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# Probing the mechanism of the bifunctional enzyme ketol-acid reductoisomerase by site-directed mutagenesis of the active site

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Ketol-acid reductoisomerase (EC 1.1.1.86) is involved in the biosynthesis of the branched-chain amino acids. It is a bifunctional enzyme that catalyzes two quite different reactions at a common active site; an isomerization consisting of an alkyl migration, followed by an NADPH-dependent reduction of a 2-ketoacid. The 2-ketoacid formed by the alkyl migration is not released. Using the pure recombinant *Escherichia coli* enzyme, we show that the isomerization reaction has a highly unfavourable equilibrium constant. The reductase activity is shown to be relatively nonspecific and is capable of utilizing a variety of 2-ketoacids. The active site of the enzyme contains eight conserved polar amino acids and we have mutated each of these in order to dissect their contributions to the isomerase and reductase activities. Several mutations result in loss of the isomerase activity with retention of reductase activity. However, none of the 17 mutants examined have the isomerase activity only. We suggest a reason for this, involving direct reduction of a transition state formed during the isomerization, which is necessitated by the unfavourable equilibrium position of the isomerization. Our mechanism explains why the two activities must occur in a single active site without release of a 2-ketoacid and provides a rationale for the requirement for NADPH by the isomerase.

Ketol-acid reductoisomerase (EC 1.1.1.86; KARI; also known as acetohydroxy acid isomeroreductase; reviewed in [1]) is a bifunctional enzyme that catalyzes two quite different reactions, acting both as an isomerase and as a reductase (Fig. 1A). In the isomerase reaction, 2-hydroxy-2-methyl-3-ketobutyrate (better known as 2-acetolactate) is rearranged via an $Mg^{2+}$-dependent methyl migration to produce 3-hydroxy-3-methyl-2-ketobutyrate (HMKB). In the reductase reaction, this 2-ketoacid undergoes an $M^{2+}$-dependent ($Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$) reduction by NADPH to yield 2,3-dihydroxy-3-methylbutyrate. This product is the precursor of both valine and leucine. The third branched-chain amino acid, isoleucine, is produced in a pathway that parallels that of valine, employing the same series of enzymes, with KARI catalyzing the conversion of 2-hydroxy-2-ethyl-3-ketobutyrate to 2,3-dihydroxy-3-ethylbutyrate. KARI is the target of the experimental herbicides Hoe704 [2] and IpOHA [3] that are thought to be transition-state intermediates of the alkyl migration step.

Both reactions occur at a common active site. One of the initial lines of evidence for a single active site was that the 2-ketoacid intermediate is not released and does not exchange with added HMKB [4]. However, the enzyme will catalyze the reduction of this intermediate if it is provided [4]. In addition to HMKB, KARI will catalyze the reduction of other

**Abbreviations**
DTNB, 5,5′-dithiobis(2-nitrobenzoate); HMKB, 3-hydroxy-3-methyl-2-ketobutyrate; KARI, ketol-acid reductoisomerase.

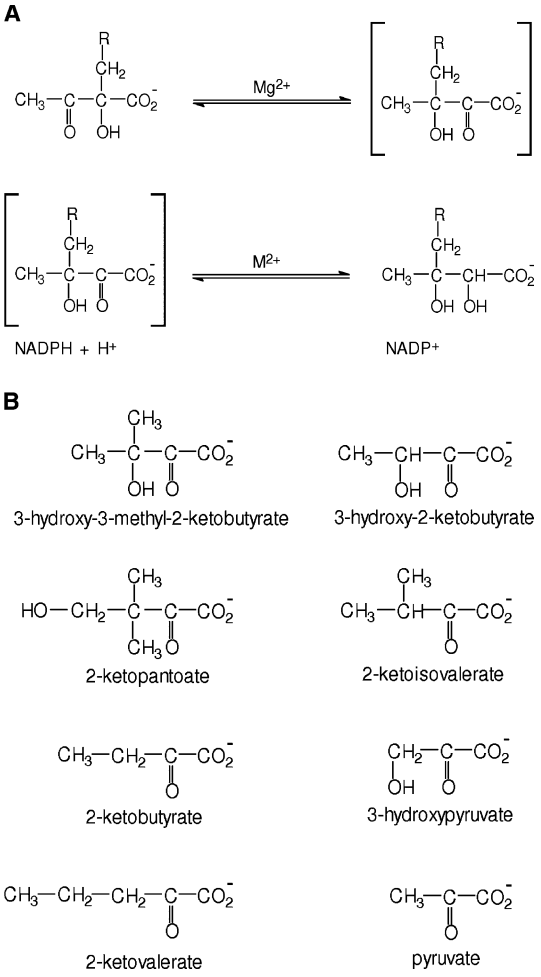


**Fig. 1.** Reactions and substrates of KARI. (A) The two reactions catalyzed by KARI. An acetohydroxyacid, where R = H (2-acetolactate) or R = $CH_3$ undergoes an $Mg^{2+}$-dependent alkyl migration to give a 2-ketoacid. This 2-ketoacid is not released but is reduced by NADPH in a reaction that requires a divalent metal ion ($M^{2+}$) that may be $Mg^{2+}$, $Mn^{2+}$ or $Co^{2+}$. The enzyme will also catalyze the reduction of externally added 2-ketoacids such as those shown in (B).

2-ketoacids. Primerano & Burns [5] described this capability for 2-ketopantoate (Fig. 1B) using the *Salmonella typhimurium* enzyme and later studies demonstrated that the *Escherichia coli* enzyme is active on 2-ketoisovalerate [6] and pyruvate [7].

