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Substrate-Induced Conformational Change and Isomerase Activity of Dienelactone Hydrolase and its Site-Specific Mutants

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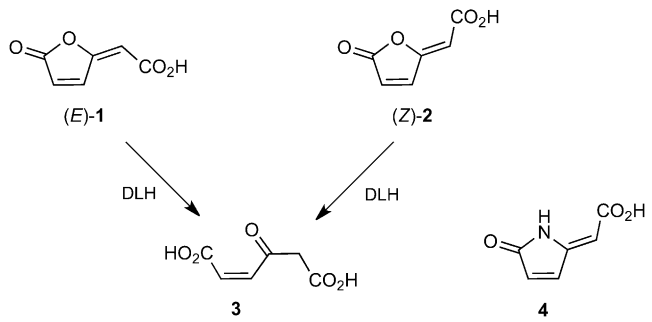
Studies of the interactions of dienelactone hydrolase (DLH) and its mutants with both *E* and *Z* dienelactone substrates show that the enzyme exhibits two different conformational responses specific for hydrolysis of each of its substrate isomers. DLH facilitates hydrolysis of the *Z* dienelactone through an unusual charge-relay system that is initiated by interaction between the substrate carboxylate and an enzyme arginine residue that activates an otherwise non-nucleophilic cysteine. The *E* dienelactone does not display this substrate–arginine binding interaction, but instead induces an alternate conformational response that promotes hydrolysis. Furthermore, the substitution of cysteine 123 for serine (C123S) in DLH, instead of inactivating the enzyme as is typical for this active-site mutation, changes the catalysis from substrate hydrolysis to isomerisation. This is due to the deacylation of the acyl–enzyme intermediates being much slower, thereby increasing their lifetimes and allowing for their interconversion through isomerisation, followed by relactonisation.

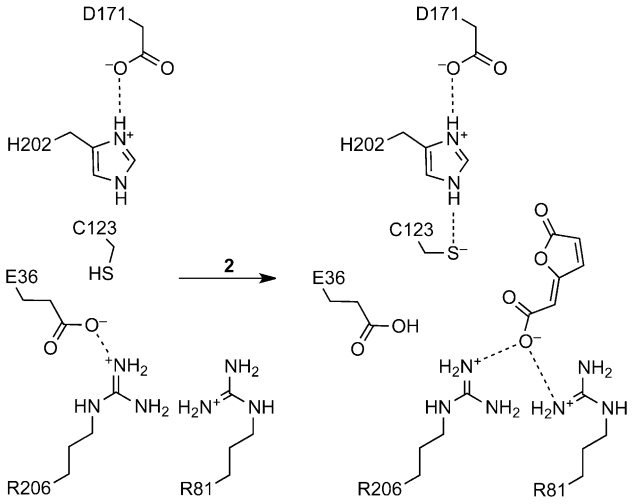
Introduction

Dienelactone hydrolase (E.C. 3.1.1.45) from *Pseudomonas* sp. B13 (DLH type III) is a monomeric enzyme found on the chlorocatechol branch of the β-ketoadipate pathway,[1] serving an important role in the biodegradation of toxic aromatic compounds.[2] It catalyses the hydrolysis of dienelactones **1** and **2** to yield maleylacetate **3** (Scheme 1).[3]



Scheme 1. Hydrolysis of *E* and *Z* dienelactones **1** and **2** catalysed by DLH, and the substrate analogue, *Z* dienelactam **4**.

Crystal-structure analysis has confirmed the presence of a DLH catalytic triad consisting of an aspartate (D171), a histidine (H202) and a nucleophilic cysteine (C123).[4,5] A mechanism of substrate hydrolysis was therefore proposed resembling that of the cysteine proteases with a resting-state ion pair between the C123 thiolate and the H202 imidazolium facilitating nucleophilic attack on dienelactone substrates **1** and **2**.[5,6] However, further crystal-structure analyses and inhibitor binding studies using *Z* dienelactam substrate analogue **4** later indicated that, in fact, C123 is present as the inactive thiol in the resting state and is only converted to the nucleophilic thiolate due to a substrate-induced activation mechanism occurring within the DLH active site (Scheme 2).[7] Accordingly, it was proposed that in the resting state, the C123 neutral thiol is positioned pointing towards the interior of the enzyme away from the active site. In the active state, this thiol is activated to the thiolate and rotated around its Cα–Cβ bond to point into the active site, aligned with the H202 imidazolium and positioned to attack the acyl carbon of substrates **1** and **2**. In the absence of substrate, an ion pair between R206 and E36



Scheme 2. Mechanism of DLH activation by *Z* dienelactone **2**.

