A Stable Miniature Protein with Oxaloacetate Decarboxylase Activity

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An 18-residue miniature enzyme, Apoxaldie-1, has been designed, based on the known structure of the neurotoxic peptide apamin. Three lysine residues were introduced on the solvent-exposed face of the apamin α-helix to serve as an active site for decarboxylation of oxaloacetate. The oxidised form of Apoxaldie-1, in which two disulfide bonds stabilise the α-helix, formed spontaneously. CD spectroscopy measurements revealed that, in its oxidised form, Apoxaldie-1 adopted a stably folded structure, which was lost upon reduction of the disulfide bonds. Despite its small size and the absence of a designed binding pocket, Apoxaldie-1 displayed saturation kinetics in its oxidised form and catalysed the decarboxylation of oxaloacetate at a rate that was almost four orders of magnitude faster than that observed with n*-butylamine. This rivals the performance of the best synthetic oxaloacetate decarboxylases reported to date. Unlike those, however, Apoxaldie-1 displayed significant stability. It maintained its secondary structure at temperatures in excess of 75 °C, in the presence of high concentrations of guanidinium chloride and at pH values as low as 2.2. Apamin-based catalysts have potential for the generation of miniature peptides that display activity under nonphysiological conditions.*

Introduction

Simple peptides decorated with functional groups rarely, if ever, display significant catalytic activities. Presentation of the same array of functional groups to a substrate within the structurally ordered environment of the active site of an enzyme, however, normally creates catalysts of high efficiency. The rational redesign of enzymes is a difficult task due to the delicate balance between stability and catalytic activity and the intrinsic instability of many naturally occurring proteins. One way to overcome this problem is to create catalysts by grafting active-site residues onto stable polypeptide scaffolds.[1–7]

A wide range of natural protein motifs have been used to create miniature enzymes that present functional groups on the surface of specific elements of secondary structure. Examples include a ββα motif,[8] α-helical bundles,[7,9–11] mixed polyproline/α-helices[4–6] and triple-stranded β-sheets.[12,13] Such scaffolds often require oligomerization for stability and functional activity.[4,6,7,9] In addition, their resistance to high temperatures,[4,6] denaturants such as guanidinium hydrochloride (Gdn·HCl) or urea,[6] and extreme pH values is normally rather low. The reduced thermal stability of miniature proteins is thought, at least in part, to result from their small size and hence low number of stabilizing interactions between different parts of the peptide chain.

We have recently described two strategies to restrict the conformational space available to small peptides with DNA binding activity. The first relied on the introduction of covalent nonpeptidic cross-linkers that can be activated by light.[14,15] The second approach made use of the stabilising effect of disulfide bonds between the DNA-recognition helix of the basic helix-loop-helix protein MyoD and an N-terminal extension derived from the bee venom peptide apamin.[16,17] Apamin is an 18-residue neurotoxic peptide, in which two turns of the C-terminal α-helix are stabilised through two disulfide bonds, thereby conferring significant stability on the peptide.[18–20] Proteolysis, CD and NMR spectroscopy revealed that, upon air oxidation, the disulfide bonds in apamin formed exclusively between Cys1 and Cys11, and Cys3 and Cys15 (Figure 1).[19,21–24] Apamin has been used to stabilise the α-helical conformation of several peptides, including S-peptide.[25] These studies revealed that the stability of the apamin motif was based mainly on the formation of the disulfide bonds, while other residues in the α-helix could be altered without significantly changing the secondary structure.

Based on a general understanding of the amine-catalysed decarboxylation of β-keto acids, a reaction that proceeds through a protonatable imine intermediate, we have previously reported the construction and characterisation of a series of peptides that folded in solution and displayed catalytic activity as oxaloacetate decarboxylases. α-Helical bundles (Oxaldie-1 and -2)[9,26] and the scaffold of the dimeric pancreatic polypeptide (Oxaldie-3 and -4)[4,6] were used to constrain a reactive amine of a lysine residue, the pK_a of which was depressed by up to two orders of magnitude due to the proximity of further lysine residues. These designs were successful in that imine formation, which is the slow step during the decarboxylation of β-keto acids by simple amines, was no longer rate determining

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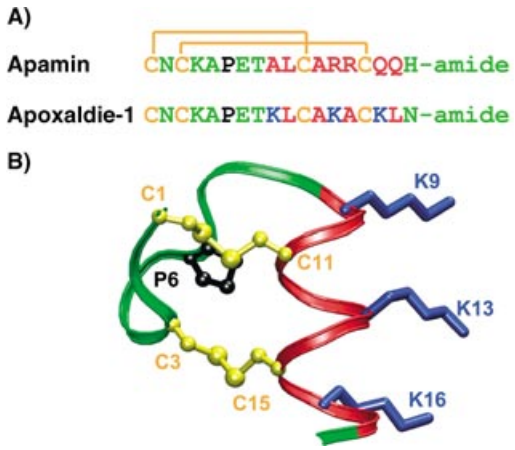


Figure 1. *Design of catalytically active peptide Apoxaldie-1. A) Alignment of the amino acid sequence of the bee venom peptide apamin and of Apoxaldie-1. The naturally produced disulfide pattern of apamin[19,21–24] is indicated in orange. B) Molecular model of Apoxaldie-1 based on the NMR structure of apamin.[20,29] Cysteine residues are shown in orange, helical residues in red, proline in black, lysines in blue and other residues in green.*

in reactions catalysed by these peptides. However, the folding and the activity of these peptides were concentration-dependent; this suggests that these scaffolds were stabilised by oligomerisation and aggregation (Table 1).[4,6,9,26] Their resistance to denaturation by elevated temperature, denaturants or changes in pH was low.