Details of the active site were revealed when the crystal structure of the spinach enzyme was determined, first in the presence of the inhibitor IpOHA [8] and later with product bound in the active site [9]. There are several interesting features revealed by these structures. First, the active site contains two bound divalent metal ions, confirming the proposal of Dumas *et al.* [10] based on site-directed mutagenesis experiments. Both metal ions are coordinated to the inhibitor/product, as well as to several amino acid side-chains and water molecules. Secondly, most of the active site is very polar, consisting of four glutamate residues (E311, E319, E492 and E496) and one each of a histidine (H226), a lysine (K252), an aspartate (D315) and a serine (S518). Only the face of the active site that accommodates the substrate side-chain is hydrophobic (L323, L324 and L501). Sequence comparison reveals that the polar active residues are highly conserved across plant, fungal and bacterial KARIs [1], suggesting that each of them plays important roles in substrate binding or catalysis. This concept is further supported by the recently determined structure of *Pseudomonas aeruginosa* KARI [11]. The tertiary and quaternary organization of this enzyme is substantially different from that of the spinach enzyme with the active site constructed from two monomers of a dodecamer. In contrast, the active site of spinach KARI is wholly contained within each monomer of a tetramer. Despite these differences, the polar active residues superimpose very closely (Table 1). We have crystallized the *E. coli* enzyme [12] and solved the structure (R Tyagi, LW Guddat & RG Duggleby, unpublished observations); the active site is organized in a similar manner to that of spinach KARI (Fig. 2).

The roles of the various polar active site residues have not been subjected to detailed scrutiny. Dumas *et al.* [10] evaluated the spinach KARI mutants E311D, D315E, E319D and E492D. E311D and E492D show diminished reductoisomerase activity towards 2-hydroxy-2-ethyl-3-ketobutyrate (with $Mg^{2+}$) and no activity was detectable for D315E and E319D. These two mutants were also unable to carry out the reductive reaction (measured with 2-ketopantoate) although the former remained fully active when $Mg^{2+}$ was replaced with $Mn^{2+}$. These results suggest that D315 participates in the isomerase reaction while E319 is involved in the reductase. However, it is also possible

**Table 1.** Corresponding active site residues of KARI of spinach, *P. aeruginosa* and *E. coli*. In the *P. aeruginosa* structure, each active site is made up of residues from two monomers and these are shown with and without the prime symbol (′).

| Spinach | *P. aeruginosa* | *E. coli* |
|---|---|---|
| H226 | H107 | H132 |
| K252 | K130 | K155 |
| E311 | E186 | E213 |
| D315 | D190 | D217 |
| E319 | E194 | E221 |
| E492 | E226′ | E389 |
| E496 | E230′ | E393 |
| S518 | S251′ | S414 |

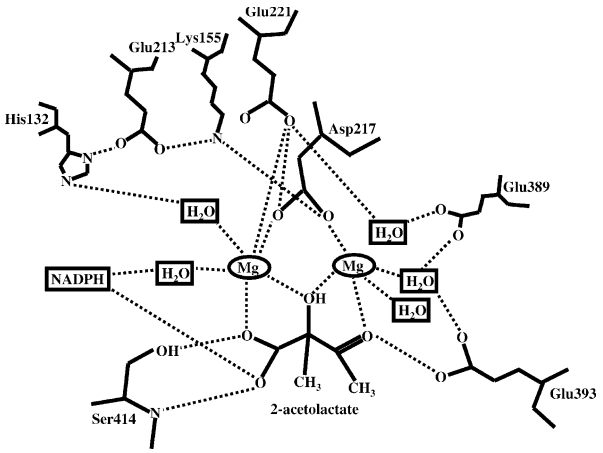


**Fig. 2.** Schematic representation of the active site of *E. coli* KARI. This representation is based on the structure of spinach KARI [9] and is shown with 2-acetolactate bound. Dotted lines represent ionic interactions and hydrogen bonds.

that E319 is required for both reactions because the isomerase reaction alone was not assayed.

Here we have constructed mutants of *E. coli* KARI at every polar residue in the active site and evaluated their kinetic properties. The obtained results lead us to propose a novel explanation of why a common active site is necessary for these two reactions.

## Results

### Wildtype

The usual assay for KARI involves measuring NADPH oxidation by 2-acetolactate. By this assay, the purified recombinant wildtype *E. coli* enzyme was found to have a specific activity of $\approx 2$ U·mg$^{-1}$ and ranged from 1.68 to 2.43 U·mg$^{-1}$. This assay depends upon both the isomerase and the reductase reactions and we would not be able to pinpoint the defect in a mutant that is affected in only one of the two activities. Therefore, we established independent assays for each activity. In addition, for inactive mutants, we developed methods to measure NADPH and $Mg^{2+}$ binding using techniques that are not reliant on catalysis.

### Reductase activity

Previous studies [4–7] had shown that the reductase activity alone could be measured with HMKB, 2-ketopantoate, 2-ketoisovalerate or pyruvate. Therefore, we compared the activity on these and several other 2-ketoacids.

In agreement with these earlier reports, KARI is capable of catalyzing the reduction by NADPH of HMKB, 2-ketopantoate, 2-ketoisovalerate and pyruvate. In addition, the *E. coli* enzyme will act on 2-ketovalerate, 2-ketobutyrate, 3-hydroxypyruvate and 3-hydroxy-2-ketobutyrate. For each substrate, data exhibit Michaelis–Menten saturation kinetics and the kinetic parameters towards each of these substrates are reported in Table 2.

The specific activity with pyruvate is $\approx 1\%$ of that with 2-acetolactate, in agreement with the value reported previously [7]. Pyruvate is the worst of the substrates tested and 2-ketovalerate is also a poor substrate. The specific activities with 2-ketopantoate, 2-ketoisovalerate and 2-ketobutyrate are all similar and each gives $\approx 8\%$ of the value with 2-acetolactate. The most remarkable result is the high $k_{cat}$ with 3-hydroxypyruvate, which is double that observed with 2-acetolactate. This high $k_{cat}$ invites the speculation that the prior alkyl transfer that is needed for 2-acetolactate reduction is rate-limiting, and that the high activity observed with 3-hydroxypyruvate results from by-passing this step. Consistent with this, HMKB, the product of 2-acetolactate rearrangement, is a somewhat better substrate than 2-acetolactate itself. Nevertheless, 3-hydroxypyruvate is an intrinsically good substrate for the reductase reaction. The activity with 3-hydroxy-2-ketobutyrate seems anomalous

**Table 2.** Kinetic parameters for the activity of *E. coli* KARI towards 2-acetolactate and various 2-ketoacids. The value of $k_{cat}$ ($s^{-1}$) is similar to the $V_m$ (U·mg$^{-1}$) because the subunit molecular mass of 59.5 (kDa) is almost equal to the number of seconds in one minute.