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prevents deprotonation of the C123 thiol by E36. Rotation of the C123 side chain around its Cα–Cβ bond in the absence of substrate is also hindered due to a close steric interaction (< 1.5 Å) between the side chain methylene of C123 and the backbone amide of L124. However, upon binding of the substrate, the ion pair between R206 and E36 is weakened, due to a new ion pair formed between R206 and the substrate carboxylate. This allows E36 to shift towards the C123 thiol and abstract its proton. Free rotation of this newly generated thiolate to align with H202 in the active conformation is also promoted by the L124 amide being displaced away from the C123 methylene by the substrate-ring carbonyl oxygen.

This mechanism was proposed based on crystal-structure analysis of both DLH and its C123S mutant.[7] DLH C123S was used as a model for inhibitor binding studies to overcome problems of C123 oxidation during X-ray crystallographic analysis of DLH.[5] Chemical evidence for this mechanism was observed in the unusual resistance of the DLH C123 side chain to chemical modification, corroborating its location in a poorly accessible region towards the interior of the enzyme, in the absence of substrate.[7] Additional theoretical analysis has also supported the occurrence of substrate-induced DLH activation.[8]

We now report a mutagenic analysis of the DLH active site that provides a clearer picture of this unusual catalytic mechanism. This has involved a detailed kinetic investigation of the catalytic activities of DLH and DLH C123S and their mutants with both *E* and *Z* dienelactones **1** and **2**. Whilst the comparative kinetic analyses of DLH and its site-specific mutants corroborate the proposed substrate-induced enzyme-activation hypothesis for hydrolysis of *Z* dienelactone **2**, we find that this mechanism does not apply with *E* dienelactone **1**, with R206 having no binding interaction with this substrate. It is instead apparent that, in order to hydrolyse *E* lactone **1**, DLH undergoes a substrate-induced conformational change different to that observed during hydrolysis of *Z* isomer **2**. Given the important role of R206 in the substrate-induced activation of DLH upon binding *Z* lactone **2**, it is surprising that DLH is at all active towards *E* isomer **1**, in the absence of a binding interaction with R206. Even more intriguing is the ability of DLH to exhibit different conformational responses specific for hydrolysis of each of its substrate isomers **1** and **2**.

The C123S mutation changes the catalytic triad of DLH to an aspartate (D171), a histidine (H202) and a serine (S123), which is also observed with some serine proteases.[9,10] Our preliminary studies of DLH C123S showed quite surprising activity. Whereas DLH catalyses only the hydrolysis of dienelactones **1** and **2**, DLH C123S promotes a completely different class of reaction and instead catalyses the isomerisation of these same substrates without hydrolysis.[11] A detailed analysis of the interactions of lactones **1** and **2** with site-specific mutants of both DLH and DLH C123S now allows us to also provide an explanation of this unusual behaviour.

Results

DLH and its mutants that retain cysteine at the active site hydrolyse *Z* dienelactone **2** to maleylacetate **3**. These interactions follow simple Michaelis–Menten kinetics, and the data show linear correlations on both Hanes and Lineweaver–Burk plots. The kinetic parameters for the interactions were determined using nonlinear-regression analysis, and the values are shown in Table 1. These proteins also hydrolyse *E* dienelactone **1** to maleylacetate **3**, but in some of these cases a pronounced deviation from Michaelis–Menten kinetics is apparent at low substrate concentrations. This is particularly marked with DLH and its R206A mutant, for which the corresponding data are illustrated in Figure 1. Nonlinear regression was used to derive the kinetic parameters for the hydrolysis of lactone **1**, which are shown in Table 2. These values were not significantly affected by either including or excluding the data obtained at low substrate concentrations.

Table 1. Kinetic parameters for interactions of DLH and a range of its active site mutants with *E* and *Z* dienelactones **1** and **2**.[a]

