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Site-directed mutagenesis of dienelactone hydrolase produces dienelactone isomerase

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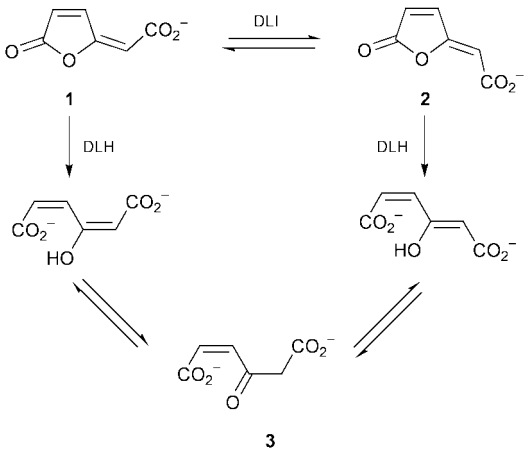
Replacing the active site Cys-123 of dienelactone hydrolase with Ser completely changes the catalysis displayed by the protein, from hydrolysis of the substrate *E*- and *Z*-dienelactones to maleylacetate by the native enzyme, to interconversion of the substrates by the mutant, dienelactone isomerase.

Dienelactone hydrolase (EC 3.1.1.45) from *Pseudomonas* sp. B13 (DLH Type III) catalyses the hydrolysis of the *E*- and *Z*-dienelactones **1** and **2** to give maleylacetate **3** (Scheme 1),[1] as part of the chlorocatechol branch of the β-ketoadipate pathway for the biodegradation of toxic aromatic compounds.[2] The activity of DLH derives from a catalytic triad of amino acids at its active site, comprising Cys-123 aligned for nucleophilic attack on the substrates by the adjacent His-202 and Asp-171 residues.[3,4] This triad is a hybrid of those found in cysteine (Cys-His-Asn)[5] and serine (Ser-His-Asp)[6] proteases, and has been observed in human and yeast ubiquitin *C*-terminal hydrolase[7] and engineered in mutant serine proteases such as thiol-subtilisin[8] and thiol-trypsin.[9] The mechanism of catalysis by DLH has been elucidated primarily through crystallographic analysis of both the native protein and several of its site specific mutants.[10] In one mutant (C123S), Cys-123 was replaced with Ser, to produce the catalytic triad found in serine proteases. We now report that this mutation completely changes the catalysis displayed by the protein, from hydrolysis of the substrates **1** and **2** by DLH to catalysis of their isomerisation by the mutant protein, dienelactone isomerase (DLI).



Scheme 1

The synthesis of the lactones **1** and **2**[11] and the procedures used to obtain the native and mutant proteins, DLH and DLI,[3,4,10] have been reported previously. The reactions of the proteins with the substrates **1** and **2** were examined in 0.02 mol dm^{-3} HEPES buffer containing 0.001 mol dm^{-3} EDTA, at pH 7.0 and 298 K. The products were characterised using HPLC and a diode array detector, thin layer chromatography and ^{1}H NMR spectrometry, by comparison with authentic samples. The kinetics of the reactions were studied by HPLC and by monitoring changes in absorbance at 280 nm by UV spectroscopy. The lactones **1** and **2** have λ_{max} values of 276 (ε 17200) and 277 (ε 17550 dm^3 mol^{-1} cm^{-1}) nm, and ε values of 17000 and 15625 dm^3 mol^{-1} cm^{-1} at 280 nm, respectively, while maleylacetate **3** shows negligible absorbance in this region.[1,12]