| Substrate | $V_m$(U·mg$^{-1}$) | $k_{cat}$($s^{-1}$) | $K_m$(mM) | $k_{cat}/K_m$ ($M^{-1}·s^{-1}$) |
|---|---|---|---|---|
| 2-acetolactate | 2.250 ± 0.099 | 2.231 ± 0.098 | 0.25 ± 0.03 | 9020 ± 877 |
| 2-Ketopantoate | 0.196 ± 0.005 | 0.194 ± 0.005 | 0.17 ± 0.02 | 1104 ± 83 |
| 2-Ketoisovalerate | 0.184 ± 0.007 | 0.182 ± 0.007 | 6.91 ± 0.58 | 26 ± 1 |
| 2-Ketovalerate | 0.050 ± 0.002 | 0.050 ± 0.002 | 3.15 ± 0.30 | 16 ± 1 |
| 2-Ketobutyrate | 0.168 ± 0.008 | 0.167 ± 0.008 | 4.56 ± 0.45 | 36 ± 2 |
| Pyruvate | 0.021 ± 0.001 | 0.021 ± 0.001 | 1.54 ± 0.18 | 14 ± 1 |
| 3-hydroxypyruvate | 5.421 ± 0.241 | 5.376 ± 0.239 | 2.96 ± 0.31 | 1818 ± 126 |
| 3-Hydroxy-2-ketobutyrate | 0.599 ± 0.023 | 0.594 ± 0.023 | 0.21 ± 0.04 | 2829 ± 448 |
| 3-Hydroxy-3-methyl-2-ketobutyrate | 3.541 ± 0.153 | 3.511 ± 0.152 | 0.27 ± 0.03 | 13199 ± 1201 |

with a $k_{cat}$ value substantially less than those of its lower and high homologues. However, it should be noted that this compound is the only 2-ketoacid tested that has a chiral centre and we have found (data not shown) that both enantiomers are active. Misorientation of one of the enantiomers might explain the low $k_{cat}$ value.

The $K_m$ values vary widely but these values cannot be interpreted simply as affinities because $K_m$ depends upon the rate constants for substrate binding, catalysis and product release. A better comparison of substrate preferences can be made using the $k_{cat}/K_m$ values, sometimes known as the specificity constant. On the basis of this quantity, 2-ketoisovalerate, 2-ketovalerate, 2-ketobutyrate and pyruvate are all very poor substrates in comparison with 2-acetolactate, while 2-ketopantoate, 3-hydroxypyruvate and 3-hydroxy-2-ketobutyrate are moderately good but still three- to eightfold worse than 2-acetolactate. Only the expected intermediate HMKB has a $k_{cat}/K_m$ value exceeding that of 2-acetolactate.

Based on these data we chose HMKB and 3-hydroxypyruvate as the substrates to measure the reductase activity of *E. coli* KARI. Our preferred substrate for these studies is 3-hydroxypyruvate because it has the highest $k_{cat}$ value of all substrates tested. Although the $K_m$ value is higher and the $k_{cat}/K_m$ value is lower, than those of HMKB, 3-hydroxypyruate has the considerable advantage of being available commercially.

### Isomerase activity

The rearrangement of HMKB to 2-acetolactate was used as an assay for the isomerase activity. It has been shown [13,14] that the kinetic mechanism for the overall (reductoisomerase) activity involves random binding of $Mg^{2+}$ and NADPH, followed by addition of 2-acetolactate. Therefore, it would be expected that the reverse isomerase reaction would require both $Mg^{2+}$ and NADPH, even though the latter is not a participant in the reaction. The presence of NADPH would create a difficulty in that it would allow the reductase reaction to proceed. We reasoned that the NADPH requirement would be purely for structural reasons and that it could be replaced by $NADP^+$. As predicted, 2-acetolactate formation was detected when *E. coli* KARI was incubated with HMKB, $NADP^+$ and $Mg^{2+}$. It appears that $NADP^+$ is not a very good surrogate for NADPH because the specific activity is quite low (Table 3). Nevertheless, the activity was readily measured and could be used for comparing the isomerase activity of mutants with that of the wildtype.

The equilibrium constant for the isomerase reaction was estimated by incubating the enzyme with $NADP^+$, $Mg^{2+}$ and either 2-acetolactate or HMKB, destroying residual (or formed) 2-acetolactate, then measuring

**Table 3.** Activities of wildtype and mutants of *E. coli* KARI. If there was measurable activity, the specific activity was determined from substrate saturation data fitted with the Michaelis–Menten equation. Where no standard error is reported, the value represents the activity at a concentration of 5 mM HMKB. Values shown as '0' are < 0.2% of wildtype for the reductoisomerase and reductase activities, and < 0.5% of wildtype for the isomerase. ND, not determined; WT, wildtype.

| Enzyme | Reductoisomerase ($U \cdot mg^{-1}$) | Reductase (hydroxypyruvate) ($U \cdot mg^{-1}$) | Reductase (HMKB) ($U \cdot mg^{-1}$) | Isomerase ($U \cdot g^{-1}$) |
|---|---|---|---|---|
| WT | 2.25 ± 0.10 | 5.42 ± 0.24 | 3.54 ± 0.15 | 120 ± 10 |
| H132K | 0 | 0.0542 ± 0.0037 | 0.053 | 0 |
| H132Q | 0.0339 ± 0.0013 | 6.41 ± 0.29 | 4.52 ± 0.11 | 5.1 ± 0.1 |
| K155R | 0.0558 ± 0.0018 | 9.30 ± 0.29 | 5.79 ± 0.20 | 2.9 ± 0.1 |
| K155E | 0 | 0.0130 ± 0.0004 | 0.037 | 0 |
| K155Q | 0 | 0.0648 ± 0.0136 | 0.024 | 0 |
| E213D | 0.561 ± 0.017 | 3.49 ± 0.26 | ND | 32.1 ± 1.2 |
| E213Q | 0 | 0.083 ± 0.003 | ND | ND |
| D217E | 0 | 0 | 0 | 0 |
| D217N | 0 | 0 | 0.258 ± 0.013 | 0 |
| E221D | 0.012 ± 0.001 | 0.0334 ± 0.0013 | 0.054 | 0 |
| E221Q | 0 | 0 | 0 | 0 |
| E389D | 0.085 ± 0.006 | 3.33 ± 0.24 | ND | 2.6 ± 0.1 |
| E389Q | 0 | 0.386 ± 0.024 | ND | ND |
| E393D | 0 | 0.188 ± 0.004 | 5.09 ± 0.24 | 0 |
| E393Q[a] | ND | 0.165 ± 0.006 | 0.096 ± 0.004 | ND |
| S414A | 0.005 ± 0.001 | 0.039 ± 0.001 | ND | 8.0 ± 0.5 |
| S414T | 0.020 ± 0.001 | 0.733 ± 0.038 | ND | 5.0 ± 0.5 |

[a] Refolded enzyme, with activities corrected for a folding efficiency of 25%.