	Z dienelactone **2**		*E* dienelactone **1**	
	k_{cat} [min^{-1}]	K_m [μM]	k_{cat} [min^{-1}]	K_m [μM]
DLH	1120 ± 15	11 ± 1	870 ± 20	180 ± 10
DLH E36D	50 ± 1	18 ± 1	44 ± 1	260 ± 10
DLH R206A	37 ± 1	350 ± 30	6.0 ± 0.2	240 ± 20
DLH R81A	1070 ± 10	160 ± 5	650 ± 15	1550 ± 90
DLH R206K	96 ± 1	25 ± 1	41 ± 1	170 ± 10
DLH R81K	1250 ± 10	16 ± 1	940 ± 20	210 ± 10
DLH Y85F	670 ± 10	19 ± 1	440 ± 10	240 ± 10
DLH W88A	690 ± 20	340 ± 20	270 ± 5	1730 ± 80
DLH S203A	820 ± 10	1.9 ± 0.1	510 ± 10	390 ± 10
DLH S203H	450 ± 10	160 ± 10	26 ± 1	6870 ± 70
DLH S203D	12 ± 0.5	5070 ± 400	4.0 ± 0.1	6190 ± 280

[a] In HEPES buffer (20 mM, pH 7.0) containing 1 mM EDTA at 25 °C.

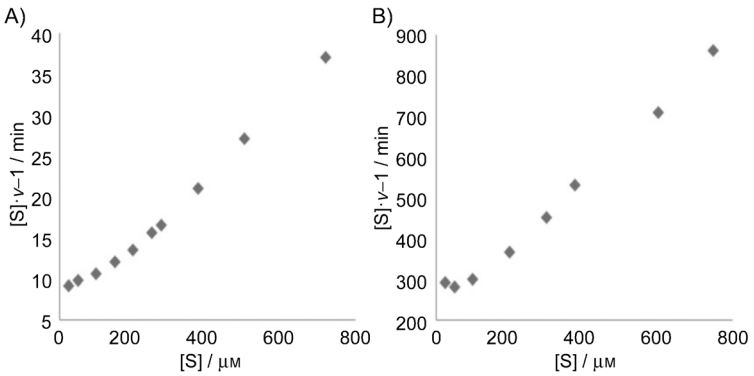


Figure 1. Hanes plots demonstrating the deviation from Michaelis–Menten kinetics for hydrolysis of *E* dienelactone **1** by A) DLH and B) DLH R206A, in HEPES buffer (20 mM, pH 7.0) containing 1 mM EDTA at 25 °C.

Interactions of DLH C123S and of the double mutants DLH C123S/R206A and DLH C123S/R81A with lactones **1** and **2** were also studied. In initial experiments, it was observed that the UV absorbance at 280 nm of a solution of *Z* lactone **2** and

DLH C123S increased with time, which is clearly inconsistent with hydrolysis of substrate **2** to maleylacetate **3**. Closer analysis using HPLC showed that instead *Z* lactone **2** was being converted to *E* isomer **1**. Consequently the ratios of lactones **1** and **2** and acetate **3** in solutions, beginning with either of the substrates (**1** or **2**) and either one of the C123S, C123S/R206A and C123S/R81A mutants of DLH, were determined using HPLC, as illustrated in Figure 2. For comparison, the corresponding data for DLH are also shown. ^{1}H NMR spectroscopy and thin layer chromatography were used to confirm the interconversion between lactones **1** and **2**, as well as their hydrolysis to maleylacetate **3**.

The interactions of *Z* lactone **2** with DLH C123S, C123S/R206A and C123S/R81A obey Michaelis–Menten kinetics, and the constants characterising this behaviour were determined using nonlinear-regression analysis and are shown in Table 2. The data for the interaction of *E* lactone **1** with DLH C123S substantially deviate from linearity when correlated using a Hanes plot (Figure 3), but the relevant kinetic parameters

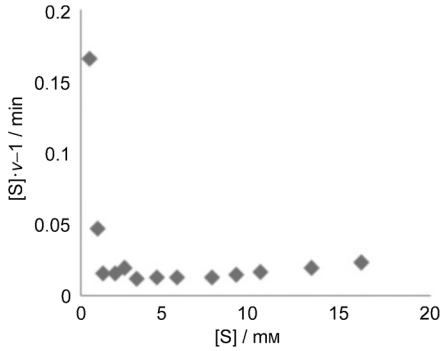


Figure 3. Hanes plot demonstrating the deviation from Michaelis–Menten kinetics for isomerisation of *E* dienelactone **1** by DLH C123S, in HEPES buffer (20 mM, pH 7.0) containing 1 mM EDTA at 25 °C.

