mM D,L-isocitrate in the presence of 5 mM free Mg^{2+}).

Kinetics assays

Kinetics assays were conducted in 25 mM MOPS, 100 mM NaCl, 1 mM dithiothreitol, pH 7.3, at 21 °C in the presence of 5 mM free Mg^{2+} . The quantity of $MgCl_2$ added was determined using

the dissociation constants of Dean and Koshland (1993). Reactions in 1-cm cuvettes were monitored at 340 nm using a Hewlett-Packard 8452A single-beam diode array spectrophotometer. The concentration of NADPH was determined using a molar extinction coefficient of 6,200 $\Delta_{340\text{ nm}}/\text{cm}$. For initial velocity studies, the concentration of one substrate was varied at several fixed levels of the second substrate. Protein concentrations were determined at 280 nm using a molar extinction coefficient of 66,330 $\Delta_{280\text{ nm}}/\text{cm}$ (Dean et al., 1989).

Data analyses

Complete kinetics data sets were fitted to the random Bi mechanism (Cleland, 1963)

$$v = VAB/(K_{i-a}K_{m-b} + K_{m-a}B + K_{m-b}A + AB), \quad (1)$$

where A and B are the concentrations of substrate and coenzyme, v is the velocity, V is the maximum velocity, K_{i-a} is the apparent dissociation constant of the $E \cdot A$ binary complex, and K_{m-a} and K_{m-b} are the Michaelis constants for A and B from the $E \cdot A \cdot B$ ternary complex. The equations describing the effects of an inhibitor I are:

$$v = VA/(K(1 + I/K_{is}) + A) \quad \text{competitive inhibition} \quad (2)$$

$$v = VA/(K + A(1 + IK_{ii})) \quad \text{uncompetitive inhibition} \quad (3)$$

$$v = VA/(K(1 + I/K_{is}) + A(1 + I/K_{ii}))$$

noncompetitive inhibition, (4)

where K is the apparent Michaelis constant of the substrate whose concentration is varied, and K_{is} and K_{ii} are the slope and intercept inhibition constants, respectively. The effects of the inhibitor when the concentration of B is varied and the concentration of A is fixed are described by a complementary set of equations. Fits to Equations 1–4 were obtained using unweighted nonlinear least-squares Gauss–Newton regressions.

pH-profiles

pH profiles were conducted in 25 mM MES, 13 mM Tris, 13 mM ethanolamine, 100 mM NaCl, 1 mM dithiothreitol, at 21 °C in the presence of 5 mM free Mg^{2+} , the pH being adjusted with NaOH or HCl. V_{max} profiles were obtained in the presence of 5 mM NADP, which is sufficient to keep all enzymes saturated below pH 9 ($K_{m\text{-NADP}}$ is typically less than 250 μM); 1 mM isocitrate was used for the wild type, the S113N, and the S113D/N115L mutant enzymes ($K_{m\text{-isocitrate}}$ are less than 20 μM), and 50 mM isocitrate was used for the S113D mutant enzyme ($K_{m\text{-isocitrate}}$ is approximately 1 mM). The Michaelis constants of isocitrate were also determined as a function of pH. The pH of the assay solutions was determined both before and after the experiments.

Table 4. Crystallographic data as a function of resolution

Enzyme	Resolution ^a (Å)	R_{merge} ^b	Reflections	Completeness ^c (%)	$\frac{\langle I \rangle}{\sigma \langle I \rangle}$ ^d	R_{cryst} ^e (%)
N115L	5.38	0.065	3,074	94.0	19.3	0.239
	4.27	0.089	3,004	97.2	20.0	0.155
	3.73	0.103	2,973	97.8	18.8	0.152
	3.39	0.127	2,970	98.5	17.3	0.174
	3.15	0.159	2,948	98.3	15.0	0.197
	2.96	0.199	2,927	97.8	12.4	0.222
	2.82	0.240	2,864	96.3	10.3	0.231
	2.69	0.278	2,791	94.2	8.1	0.249
	2.59	0.290	2,496	84.1	6.0	0.274
	2.50	0.298	1,957	66.1	4.9	0.297
	2.50	0.118	28,004	92.5	15.6	0.199
S113E/N115L	5.38	0.053	2,765	85.0	25.1	0.243
	4.27	0.070	2,731	88.9	21.7	0.158
	3.73	0.082	2,708	90.2	17.6	0.145
	3.39	0.101	2,725	90.9	12.1	0.171
	3.15	0.126	2,774	92.8	8.3	0.196
	2.96	0.160	2,722	92.1	5.8	0.214
	2.82	0.187	2,704	91.4	4.3	0.230
	2.69	0.223	2,636	89.9	3.3	0.239
	2.59	0.244	2,361	80.2	2.4	0.252
	2.50	0.265	1,921	65.7	2.0	0.269
	2.50	0.099	26,055	86.7	9.7	0.195

^a The indicated resolution is the upper limit for each bin. All low-resolution data are included in the first bin.

^b $R_{\text{merge}} = \sum |I - \langle I \rangle| / \sum \langle I \rangle$, where the summation is over all observations. Each data set was obtained from a single crystal.

^c Completeness is the percentage of all reflections with non-negative intensities within a resolution bin.

^d $\langle I \rangle / \sigma \langle I \rangle$ is the ratio, within a resolution bin, of the mean intensity of reflections to the mean standard deviation.

^e All measured reflections within a resolution bin are used to calculate $R_{\text{cryst}} = \sum F_{\text{calc}} - F_{\text{obs}} / \sum F_{\text{obs}}$, where F_{calc} is scaled to F_{obs} using four parameters.

X-ray structure determinations

IDH crystals (Hurley et al., 1989) were presoaked for 3 h in the presence of 100 mM isocitrate and 10 mM Mg^{2+} in 100 mM NaCl, 35 mM Na_2HPO_4 , 2 mM dithiothreitol, and 0.02% NaN_3 in 45% saturated $(NH_4)_2SO_4$, pH 6.0, adjusted with NH_4OH (Hurley et al., 1990a). X-ray reflection data were collected at room temperature on an R-AXIS IIC area detector using monochromated Cu K α radiation from a Rigaku RU-200 rotating anode X-ray generator run at 5 kW. Data were collected in 1° oscillations and processed with DENZO and scaled with Scalepack (Otwinowski, 1988). Data for the N115L structure were 92.5% complete to 2.5 Å with the unweighted R_{merge} (intensity) = 0.118 for 28,004 unique reflections (Table 4). Data for the S113E/N115L structure were 86.7% complete to 2.5 Å with the unweighted R_{merge} = 0.099 for 26,055 unique reflections (Table 4). The relatively high R_{merge} values are typical for these radiation-sensitive crystals. Structures were refined by iterative minimization and restrained B-factor refinement with XPLOR (Brünger et al., 1987; Brünger & Krukowski, 1990) and rebuilding with CHAIN (Sack, 1988). Starting points for the refinements were models of wild-type IDH with the mutated residues replaced by alanines. No waters are included in the models. Bond angles and lengths deviate from ideal values by 2.88° RMS and 0.012 Å RMS. The crystallographic R-factor is 0.199 for the N115L structure and 0.195 for the S113E/N115L structure (Table 4).

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