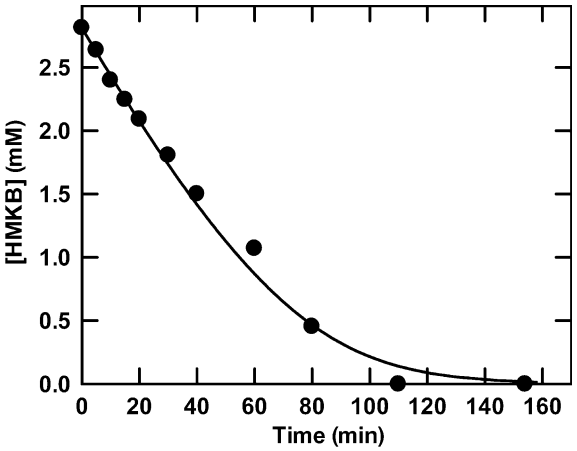


**Fig. 3.** The isomerase activity of wildtype *E. coli* KARI towards HMKB. KARI was incubated at 37 °C with 2 mM $NADP^+$, 10 mM $MgCl_2$ and 2.8 mM HMKB in 0.1 M Tris/HCl buffer (pH 8.0). At intervals, samples were removed and assayed for HMKB as described in Experimental procedures. After 2.5 hours, the residual HMKB is 17.2 μM.

formed (or residual) HMKB. An experiment starting from HMKB is illustrated in Fig. 3. The residual HMKB is less than 0.64% of the starting concentration, indicating an equilibrium constant of at least 150 in favour of 2-acetolactate. When 2-acetolactate was used as the substrate, the highest concentration of HMKB formed in several experiments was 0.22% of the 2-acetolactate added, corresponding to an equilibrium constant of 450. Allowing for the fact that neither reaction may have reached equilibrium, these results suggest that the equilibrium position of the isomerase reaction favours 2-acetolactate by a large factor, on the order of 300. We are aware that this equilibrium constant is inconsistent with the reported purification of a mycobacterial enzyme catalyzing the isomerase reaction only [15].

## NADPH binding

The fluorescence ($\lambda_{ex} = 370$ nm; $\lambda_{em} = 460$ nm) of NADPH is enhanced upon binding to *E. coli* KARI and we followed the published procedure [14] for performing and analyzing NADPH binding experiments. In addition, we examined the use of fluorescence resonance energy transfer to monitor NADPH binding. In these experiments, tryptophan residues are excited at 295 nm and nonradiative energy transfer to NADPH is detected by its fluorescence at 460 nm. This method gave similar results to direct measurements of enhanced NAPDH fluorescence.

## $Mg^{2+}$ binding

No useful absorbance or fluorescence signals could be detected when $Mg^{2+}$ was added to *E. coli* KARI, in the absence or presence of NADPH. Therefore, an indirect method was developed based on the observation that KARI undergoes significant conformational changes upon binding of $Mg^{2+}$ [16]. We exploited this property by measuring the release of the coloured nitrothiobenzoate ion upon reaction of 5,5′-dithiobis (2-nitrobenzoate) (DTNB) with cysteine residues (Fig. 4A). The stoichiometry and kinetics of this process are quite complex, with two of the six cysteine

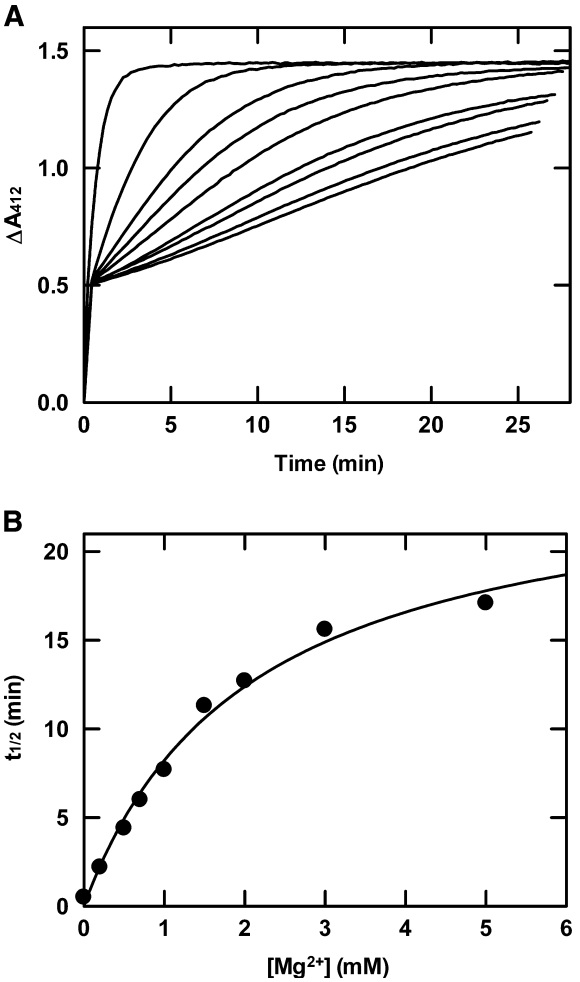


**Fig. 4.** Protection of wildtype *E. coli* KARI against reaction with DTNB by $Mg^{2+}$. (A) The reaction of KARI with DTNB, followed by the increase in absorbance at 412 nm. There is a fast initial burst followed by a slower reaction that is affected by the concentration of $Mg^{2+}$ (0, 0.2, 0.5, 0.7, 1.0, 1.5, 2.0, 3.0 and 5.0 mM, from left to right). The half-time for this slower phase shows an hyperbolic dependence upon [$Mg^{2+}$] (B) and was used to estimate an apparent $K_d$ for [$Mg^{2+}$] of 2.06 ± 0.38 mM.

residues in the *E. coli* enzyme reacting within a few seconds, a further three reacting over a period of several minutes, and one not reacting at all. Addition of $Mg^{2+}$ partially protects the three slowly reacting cysteine residues. These three appear to react with DTNB at different rates so the formation of the nitrothiobenzoate ion does not follow first-order kinetics. However, the half-time measured from these curves shows a hyperbolic dependence on [$Mg^{2+}$] (Fig. 4B) from which an apparent dissociation constant can be derived. We readily concede that this method is entirely empirical and the measured apparent dissociation constant may have no strict physicochemical meaning. However, it does allow a crude measure of the affinity of KARI for $Mg^{2+}$ and would certainly identify any mutant that has lost its ability to bind this metal ion.