Table 2. Kinetic parameters for the interactions of DLH C123S, DLH C123S/R206A and DLH C123S/R81A with *E* and *Z* dienelactones **1** and **2**.[a]

	k_{cat} (isomerisation) [min^{-1}]	k'_{cat} (hydrolysis) [min^{-1}]	K_m[b] [mM]	Ratio k_{cat}/k'_{cat}	v (isomerisation)[c] [×10^2 min^{-1}]	v' (hydrolysis)[c] [×10^2 min^{-1}]	Ratio v/v'
Z dienelactone **2**							
DLH C123S	11 ± 0.5	< 0.01	2.7 ± 0.1	> 1100	660 ± 1	< 0.1	> 6600
DLH C123S/R206A	1.4 ± 0.1	0.4 + 0.1	36 ± 4	3.5	5.0 ± 0.1	1.3 ± 0.05	4
DLH C123S/R81A	4.6 ± 0.3	0.35 + 0.03	8.6 ± 1.5	13	12 ± 0.5	0.8 ± 0.05	15
E dienelactone **1**							
DLH C123S	38 ± 1.0	< 0.01	4.4 ± 0.2	> 3800	620 ± 80	< 0.1	> 6200
DLH C123S/R206A					9.6 ± 0.1	0.60 ± 0.01	16
DLH C123S/R81A					41 ± 2	3.0 ± 0.1	14

[a] In HEPES buffer (20 mM, pH 7.0) containing 1 mM EDTA at 25 °C. [b] Isomerisation and hydrolysis occur with the same K_m, implying that both processes involve a common binding event. For lactone **1**, K_m was determined as $K^h_{0.5}$ by nonlinear regression to best fit the data to the Hill equation. [c] Determined by using an initial substrate concentration of 6.8 mM.

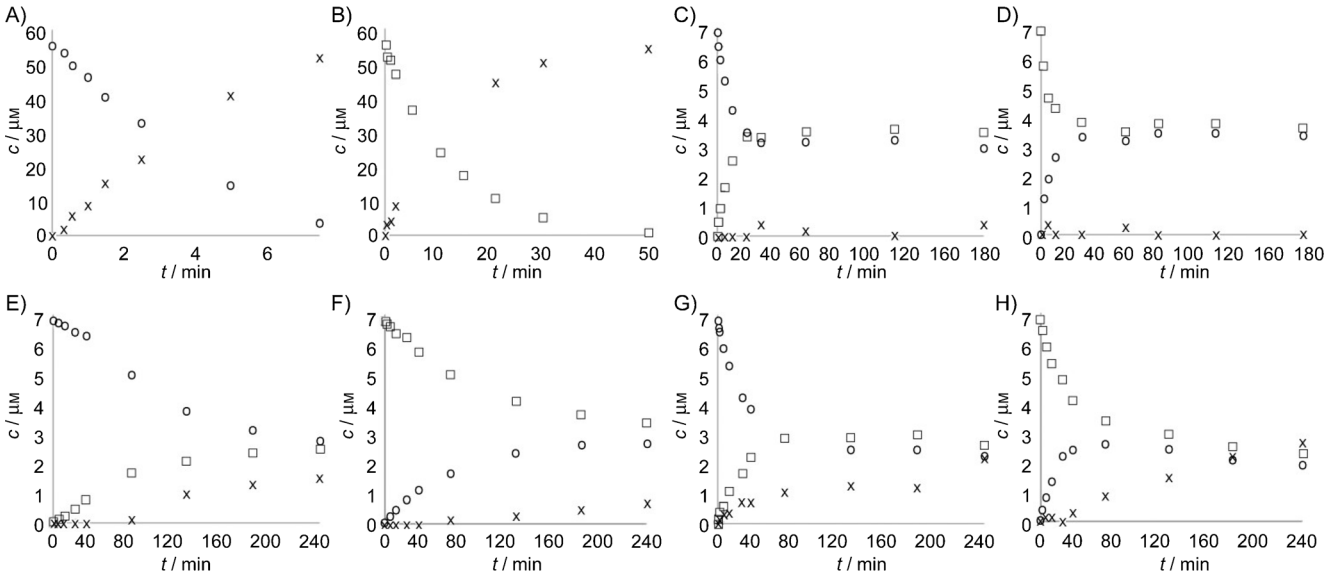


Figure 2. Concentrations[a] of *E* and *Z* dienelactones **1** (□), **2** (○) and maleylacetate **3** (×) in mixtures obtained by treatment[b] of A) lactone **2** with DLH (12 nM), B) lactone **1** with DLH (20 nM), C) lactone **2** with DLH C123S (10.7 μM), D) lactone **1** with DLH C123S (10.7 μM), E) lactone **2** with DLH C123S/R206A (80 μM), F) lactone **1** with DLH C123S/R206A (80 μM), G) lactone **2** with DLH C123S/R81A (59.7 μM), H) lactone **1** with DLH C123S/R81A (59.7 μM). [a] Determined by HPLC. [b] In HEPES buffer (20 mM, pH 7.0) containing 1 mM EDTA at 25 °C.

