Active site mutagenesis of the putative Diels–Alderase macrophomate synthase†

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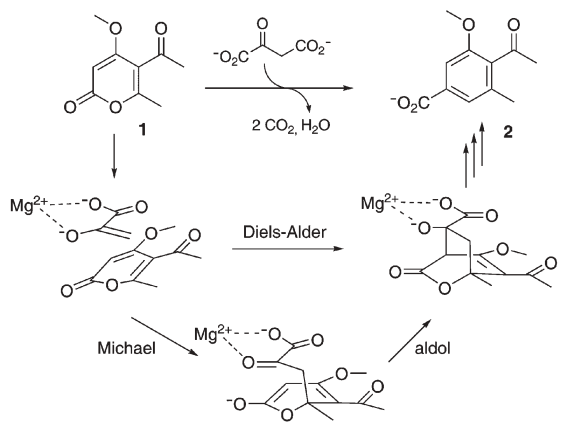
Although the macrophomate synthase active site is rich in potential functional groups, site-directed mutagenesis shows that only three residues are absolutely required for catalysis of oxaloacetate decarboxylation and trapping of the resulting enolate with a 2-pyrone; the other residues that line the binding pocket are surprisingly tolerant to substitution.

A variety of antibodies[1] and oligonucleotides[2] have been engineered to catalyze Diels–Alder reactions, but only three natural enzymes with possible Diels–Alderase activity[3] – lovastatin nonaketide synthase (LNS),[4] solanapyrone synthase (SPS),[5] and macrophomate synthase (MPS)[6,7] – have been isolated to date. The recent determination of the crystal structure of MPS,[8] which promotes the conversion of 2-pyrones to benzoates (Scheme 1), provides a valuable opportunity to examine the mechanism of one of these catalysts in greater detail. Identification of the key catalytic residues in the MPS active site is particularly important in light of QM/MM calculations which suggest that a two-step Michael–aldol sequence is energetically preferred over a concerted pericyclic reaction (Scheme 1).[9] To that end we have prepared 16 active site variants of this catalyst and characterized them kinetically.



Scheme 1 The MPS-catalyzed formation of macrophomate (**2**) from 2-pyrone **1** and oxaloacetate may proceed *via* a concerted Diels–Alder reaction or a two-step Michael–aldol sequence.

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We produced MPS with an N-terminal hexahistidine tag in *E. coli* under the control of a T7 promoter. The protein was purified to homogeneity by affinity chromatography on a Ni(II)-chelating NTA resin at pH 7.0 and 4 °C. This procedure, which yields up to 260 mg of pure protein per litre of cell culture, is more efficient than the original purification strategy,[10] which entailed three chromatography steps. In agreement with the crystallographic data,[8] analytical ultracentrifugation measurements show that the enzyme adopts a hexameric quaternary structure in solution. Importantly, the hexahistidine tag does not significantly affect its catalytic properties. The decarboxylation of oxaloacetate is catalyzed with a k_{cat} of 12 s^{-1} and a K_m of 80 μM, in good agreement with literature values.[6] When 2-pyrone **1** is included in the reaction mixture, macrophomate (**2**) is formed at a rate that is consistent with the reported k_{cat} value of 0.6 s^{-1}.

The MPS active site is a spacious cavity at the interface of two subunits (Fig. 1).[8] It contains a catalytically essential magnesium ion, which activates oxaloacetate for decarboxylation and coordinates the resulting enolate that subsequently reacts with 2-pyrones. One side of the pocket is formed by Trp68, an extended network of polar residues (Asp70, Glu72, His73, Arg101, His125 and Gln183), and the Mg^{2+}-chelating amino acids Glu185 and Asp211. The hydrophobic side chains of Phe149′, Pro151′, Trp152′ and Tyr169′ from a different subunit define an adjacent apolar surface below the pyruvate binding site, creating a cleft into which the 2-pyrone can bind. We systematically mutagenized ten