## KARI mutants

### Expression and purification

All *E. coli* KARI mutants were expressed and purified successfully, with one exception. E393Q is insoluble and we were unable to find conditions where it could be expressed in a soluble form. However, after denaturation and refolding some reductase activity was observed. Several of the mutants had a very low, but measurable, activity and we were concerned that this might represent a background of native wildtype KARI from the host cells. Although we would not expect the native wildtype enzyme to be retained by the immobilized nickel that was used for affinity chromatographic purification, we could not rule this out. Moreover, trace amounts of oligomers of hexahistidine-tagged recombinant KARI mutant subunits and the native wildtype *E. coli* protein might form and be responsible for the measured activity. Therefore, we expressed such mutants using the *E. coli* host strain CU505 in which the *ilv*GMEDA and *ilv*YC operons are deleted. Because this strain does not contain the T7 RNA polymerase gene, we cloned the KARI gene (*ilv*C) from our usual expression vector pET-C [7] into a different vector, pProExHT, where expression is under the control of the *lac* promoter. The protein expressed by this vector has an N-terminal hexahistidine tag, and it was purified in the same way as that expressed by pET-C.

### Catalytic properties

The specific activities of the wildtype and mutants in the reductoisomerase, reductase, and reverse isomerase assays are summarized in Table 3. As mentioned above, E393Q was obtained in a soluble form only after denaturation and refolding whereupon some reductase activity was observed. When wildtype *E. coli* KARI was denatured and refolded in the same manner, 25% (reductoisomerase) and 26% (reductase) activity was recovered. The reductase activities reported in Table 3 for E393Q are calculated assuming a refolding efficiency of 25%. Table 4 summarizes the Michaelis constants for all mutants that showed activity in at least one of the assays.

Without exception, all mutants have impaired reductoisomerase activity. For E213D there is a 75% reduction while for all other mutants the residual activity is less than 4%. Based on these results we conclude that all eight residues investigated here contribute to the overall reaction. The reason for the activity loss was investigated further by separate measurements of the reductase activity. H132Q, K155R, E213D and E389D all have nearly normal activity with 3-hydroxypyruvate, S414T and E389Q have 14% and 7% of wildtype activity, respectively, and all other mutants have little or no reductase activity.

It is of interest that of the four mutants that have high activity, all involve no change in charge (at the assay pH

**Table 4.** $K_m$ values of wildtype and mutants of *E. coli* KARI. The $K_m$ values for $Mg^{2+}$ and NADPH were measured for the reductoisomerase reaction except where the activity is very low, where it was measured for the reductase reaction (shown in italics). ND, not determined, usually because there is little or no activity for this mutant (Table 3). WT, wildtype.

| Enzyme | 2-acetolactate (μM) | 3-hydroxy-pyruvate (mM) | $Mg^{2+}$ (μM) | NADPH (μM) |
|---|---|---|---|---|
| WT | 247 ± 33 | 2.96 ± 0.31 | 831 ± 81 | 2.53 ± 0.30 |
| | | | 2060 ± 380[a] | 16.0 ± 2.0[a] |
| H132K | ND | 0.818 ± 0.174 | *11.6 ± 5.3* | *3.12 ± 0.47* |
| H132Q | 929 ± 68 | 7.43 ± 0.62 | 856 ± 71 | 69.6 ± 2.8 |
| K155R | 1218 ± 66 | 13.6 ± 0.7 | 6244 ± 431 | 7.27 ± 0.44 |
| K155E | ND | 2.66 ± 0.23 | *23.3 ± 2.9* | *8.04 ± 1.14* |
| K155Q | ND | 15.3 ± 6.0 | 9.78 ± 0.42 | 9.30 ± 2.11 |
| E213D | 922 ± 91 | 3.67 ± 0.70 | 2079 ± 245 | 16.0 ± 2.0 |
| E213Q | ND | 0.441 ± 0.050 | *197 ± 10* | ND |
| D217E | ND | ND | ∞[a] | 80.0 ± 12.0[a] |
| D217N[b] | ND | 7.64 ± 0.68 | *114 ± 7* | *5.08 ± 0.52* |
| E221D | 356 ± 18 | 1.37 ± 0.15 | 2038 ± 171 | 20.3 ± 2.8 |
| E221Q | ND | ND | 470 ± 100[a] | 20.0 ± 3.0[a] |
| E389D | 2028 ± 357 | 8.50 ± 1.49 | 2156 ± 225 | 23.0 ± 5.0 |
| E389Q | ND | 8.88 ± 1.36 | *2432 ± 308* | ND |
| E393D | ND | 3.32 ± 0.16 | *5380 ± 340* | *4.76 ± 0.76* |
| E393Q | ND | 0.588 ± 0.063 | ND | ND |
| S414A | 711 ± 119 | 0.334 ± 0.030 | *796 ± 96* | *8.37 ± 1.11* |
| S414T | 414 ± 90 | 1.101 ± 0.162 | 2400 ± 444 | 5.07 ± 0.76 |

[a] Apparent $K_d$ values, measured by fluorescence enhancement (NADPH) or by protection against reaction with DTNB ($Mg^{2+}$). [b] For HMKB only.

of 8.0). When each of these residues is mutated to produce a change in charge (H132K, K155E, K155Q, E213Q and E389Q) reductase activity is decreased substantially. It is clear that the ionic property of these four residues is crucial for the reductase. Measurements of the reductase activity with HMKB gave similar results to those obtained with 3-hydroxypyruvate. The sole exception is E393D, which has normal activity with HMKB but low activity with 3-hydroxypyruvate.