were obtained using the Hill equation (Table 2).[12] DLH C123S/R206A and C123S/R81A bind lactone **1** only weakly, and it was therefore impractical to prepare solutions containing sufficient concentrations of lactone **1** to be able to determine the catalytic rate constant (k_{cat}) and Michaelis–Menten constant (K_m) values for these interactions. Instead, the specific activities of these enzymes in isomerising and hydrolysing lactone **1** were determined using an initial substrate concentration of 6.8 mM. For comparison, the corresponding data were obtained for the interactions of DLH C123S with lactone **1**, and DLH C123S, C123S/R206A and C123S/R81A with lactone **2** (Table 2).

Discussion

The k_{cat} and K_m values in Table 2 are consistent with the mechanism proposed for the hydrolysis of *Z* dienelactone **2** by DLH (Scheme 2). Both R81 and R206 are shown to be important for substrate binding, since their replacement with alanine in DLH R81A and R206A increases the K_m value more than tenfold. The role of R206 and E36, but not R81, in catalysing reaction of the bound substrate is illustrated by the close similarity in the k_{cat} values for DLH and the R81A mutant, and the much lower values for DLH R206A and E36D. This is consistent with R206 and E36 being involved in the activation of C123 for nucleophilic attack on lactone **2**. By contrast, the kinetic parameters summarised in Table 1 are not in accord with the same mechanism operating for the hydrolysis of dienelactone (*E*)-**1** by DLH. Whilst the binding role of R81 observed for lactone **2** is also shown for lactone **1**, R206 does not retain substrate-binding properties. This is apparent, since the K_m values of DLH and DLH R206A are similar, while that of DLH R81A is substantially higher. R206 does however retain its catalytic role for reaction with lactone **1** once bound, which is shown by the k_{cat} value for DLH R206A being less than 1% of that of DLH. A large reduction in k_{cat} for DLH E36D confirms this glutamate is also important for reaction of lactone **1**.

These results indicate that the mechanism illustrated in Scheme 2 is not adequate to explain the hydrolysis of *E* lactone **1**. For reaction of *Z* isomer **2**, its binding to R206 is coupled to the role of R206 and E36 in deprotonating C123, and the realignment of the resultant thiolate with H202 and D171 of the catalytic triad for hydrolysis of the bound substrate. However, this is not the case for *E* isomer **1**, where binding seems not to involve R206. Nevertheless, for hydrolysis of this substrate, there must be a rearrangement of the active-site residues from their orientations observed in the crystal structure of free DLH to those required for catalysis. The deviations from Michaelis–Menten kinetics observed for interactions of *E* lactone **1** with DLH and its mutants (Figure 1) indicate that this occurs through the enzyme undergoing a conformational transition on substrate binding. These deviations only occur at low substrate concentrations, where the enzyme reverts to its inactive form between binding events. It seems likely that this substrate-induced conformational transition disrupts the ion pair between R206 and E36, thus allowing C123 activation without formation of an R206–substrate carboxylate ion pair, since the k_{cat} values of DLH, DLH E36D and DLH R206A show an important contribution of E36 and R206 to the hydrolysis of lactone **1**. In any case, it is clear that events facilitating hydrolysis of *E* dienelactone **1** by DLH are different from those for *Z* isomer **2**.