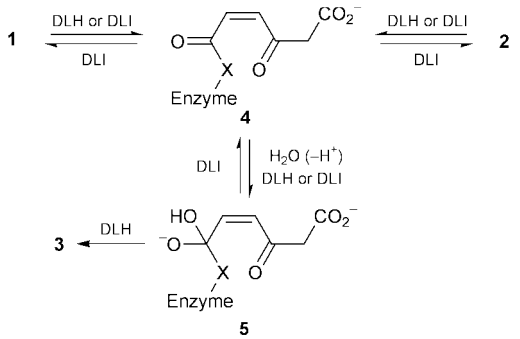
The effects of DLH and DLI on the dienelactones **1** and **2** are illustrated in Fig. 1 and the K_m and k_{cat} values characterising these interactions are summarised in Table 1. Hydrolysis of each of the lactones **1** and **2** is catalysed efficiently by DLH and there is no evidence of isomerisation of the substrates in the presence of this enzyme. In complete contrast, DLI catalyses the interconversion of the lactones **1** and **2**, to the equilibrium mixture where they are present in the ratio 53 : 47, and little hydrolysis of either substrate **1** or **2** is discernible, even long after equilibration of the substrates **1** and **2** is complete. On this basis, the k_{cat} values for hydrolysis of the lactones **1** and **2** by DLI are at least 100-fold less than those for substrate isomerisation.

Table 1 Kinetic parameters for interaction of DLH and DLI with the lactones **1** and **2**[a]

Enzyme	Substrate	k_{cat}/s^{-1}	10^3 K_m/mol dm^{-3}
DLH	**1**	14 ± 1 (9.2)[b]	0.20 ± 0.015 (0.17 ± 0.007)[b]
DLH	**2**	19 ± 0.2 (30)[c]	0.011 ± 0.001 (0.016)[c]
DLI	**1**	0.63 ± 0.03	4.4 ± 0.10
DLI	**2**	0.18 ± 0.003	2.7 ± 0.13

[a] In 0.02 mol dm^{-3} HEPES buffer containing 0.001 mol dm^{-3} EDTA, at pH 7.0 and 298 K. [b] Data from ref. 4. [c] Data from refs. 1 and 13.

The contrasting behaviour of DLH and DLI is not due simply to substrate isomerisation and hydrolysis by DLH and substrate isomerisation without hydrolysis by DLI. Hydrolysis of the *Z*-lactone **2** by DLH is catalysed *ca.* 25 times more efficiently (k_{cat}/K_m) than that of the *E*-isomer **1** under the conditions of this study. Isomerisation accompanying hydrolysis of the *E*-lactone **1** by DLH would therefore be expected to result in a build-up in the concentration of the *Z*-isomer **2**, until its proportion of the residual lactones **1** and **2** approached at least 4%. Isomerisation



Scheme 2 (a) DLH, X = S; (b) DLI, X = O.

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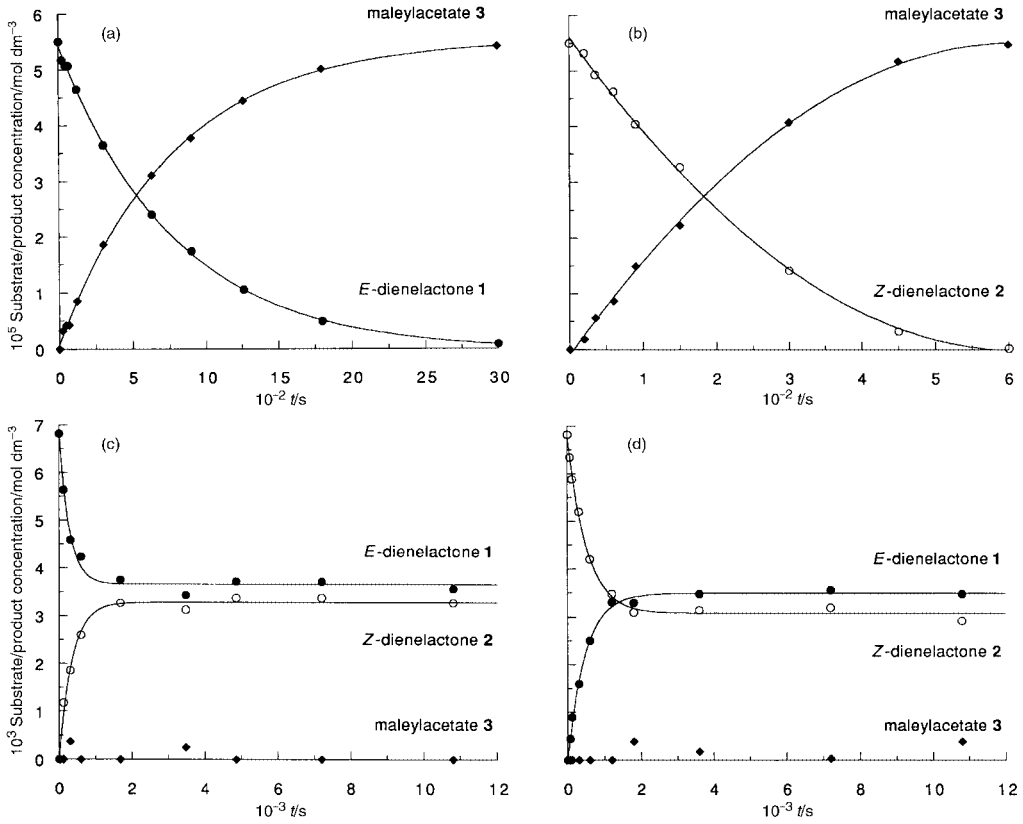