For those mutants with little or no reductase activity, a low reductoisomerase activity is inevitable. Therefore, we tested most of the mutants for isomerase activity. The pattern is quite similar to the results obtained for the reductoisomerase with little or no activity observed in any mutant in which reductoisomerase activity is low. E213D, with 25% of the wildtype reductoisomerase activity also retains a similar fraction (27%) of isomerase activity. Thus, while it is possible to obliterate the isomerase activity but leave the reductase activity largely unimpaired, mutations that affect the reductase invariably result in a major decrease in the isomerase activity. The implications for this finding on the mechanism of the enzyme are discussed later.

E496 of spinach KARI (*E. coli* E393) has been proposed to play a key role in isomerization reaction [17] and it is relevant that E393D has no isomerase activity but shows partial retention of the reductase (Table 2). However, this is a pattern that is observed for several other mutants and no special function of E393 can be proposed on the basis of the results presented here.

Two of the mutants (D217E and E221Q) showed no activity in any of the assays. These mutants (and wildtype) were assessed for their ability to bind $Mg^{2+}$ and NADPH (Table 4). Both mutants could bind NADPH, with $K_d$ values of 80 ± 12 and 20 ± 3 μM, respectively, compared to the wildtype value of 16 ± 2 μM. For $Mg^{2+}$, E221Q has a $K_d$ value of 0.47 ± 0.10 mM, somewhat smaller than the wildtype value of 2.06 ± 0.38 mM. However, D217E appeared to be incapable of binding this cofactor so it is not surprising that it is devoid of any activity. For E221Q, we have not ruled out the possibility that it will not bind any of the carbon substrates.

## Discussion

The geometry [11] and identity (Table 1) of eight polar amino acid residues forming the active site of KARI are conserved across species, despite major differences in the structural organization of the enzyme. This high degree of conservation implies that each amino acid plays an essential role, and we have attempted to understand these roles by mutagenesis of *E. coli* KARI.

Most of the mutations abolish the overall reductoisomerase, with only E213D retaining substantial (25%) activity (Table 3). This residue does not interact directly with the carbon substrate, the metal ion cofactor, NADPH or active site water molecules, and its sole function appears to be in positioning H132 and K155 (Fig. 2). Evidently, shortening the side-chain by one methylene group does not interfere greatly in this function. This mutant also has the highest isomerase activity of all mutants tested and nearly normal reductase activity. The most notable effect of this mutation is the sixfold increase in the $K_m$ for NADPH (Table 4), evidently caused by repositioning of H132 which is reasonably close (3.2 Å) to NADPH. That the effect of E213D on the $K_m$ for NADPH is mediated through H132 is supported by the observation that mutating H132 to glutamine has by far the greatest effect on the $K_m$ for NADPH, increasing it by 28-fold (Table 4).

Several of the mutations leave the reductase activity largely intact (Table 3). For H132, K155, E213 and E389 it is clear that maintaining the same ionic form is important here, because there are obvious differences between the effects of mutations that retain and those that alter the charge. It may be significant that none of these amino acid side-chains make contact with the carbon substrate or the metal ion cofactor (Fig. 2). In contrast, mutation of the three anionic residues that contact the carbon substrate or the metal ion cofactor (D217, E221 and E393) each causes a major decrease in reductase activity, irrespective of whether the change maintains or alters the charge. The eighth residue, S414, forms a hydrogen bond with the substrate through the side-chain hydroxyl and can be replaced by threonine but not alanine with retention of reductase activity. Curiously, the S414T mutant has a low reductoisomerase activity, possibly because the larger size of 2-acetolactate (compared to 3-hydroxypyruvate) is less able to accommodate the increased bulk of threonine. This is not reflected in binding per se, if the $K_m$ for 2-acetolactate is any guide (Table 4).

Proust-De Martin *et al.* [17] have emphasized the importance of the two magnesium ions in the active site of KARI. It is therefore of interest that the $K_m$ for $Mg^{2+}$ exhibits the largest variation in response to mutation (Table 4). These values range from approximately sevenfold increases (K155R and E393D) to over 70-fold decreases (H132K and K155Q). These effects do not seem to be related to the position of the residue, as the two most extreme values both involve K155. Neither do they seem related to charge, because H132K and K155Q would be expected alter charge in opposite directions (assuming that H132 is neutral at

pH 8.0). However, a low $K_m$ for $Mg^{2+}$ is clearly not conducive to KARI activity, because in every case there is no detectable reductoisomerase and quite low (< 2% of wildtype) reductase activity.

KARI is a bifunctional enzyme catalyzing two quite different but sequential reactions at a single active site [1]. One of the main purposes of this study was to try to dissect the two reactions by mutagenesis of active site residues, expecting to find mutants of the *E. coli* enzyme in which one activity was abolished while the other was retained. In part this expectation was fulfilled in that we found mutants with little or no isomerase activity but high reductase activity. Strikingly, the reverse is not true and all mutations that eliminate the reductase also eliminate the isomerase activity. This suggests a linkage between the two reactions.

Earlier observations had also implied a linkage. Arfin & Umbarger [4] showed that when the enzyme acts on 2-acetolactate, the 2-ketoacid intermediate is not released and does not exchange with this intermediate if it is added externally. However, the enzyme is perfectly capable of using the intermediate in either the reverse isomerase or reductase reaction. Reasons for these apparently contradictory results have not been established previously. Indeed, the reasons why the enzyme is bifunctional have not been properly addressed in previous studies. Why could there not be two separate enzymes?

The answer to this question appears to lie in the isomerase equilibrium constant that we have measured, which favours 2-acetolactate by a considerable margin. A separate isomerase would form too little of the reductase substrate to constitute an efficient system. Combining the two reactions at a single active site overcomes this difficulty and implies that the 'intermediate' does not actually exist. We suggest that reduction occurs at the level of an isomerase transition state rather than after formation of the 2-ketoacid (Fig. 5). A similar proposal was made by Arfin & Umbarger [4].