The roles of R81 and R206 of DLH to bind *Z* dienelactone **2** and of R81 to bind *E* dienelactone **1**, appear to be maintained by lysine incorporated in DLH R81K and DLH R206K. The K_m values for the three proteins interacting with lactone **2** are quite similar, as are those for DLH and DLH R81K with lactone **1**. However, the lysine mutant DLH R206K does not retain the catalytic activity, as demonstrated by its much lower k_{cat} values than those for DLH for processing both substrates. Previous crystallographic analysis of DLH and DLH C123S with the bound inhibitor, *Z* dienelactam **4**, indicated that Y85 is positioned with its hydroxyl group in the active site, 3.74 Å from the exocyclic methine of the inhibitor.[13] In this position, Y85 might therefore stabilise enolate formation during ring opening of lactones **1** and **2** by proton donation. However, substituting this tyrosine for phenylalanine and thereby removing the hydroxyl group had little effect on either the k_{cat} or K_m values of DLH Y85F compared to those of DLH with either substrate. Therefore, should this tyrosine be involved in the reaction as an acid catalyst, it is not during a rate-determining step. A substrate-binding role for W88 is also apparent, as a decrease in binding affinities is observed when that tryptophan is replaced with alanine in DLH W88A. W88 is the only residue found in close proximity to the substrate ring hydrogens of *Z* dienelactam **4** bound in the DLH active site. It therefore appears that W88 is able to stabilise the Michaelis complex through the formation of π–π interactions with substrates **1** and **2**. Previously it has been proposed that the S203 hydroxyl group is positioned to bind to the carboxylate of *Z* dienelactone **2**.[7] Under these circumstances, substituting the serine for alanine in DLH S203A would have been expected to lead to a decrease in catalytic efficiency. However, in terms of k_{cat}/K_m, the mutation has produced an enzyme that is four times more efficient in processing lactone **2**. It appears that rather than providing a substrate-binding role, S203 instead weakens substrate binding through interaction with the side chain of R206. Mutation of S203 to alanine therefore releases the R206 guanidinium to enhance binding of lactone **2**, as seen in the tenfold reduction in K_m for DLH S203A with this substrate (Table 1). This binding effect is not observed during hydrolysis of *E* dienelactone **1**, since this substrate does not interact with R206.

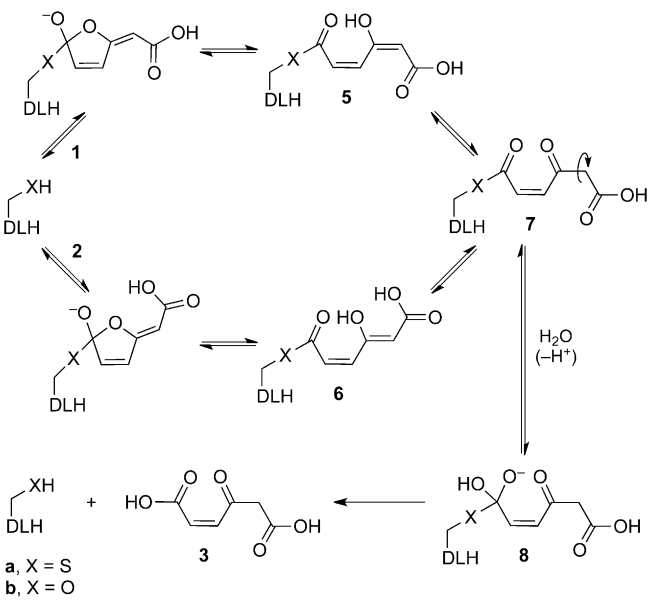
The only reaction of lactones **1** and **2** observed with DLH and its mutants that retain C123 is hydrolysis to maleylacetate **3**. There is no evidence of interconversion between substrates **1** and **2** in the presence of these enzymes (Figure 2 A and B for DLH). In complete contrast, DLH C123S catalyses the interconversion of lactones **1** and **2** to the equilibrium position, where they are present in a 53:47 ratio, and negligible hydrolysis of either substrate is discernible, even long after equilibration of substrates **1** and **2** is complete (Figure 2 C and D). On this basis, the k_{cat} values for hydrolysis of lactones **1** and **2** by DLH C123S are at least 1000 times less than those for isomerisation. DLH C123S/R206A and C123S/R81A also catalyse the interconversion of dienelactones **1** and **2** (Figure 2 E–H). Again this is the dominant reaction in each case, but hydrolysis to maleyla-

cetate **3** is also observed as a competing process. The ratios of the k_{cat} values for isomerisation and hydrolysis are 3.5 and 13 for the interaction of lactone **2** with DLH C123S/R206A and C123S/R81A, respectively. The corresponding ratios of the isomerisation and hydrolysis rates for reactions of a 6.8 mM solution of *E* lactone **1** with DLH C123S/R206A and C123S/R81A are 16 and 14, respectively. Compared to DLH C123S, DLH C123S/R206A exhibits a 13-fold increase in K_m and an eightfold decrease in k_{cat} for isomerisation of lactone **2**, whilst DLH C123S/R81A exhibits a threefold increase in K_m and a twofold decrease in k_{cat} for isomerisation of this same substrate. Though more modest, these effects of the DLH C123S mutation on the K_m values are in line with those observed with identical mutations of DLH (Table 1), in that they indicate the roles of R206 and R81 in facilitating binding of lactone **2** are shared by both DLH and DLH C123S. As observed during the reaction of lactone **1** catalysed by DLH, the deviations from Michaelis–Menten kinetics (Figure 3) indicate that DLH C123S-catalysed isomerisation of *E* dienelactone **1** is dependent on a substrate-induced conformational change occurring in the enzyme active site, and this most likely corresponds to a rearrangement of the active site residues from their orientations in the most stable, catalytically inactive state to those required for catalysis.