Fig. 1 Concentrations[a] of the *E*-dienelactone **1** (●), the *Z*-isomer **2** (○) and maleylacetate **3** (◆) in mixtures obtained by treatment[b] of (a) the lactone **1** with DLH (2×10^{-8} mol dm^{-3}), (b) the lactone **2** with DLH (1.2×10^{-8} mol dm^{-3}), (c) the lactone **1** with DLI (1.07×10^{-5} mol dm^{-3}), and (d) the lactone **2** with DLI (1.07×10^{-5} mol dm^{-3}). [a] Determined by HPLC. [b] In 0.02 mol dm^{-3} HEPES buffer containing 0.001 mol dm^{-3} EDTA, at pH 7.0 and 298 K.

of the *Z*-lactone **2** by DLH would be expected to lead to the accumulation of an even larger proportion of the *E*-isomer **1**, at least up to the equilibrium value of 53%. Such proportions (even the 4%) are well above the limits of detection, so there is no indication that DLH catalyses substrate isomerisation, only substrate hydrolysis. With DLI, substrate isomerisation occurs without hydrolysis.

The reactions of the lactones **1** and **2** with DLH involve formation of the thioester intermediate **4a** (Scheme 2).[10] In principle, DLI could react in a quite different manner, by reversible Michael addition of the enzyme's active site serine to the enolic carbon of the substrates **1** and **2**, with isomerisation. However, this mechanism seems implausible, since a similar process involving the thiolate of DLH would be more likely to lead to isomerisation if that was the case, and the distance between the serine oxygen of DLI and the enolic carbon of the substrates **1** and **2** is likely to be too great. The crystal structure of the lactam analogue of the lactone **2** bound in the active site of DLI indicates that this distance is 4.2 Å.[10] Thus it seems more reasonable that DLI reacts *via* the ester intermediate **4b** (Scheme 2). The lack of hydrolysis of this species may indicate that water is not available, at least in the necessary orientation, in the active site of the mutant protein. Alternatively, it could be due to non-productive collapse of the tetrahedral intermediate **5b**.[14] Whereas the intermediate **5a** will readily collapse with loss of the thiolate group, to give maleylacetate **3**, the analogous serine derivative **5b** should most readily lose hydroxide to revert to the ester **4b**, which could then recyclise. By analogy, with UDP-glucose dehydrogenase it has been found that the Ser mutant, obtained by displacing the active site Cys-260, reacts with the substrate to give an acyl enzyme, that is less labile towards hydrolysis than the corresponding putative thioester formed from the native protein.[15] However, the UDP-glucose dehydrogenase mutant is not active catalytically, unlike the situation with DLH and DLI, where both proteins are catalysts but of different processes. Irrespective of the mechanism, this difference between DLH and DLI is clear. The reaction of DLI with the lactone **1** affords ready access to the isomer **2**, and the general utility of this interconversion is currently being explored.

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