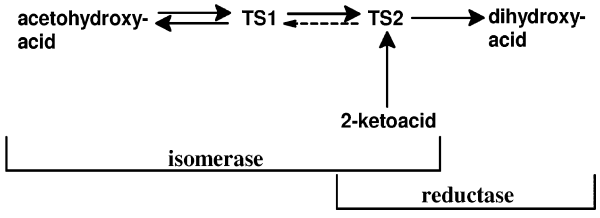


**Fig. 5.** Proposed model for the reactions catalyzed by KARI. The acetohydroxyacid substrate is converted via a first transition state (TS1) to a second (TS2) that is reduced to the dihydroxyacid product. Externally supplied 2-ketoacids can be converted to TS2 and then participate in the reductase reaction. The reverse of the isomerase reaction is thermodynamically favoured by the near irreversibility of the conversion of the 2-ketoacid to TS2. However the reaction is inefficient due to slow conversion of TS2 to TS1.

The kinetic mechanism for the reductoisomerase activity involves random binding of $Mg^{2+}$ and NADPH, followed by addition of 2-acetolactate [13,14]. The requirement for $Mg^{2+}$ binding to precede that of 2-acetolactate is expected because the metal ion acts as a bridging ligand between the protein and the substrate [9]. Previously, the reason that NADPH is required to bind prior to 2-acetolactate was not clear. Our proposal that an isomerase transition state moves directly into the reductase reaction provides a rational explanation for this NADPH requirement.

The reductase specific activity of wildtype *E. coli* KARI with HMKB is 57% higher than that of the overall reductoisomerase activity with 2-acetolactate. Thus, a 2-ketoacid has no difficulty in accessing the transition state. However, the reverse isomerase activity is quite low, only 5% of the reductoisomerase activity. While this might be due to the limitations of the assay, where we must substitute $NADP^+$ as a surrogate for NADPH, an alternative explanation is that there are two isomerase transition states (Fig. 5). The second is readily accessible from a 2-ketoacid and provides the starting point for the reductase. The first, which must be formed from the second for the reverse isomerase reaction to proceed, is less accessible from a 2-ketoacid, accounting for the low activity.

In summary, we suggest that describing KARI as catalysing a two-stage reaction is somewhat misleading. Substrate isomerization and reduction are coordinated processes that are conceptually inseparable. While the enzyme can display a separate reductase activity, this should be regarded as a laboratory artefact with little or no biological significance.

## Experimental procedures

### Materials

HMKB and racemic 3-hydroxy-2-ketobutyrate were prepared by alkaline hydrolysis of the corresponding esters, which were obtained as follows. Ethyl HMKB was synthesized [18] from ethyl 3-methyl-2-ketobutyrate as described by Chunduru *et al.* [13]. Racemic ethyl 3-hydroxy-2-ketobutyrate was prepared using a similar procedure [18], starting with ethyl 2-ketobutyrate that was synthesized from ethyl bromide and diethyl oxalate as described by Weinstock *et al.* [19]. 2-Ketopantoate and racemic 2-acetolactate were prepared by alkaline hydrolysis of dihydro-4,4-dimethyl-2,3-furandione and methyl 2-hydroxy-2-methyl-3-ketobutyrate, respectively, both of which were purchased from

Sigma-Aldrich (Castle Hill, Australia). Other reagents were obtained from common commercial suppliers.

### Enzyme expression, purification and mutagenesis

Wildtype hexahistidine-tagged recombinant *E. coli* KARI was expressed using the plasmid pET-C, which contains the *ilv*C gene that encodes KARI, cloned into the pET30a(+) plasmid [7]. Expression is under the control of the T7 promoter and therefore requires a host cell containing the T7 RNA polymerase gene. We used the *E. coli* strain BL21 (DE3) for this purpose. The expressed enzyme has an N-terminal hexahistidine tag and was purified by immobilized metal affinity chromatography as described previously [7]. The purified enzyme has a specific activity of $\approx 2$ U·mg$^{-1}$ when 2 mM 2-acetolactate is used as the substrate. It was stored at −70 °C in 20 mM Hepes/KOH buffer, pH 7.5.

Mutations were introduced by PCR using the megaprimer method [20] or a modification of this procedure [21]. For certain mutants, the KARI gene was cloned into the plasmid pProEXHT (Gibco BRL, Invitrogen, Mount Waverley, Australia) and expressed in the KARI-deficient *E. coli* strain, CU505. The purification procedure was identical to that for the wildtype enzyme.

### Activity assays

Reductoisomerase and reductase activity measurements were conducted at 37 °C in 0.1 M Tris/HCl buffer (pH 8.0) containing 0.22 mM NADPH, 10 mM $MgCl_2$ and various concentrations of 2-acetolactate or 2-ketoacid substrates. The change in absorbance at 340 nm was followed in a Cary 50 spectrophotometer. Measurements of the reverse isomerase activity were conducted at 37 °C in 0.1 M potassium phosphate buffer (pH 7.3) containing 4 mM $NADP^+$, 5 mM $MgCl_2$ and 5 mM HMKB. After 30 min, the reaction was stopped by addition of 0.5% (v/v) $H_2SO_4$ and 2-acetolactate was estimated using creatine and α-naphthol [22].

Substrate and cofactor saturation curves were determined by measuring the steady-state rate over a range of concentrations of each varied component. Nonvaried components were held fixed at the concentrations stated above (2 mM for acetolactate). However, some mutants have elevated $K_m$ values for a substrate and/or cofactor and in these cases the concentration of the nonvaried components were increased so that they would be at least 90% saturating. For the varied component, a preliminary estimate of the half-saturating concentration was calculated using a few widely spaced concentrations. This estimate was then used to design a more precise experiment with 12–20 assays at a series of concentrations, generally spanning the range from 10 to 90% saturation. Data fitted the Michaelis–Menten equation, which was used to estimate, by nonlinear regression, values and standard errors for the Michaelis constant and the maximum velocity. The latter was converted to a specific activity or a $k_{cat}$ value from the known protein concentration and the subunit molecular mass of 59.5 kDa.

The equilibrium constant for the isomerase activity was measured by incubating the enzyme at 37 °C with 2 mM $NADP^+$, 10 mM $MgCl_2$ and 2.8 mM HMKB in 0.1 M Tris/HCl buffer (pH 8.0). At intervals, 100 μL samples were mixed with an equal volume of 2% (v/v) $H_2SO_4$ and heated at 60 °C for 15 min to destroy any 2-acetolactate formed by the isomerase reaction. These samples were then neutralized with 20 μL of 1 M Tris/HCl buffer (pH 8.0) and 20 μL of 4 M NaOH, and the residual HMKB was estimated using the reductase activity of KARI. In addition, similar experiments performed with 2.11 mM 2-acetolactate as the substrate allowed the estimation of HMKB formed by the isomerase.