Any isomerisation of lactones **1** and **2** catalysed by DLH and its mutants that retain C123 would have been detectable, particularly in the reactions of *Z* lactone **2**. With most of these enzymes, *E* lactone **1** is hydrolysed much less efficiently (k_{cat}/K_m) than *Z* isomer **2**. Consequently, if lactone **1** formed through simultaneously occurring isomerisation and hydrolysis of *Z* isomer **2**, it would accumulate as a proportion of lactones **1** and **2** remaining, at least up to the equilibrium value of 53%. None was observed, even when mixtures were analysed after more than 90% of lactone **2** had hydrolysed, and the limits of detection of lactone **2** as a fraction of total residual substrate concentration were then less than 1%. This indicates that, should DLH and its mutants that retain C123 catalyse substrate isomerisation, the rate of this process is negligible in comparison to that of substrate hydrolysis. This is completely different to DLH C123S, with this enzyme instead catalysing substrate isomerisation with no evidence of substrate hydrolysis.

There have been other reports of the conversion of an enzyme's active-site cysteine to serine.[14–19] The resultant proteins showed greatly reduced catalytic activity or were inactive. With UDP-glucose dehydrogenase,[17] it was found that replacing the active-site C260 with serine gave a modified protein, that formed an ester instead of a thioester with the substrate, but the ester was not labile and, therefore, the mutant protein showed no substrate turnover. Formation of an unreactive covalent adduct was also indicated as a result of replacing the active-site C55 with serine in nitrile oxidoreducatase QueF.[18] In these cases, the wild-type enzymes and their serine mutants both form covalent enzyme–substrate intermediates, with adduct breakdown being slowed or stopped with the mutants. Nevertheless, there is a similarity in mechanism, whereas the change of reaction pathway from hydrolysis to isomerisation, observed with DLH and DLH C123S, is unprecedented.

It is conceivable that DLH C123S, DLH C123S/R206A and DLH C123S/R81A catalyse the isomerisation by a reversible Michael addition of the S123 nucleophile onto the exocylic acrylate moiety of lactones **1** and **2**. However, this seems unlikely, because a similar process involving the thiolate of DLH and its mutants that retain C123 would also be expected. Further, analysis of the crystal structure of *Z* dienelactam **4**-bound DLH C123S indicates that the distance between the serine and the acrylate is too great for nucleophilic attack (4.2 Å). Alternatively, it seems more reasonable that, whereas the reactions catalysed by DLH and its mutants that retain C123 are understood to proceed via thioesters **5a**–**7a**, DLH C123S, DLH C123S/R206A and DLH C123S/R81A are instead acylated by lactones **1** and **2** to give the corresponding esters **5b**–**7b** (Scheme 3). Hydrolysis of thioesters **5a**–**7a** then proceeds through formation of the tetrahedral intermediate **8a**, which collapses with preferential loss of the best leaving group, the enzyme thiol or thiolate, to give maleylacetate **3**. By contrast, the tetrahedral intermediate **8b** for hydrolysis of the esters **5b**–**7b** preferentially expels water or hydroxide, thus reforming the esters **5b**–**7b** and making them more resistant to hydrolysis, as observed with the acylated serine mutants of UDP-glucose dehydrogenase.[17] This increases the lifetime of esters **5b**–**7b**, allowing more time for their interconversion, leading to isomerisation, before recyclisation.



Scheme 3. Proposed mechanisms for the reactions of lactones **1** and **2** catalysed by DLH and its mutants.

Consistent with this rationale, the rates of hydrolysis of lactones **1** and **2** by DLH C123S, DLH C123S/R206A and DLH C123S/R81A are at least five orders of magnitude lower than those with DLH. The totality of the data shown in Tables 1 and 2 also strongly supports this proposed mechanism. The K_m values demonstrate that R206 and R81 are important in bind-

ing lactones **1** and **2**, presumably through interactions with the substrate carboxylate groups. It is likely that analogous interactions constrain the conformation of esters **5b**–**7b** to maintain the keto–enol moiety near the ester-group carbon, thereby facilitating the recyclisation with DLH C123S. With the double mutants, DLH C123S/R206A and DLH C123S/R81A, there are less of these interactions, resulting in a less constrained conformation. As a consequence, cyclisation is less favoured and the isomerisation rates are lower with the R206A and R81A mutants, while the ester-group carbon is more exposed to attack by water, and the hydrolysis rates are higher. This explains why isomerisation of lactones **1** and **2** is the only process observed with DLH C123S, while hydrolysis and isomerisation are competing processes catalysed by DLH C123S/R206A and C123S/R81A.