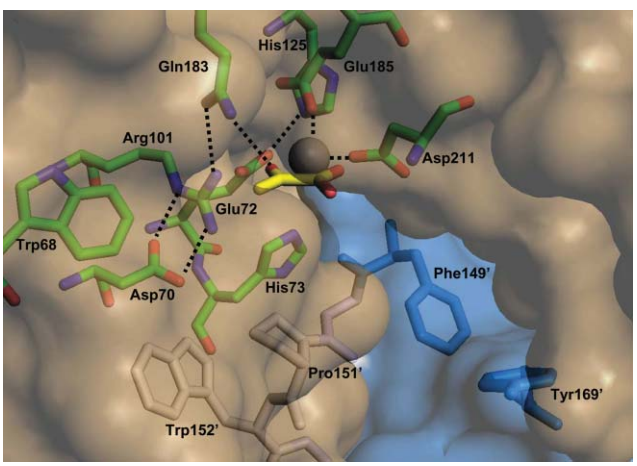

Fig. 1 View into the MPS active site with Mg^{2+}-bound pyruvate (yellow). The catalytic pocket is formed at the interface of two protein subunits (brown and blue surfaces). Hydrogen bonding interactions between the catalytic residues and pyruvate are shown as dashed lines. This figure was prepared using PyMOL.[11]

of these amino acids using standard procedures to probe their contribution to catalysis. Mutant genes were constructed using the QuickChange method[12] and verified by DNA sequencing. The protein variants were then produced and purified as described for the parent enzyme. Their structural integrity was confirmed by ESI-MS and circular dichroism spectroscopy. All variants were tested for oxaloacetate decarboxylase activity in the absence of 2-pyrone **1** and, independently, for their ability to form **2** in its presence. Pyruvate, which is an alternative substrate for the enzyme,[10] was used to assay MPS activity with variants that lack decarboxylase activity. The steady-state kinetic parameters for the decarboxylation reaction and the relative MPS activities are summarized in Table 1.

Our results show that three residues, Asp70, His73 and Arg101, are absolutely essential for efficient enzyme-catalyzed decarboxylation of oxaloacetate. Even conservative replacement of aspartate and histidine by asparagine, or arginine by lysine, reduces decarboxylase activity more than 10^4-fold. In contrast, substitution of Glu72, His125, Gln183, and the various hydrophobic residues leads to relatively modest reductions in activity (< 9-fold changes in k_{cat}, and 3–90-fold decreases in k_{cat}/K_m). The large increase in K_m for some of these mutants points to disrupted oxaloacetate binding, perhaps due to subtle changes in the coordination sphere of the magnesium ion or to an otherwise distorted active site geometry. Given the location of most of these residues at the subunit interface, altered quaternary interactions may be the ultimate origin of these effects.

Table 1 Steady state parameters for the MPS-catalyzed decarboxylation of oxaloacetate and relative rates of formation of macrophomate (**2**)[a]

Mutant	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [M^{-1} s^{-1}]	$k_{rel,ox}^{MPA}$ [b]	$Yield_{rel,py}^{MPA}$ [c]
wt MPS	12.0 ± 0.6	80 ± 10	150000	100	100
Trp68Tyr	13 ± 1	2000 ± 120	6500	18	21
Asp70Ala	—	—	< 10	—	—
Asp70Asn	—	—	< 10	—	—
Glu72Ala	1.3 ± 0.1	800 ± 110	1600	9	13
Glu72Gln	1.0 ± 0.1	160 ± 20	6300	92	93
His73Ala	—	—	< 10	—	2
His73Asn	—	—	< 10	—	3
Arg101Ala	—	—	< 10	—	—
Arg101Ser	—	—	< 10	—	—
Arg101Lys	—	—	< 10	—	10
His125Ala	7.7 ± 0.2	1000 ± 100	7700	31	15
Pro151Ala	9.5 ± 1.4	3700 ± 900	2600	21	33
Trp152Tyr	7.3 ± 0.3	200 ± 30	37000	67	97
Tyr169Phe	5.0 ± 0.1	120 ± 10	42000	110	100
Gln183Ala	4.1 ± 0.2	2900 ± 250	1400	4	6
Gln183Asn	8.0 ± 0.4	3000 ± 330	2700	16	11