### Binding studies

$Mg^{2+}$ binding was assessed by observing changes in the reactivity of cysteine residues with DTNB. Reaction mixtures were prepared containing 0.5 mg·mL$^{-1}$ DTNB and various concentrations of $MgCl_2$ in 0.1 M Na/Tes buffer (pH 7.5) and equilibrated at 37 °C. KARI was added to a final concentration of $\approx 1$ mg·mL$^{-1}$ and the absorbance at 412 nm was followed. The data were analyzed as described in Results. NADPH binding was measured as described by Dumas *et al.* [14].

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## References

1 Dumas R, Biou V, Halgand F, Douce R & Duggleby RG (2001) Enzymology, structure and dynamics of acetohydroxy acid isomeroreductase. *Acc Chem Res* **34**, 399–408.

2 Schulz A, Sponemann P, Kocher H & Wengenmayer F (1988) The herbicidally active experimental compound Hoe 704 is a potent inhibitor of the enzyme 2-acetolactate reductoisomerase. *FEBS Lett* **238**, 375–378.

3 Aulabaugh A & Schloss JV (1990) Oxalyl hydroxamates as reaction-intermediate analogues for ketol-acid reductoisomerase. *Biochemistry* **29**, 2824–2830.

4 Arfin SM & Umbarger HE (1969) Purification and properties of the acetohydroxy acid isomeroreductase of *Salmonella typhimurium*. *J Biol Chem* **244**, 1118–1127.

5 Primerano DA & Burns RO (1983) Role of acetohydroxy acid isomeroreductase in biosynthesis of pantothenic acid in *Salmonella typhimurium*. *J Bacteriol* **153**, 259–269.
6 Mrachko GT, Chunduru SK & Calvo KC (1992) The pH dependence of the kinetic parameters of ketol acid reductoisomerase indicates a proton shuttle mechanism for alkyl migration. *Arch Biochem Biophys* **294**, 446–453.
7 Hill CM & Duggleby RG (1999) Purified recombinant *Escherichia coli* ketol-acid reductoisomerase is unsuitable for use in a coupled assay of acetohydroxyacid synthase activity due to an unexpected side reaction. *Protein Expr Purif* **15**, 57–61.
8 Biou V, Dumas R, Cohen-Addad C, Douce R, Job D & Pebay-Peyroula E (1997) The crystal structure of plant acetohydroxy acid isomeroreductase complexed with NADPH, two magnesium ions and a herbicidal transition state analog determined at 1.65 Å resolution. *EMBO J* **16**, 3405–3415.
9 Thomazeau K, Dumas R, Halgand F, Forest E, Douce R & Biou V (2000) Structure of spinach acetohydroxyacid isomeroreductase complexed with its reaction product dihydroxymethylvalerate, manganese and (phospho)-ADP-ribose. *Acta Crystallogr D* **56**, 389–397.
10 Dumas R, Butikofer MC, Job D & Douce R (1995) Evidence for two catalytically different magnesium-binding sites in acetohydroxy acid isomeroreductase by site-directed mutagenesis. *Biochemistry* **34**, 6026–6036.
11 Ahn HJ, Eom SJ, Yoon H-J, Lee BI, Cho H & Suh SW (2003) Crystal structure of class I acetohydroxy acid isomeroreductase from *Pseudomonas aeruginosa*. *J Mol Biol* **328**, 505–515.
12 McCourt JA, Tyagi R, Guddat LW, Biou V & Duggleby RG (2004) Facile crystallization of *Escherichia coli* ketol-acid reductoisomerase. *Acta Crystallogr D* **60**, 1432–1434.
13 Chunduru SK, Mrachko GT & Calvo KC (1989) Mechanism of ketol acid reductoisomerase steady-state analysis and metal ion requirement. *Biochemistry* **28**, 486–493.
14 Dumas R, Job D, Ortholand JY, Emeric G, Greiner A & Douce R (1992) Isolation and kinetic properties of acetohydroxy acid isomeroreductase from spinach (*Spinacia oleracea*) chloroplasts overexpressed in *Escherichia coli*. *Biochem J* **288**, 865–874.
15 Allaudeen HS & Ramakrishnan T (1970) Biosynthesis of isoleucine and valine in Mycobacterium tuberculosis $H_{37}R_v$. II. Purification and properties of acetohydroxy acid isomerase. *Arch Biochem Biophys* **140**, 245–256.
16 Halgand F, Dumas R, Biou V, Andrieu JP, Thomazeau K, Gagnon J, Douce R & Forest E (1999) Characterization of the conformational changes of acetohydroxy acid isomeroreductase induced by the binding of $Mg^{2+}$ ions, NADPH, and a competitive inhibitor. *Biochemistry* **38**, 6025–6034.
17 Proust-De Martin F, Dumas R & Field MJ (2000) A hybrid-potential free-energy study of the isomerization step of the acetohydroxy acid isomeroreductase reaction. *J Am Chem Soc* **122**, 7688–7697.
18 Wang B-L, Duggleby RG, Li Z-M, Wang J-G, Li Y-H, Wang S-H & Song H-B (2004) Synthesis, crystal structure and herbicidal activity of intermediate mimics of the KARI reaction. *Pest Manag Sci* doi: 10.1002/ps.972
19 Weinstock LM, Currie RB & Lovell AV (1981) A general, one-step synthesis of alpha-keto esters. *Synthetic Comm* **11**, 943–946.
20 Brøns-Poulsen J, Petersen NE, Hørder M & Kristiansen K (1998) An improved PCR-based method for site directed mutagenesis using megaprimers. *Mol Cell Probes* **6**, 345–348.
21 Tyagi R, Lai R & Duggleby RG (2004) A new approach to 'megaprimer' polymerase chain reaction mutagenesis without an intermediate gel purification step. *BMC Biotechnol* **4**, 2.
22 Singh BK, Stidham MA & Shaner DL (1988) Assay of acetohydroxyacid synthase. *Anal Biochem* **171**, 173–179.