Conclusions

In summary, our studies of the interactions of lactones **1** and **2** with DLH and a range of its site-specific mutants have resulted in the identification of several unusual features. While our data are consistent with the mechanism proposed for catalysis of the hydrolysis of *Z* lactone **2** by DLH, they show that hydrolysis of *E* isomer, **1**, requires a different active-site conformational change in the enzyme to align the catalytic residues in their active orientation. A conformational change is also apparent in the kinetics of the interaction of DLH C123S with lactone **1**. In addition, our kinetic analyses with mutants of DLH and DLH C123S now allow us to explain why the cysteine-to-serine substitution in DLH C123S changes the catalysis displayed by the proteins from hydrolysis of lactones **1** and **2** to interconversion of these same species.

Experimental Section

The synthesis of lactones **1** and **2** has been reported previously.[20] Each was obtained in analytically pure form. An authentic sample of maleylacetate **3** was prepared by treatment of lactone **1** with aqueous base. DLH and DLH C123S were obtained using reported methods.[4] Other mutants of DLH were prepared from either DLH or DLH C123S, through either PCR-based mutagenesis involving amplification of the reverse complement oligonucleotide strand (5′–3′) incorporating the desired mutated codon (DLH E36D, R81K and R206A), or by using the Quick-Change site-directed mutagenesis kit (Stratagene). The oligonucleotides consisting of the 5′–3′ primer with the replaced codon underlined are shown in Table 3, and these were used with the exact complement in the Quick-Change protocol. The oligonucleotides and primers were obtained from Bresatec Pty. Ltd. (Adelaide–Thebarton, Australia) or GeneWorks (Hindmarsh, Australia). The plasmid pND704[21] and the *E. coli* strain AN1459 for these experiments were provided by Prof. Nick Dixon of the School of Chemistry, University of Wollongong. The gene encoding DLH (clcD) was cloned into the pND704 plasmid, comprising a heat-inducible overexpression system, and transformed into the *E. coli* strain AN1459. To confirm the constructs, the full coding region of each mutant plasmid was sequenced using the Big Dye Terminator reaction at the Biomolecular Resource Facility of the John Curtin School of Medical Research, Australian National University. The mutant proteins were purified and characterised using procedures similar to those employed with DLH.[4]

Table 3. Oligonucleotides used for site-directed mutagenesis of DLH and DLH C123S. The replaced codon is underlined.

Mutation	Oligonucleotide
E36D	CATGAACGCGTTCACACCAAATATTTGTTGAGCGATCAC
R206A	CAACGCCGCGGCACTCGCCACATAGCCCGAACTGCTCGTCGC GGCGAACGAG
R81A	CAGGATGAGGCGCAGGCAGAGCAAGCCTAC
R206K	GAGGCCGGACACTCGTTCGCCAAGACGAGCAGTTC
R81K	GTCGAAGGCCTGCCAGAGCTTGTAGGCTTGCTCTTTCTGCGCTC
Y85F	AGAGAGCAAGCCTTCAAGCTCTGGCAG
W88A	GCCTACAAGCTCGCGCAGGCCTTCGAC
S203A	GAGGCCGGACACGCGTTCGCCAGGACG
S203H	GAGGCCGGACACCATTTCGCCAGGACG
S203D	GAGGCCGGACACGATTTCGCCAGGACG

The interactions of the proteins with substrates **1** and **2** were examined in HEPES buffer (20 mM, pH 7.0) containing EDTA (1 mM) and BSA (25 $\mu g\,mL^{-1}$) at 25 °C. The products were characterised using HPLC and a diode array detector, thin layer chromatography and ^1H NMR spectroscopy, by comparison with authentic samples. The kinetics of the reactions were studied by monitoring changes in absorbance at 280 nm by UV spectroscopy, taking into account the formation of different products as determined by HPLC. Lactones **1** and **2** have λ_{max} values of 276 and 277 nm ($\varepsilon = 17\,200$ and $17\,550\,M^{-1}cm^{-1}$ respectively), while maleylacetate **3** shows negligible absorbance in this region.[1,22]

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