[a] Assays were performed in 50 mM PIPES (pH 7.0) containing 5 mM $MgCl_2$ at 30 °C. The disappearance of oxaloacetate was monitored at 305 nm in the absence of 2-pyrone **1**. Under these conditions, the spontaneous rate of decarboxylation is $k_{uncat} = 1.5 \times 10^{-5}$ s^{-1} at pH 7.0 and 25 °C,[13] giving a net rate acceleration for the enzyme (k_{cat}/k_{uncat}) of *ca.* 8×10^5. The formation of macrophomate (MPA, **2**) was monitored by reverse phase HPLC as a function of time. [b] $k_{rel,ox}^{MPA}$ represents the relative rate of macrophomate formation in reactions of 2-pyrone ([**1**] = 1 mM) and oxaloacetate (1.0 mM) with MPS (0.7 μM). The standard error on the relative rates is ⩽ 15%. [c] The relative yield of macrophomate formed in reactions of pyrone ([**1**] = 1 mM) and pyruvate (10 mM) after incubation with MPS (20 μM)for 72 h at pH 7.0 and 30 °C. The error on the HPLC measurements is ⩽ 15%.

The same three residues that are required for the decarboxylation of oxaloacetate also strongly influence the subsequent conversion of 2-pyrones to benzoates. Thus, no MPS activity is detected for the Asp70Ala/Asn or Arg101Ala/Ser variants, and only small amounts of **2** are formed when the His73Ala/Asn and Arg101Lys variants are incubated with pyruvate and **1** (Table 1). Arg101 is within hydrogen bonding distance of the carboxylate group of pyruvate, and the fact that Arg101Lys, but not Arg101Ala/Ser, retains low levels of activity supports the suggestion[8] that a long cationic residue at this position may also be important for orienting and activating the 2-pyrone *via* a hydrogen bonding interaction. Asp70, which forms a tight salt bridge with Arg101 but is too distant to interact with substrate directly, probably fulfils a structural role, ensuring that the guanidinium cation is appropriately positioned for catalysis. Similarly, although His73 could conceivably function as an acid or base in the complex reaction sequence leading to the final product,[14] its location within the active site makes a structural role more plausible: it mediates intersubunit packing through extensive van der Waals contacts through its imidazole ring and by donating a hydrogen bond to a carboxylate group on the adjacent subunit. Disruption of this set of interactions would be expected to perturb local active site geometry significantly.

Changes in MPS activity upon mutating the other active site residues are minor (less than 6-fold reductions in rate) and correlate roughly with the (larger) effects on the initial decarboxylation step (Table 1). These results are consistent with previous observations that pyruvate formation from oxaloacetate is more efficient than formation of **2**.[6] As shown with the Glu72Gln variant, decarboxylase activity can be reduced by as much as a factor of 20 without impacting MPS activity. Interestingly, generation of the reactive enolate by deprotonation of pyruvate can be more or less sensitive to specific mutations than the decarboxylation of oxaloacetate (Table 1). Finally, we find no evidence to support the suggestion that Tyr169, which sits at the entrance of the binding pocket, engages in a productive hydrogen bond with the exocyclic C5 carbonyl group of the 2-pyrone.[8] In our hands, the Tyr69Phe variant is fully active, perhaps because of the improved purification protocol.

Despite containing a large number of potentially useful functional groups, the MPS active site is notable for its relative insensitivity to mutation. Aside from the catalytically essential magnesium ion, generation of the reactive pyruvate enolate and its reaction with 2-pyrones appear to be facilitated primarily by Arg101, which may preorganize the reactants through hydrogen bonds and also electrostatically stabilize negative charges that develop in the course of the reaction. The other essential amino acids identified by mutagenesis, Asp70 and His73, more likely serve structural roles. Although these findings do not resolve the central question regarding the concertedness of the key C–C bond forming step(s),[6,9] it is striking that these same residues are strictly conserved in a structurally homologous enzyme, 2-dehydro-3-deoxygalactarate (DDG) aldolase, which catalyzes the reversible aldol addition of pyruvate to tartronic semialdehyde.[15] Based on the relatively minor effects associated with their mutation, the other active site residues appear to play a more indirect role in catalysis, fine tuning the reactivity of the magnesium ion or influencing, through steric interactions, the choice of reaction

partner for the pyruvate enolate and the fate of the intermediates leading to the final product.

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