Table 1. *Kinetic parameters of the catalytic decarboxylation of oxaloacetate.[a]*

Peptide	k_{cat} [10^{-3} s^{-1}]	K_M [mM]	k_{cat}/K_M [$M^{-1}s^{-1}$]
Oxaldie-2 (200 μM)[9]	7.5	48.0	0.16
Oxaldie-2 (100 μM)[9]	15.0	210.0	0.07
Oxaldie-3[4]	86.0	49.4	1.74
Oxaldie-4[6]	229.0	64.8	3.53
Apoxaldie (oxidised)	66.0	29.8	2.22
Butylamine[9]	–	–	0.0005
Phe-OEt[26]	–	–	0.0061
Spontaneous[9]	0.013	–	–

[a] For details, see the main text and the Experimental Section.

Here we describe the suitability of an apamin-based scaffold for the creation of monomeric peptides with catalytic activity and high stability. To this end we have designed an oxaloacetate decarboxylase based on the peptide venom apamin (Apoxaldie-1) and containing three lysine residues on the solvent-exposed face of the α-helix. While the catalytic efficiency of Apoxaldie-1 was comparable to that of the best designed peptide-based oxaloacetate decarboxylases described to date, its thermal and chemical stability were dramatically improved.

Results and Discussion

Design and synthesis of Apoxaldie-1

Our previous work had revealed that peptides containing lysine residues in close proximity within the structural context of an α-helix could act as efficient catalysts for the decarboxylation of β-keto acids. Amino acid residues 9, 13 and 16 of apamin, which are solvent-exposed in consecutive turns of the α-helix and in close proximity, were therefore replaced with lysine residues to provide an active site similar to that of Oxaldies-2 to -4 (Figure 1).[4,6,9] A further three residues were changed from the original apamin sequence (Arg14Ala, Gln17Leu, His18Asn) to facilitate the inclusion of the three lysine residues by minimising potential side-chain conflicts.

Apoxaldie-1 was synthesized by solid-phase peptide synthesis by using standard Fmoc chemistry, purified by C18 reverse-phase HPLC and identified by MALDI-TOF and electrospray mass spectrometry. The experimentally observed mass of 1937 Da for reduced Apoxaldie-1, was identical to the calculated mass.

The oxidised form of Apoxladie-1 could be generated by simple air-oxidation of a dilute solution of the peptide. Both MALDI-TOF and electrospray mass spectrometry revealed the correct mass of 1933 Da for oxidised Apoxaldie-1. No free thiols could be detected with Ellman's reagent.[27] As with apamin,[22,28] intramolecular disulfides formed spontaneously, and no evidence for intermolecular disulfide formation was found.

Folding of Apoxaldie-1 is dependent on redox state

The structural properties of Apoxaldie-1 were characterised by CD spectroscopy. At 20 °C, the spectrum of Apoxaldie-1 possessed a minimum at 205 nm and a shoulder at 222 nm (Figure 2) and closely resembled those of unmodified

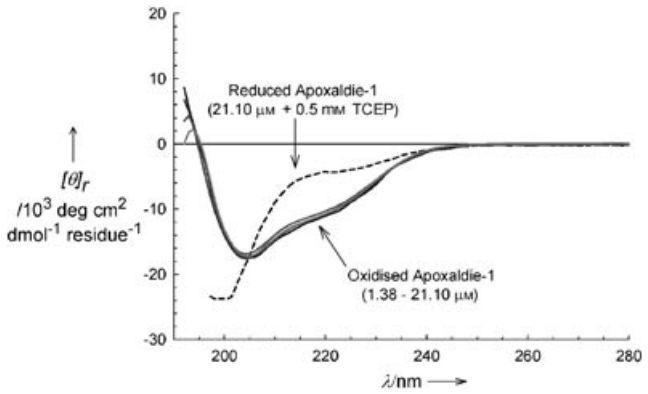


Figure 2. *Dependence of the CD spectrum of Apoxaldie-1 both on the concentration of the peptide and on its redox state. The range of peptide concentrations used was 1.38–21.10 μM. The CD spectrum of 21.1 μM Apoxaldie-1 in the presence of 0.5 mM TCEP is indicated (dotted line). Potassium phosphate buffer (10 mM , pH 7.0) at 20 °C was used. Note that the reduction in the intensity of the CD signal below 190 nm was due to an increase in absorbance at these wavelengths both from the buffer and from the peptide. The CD spectrum of the TCEP-treated peptide has been truncated at 198 nm due to the absorbance of TCEP in this region.*

apamin[18] and its diselenide analogue[28] (Figure 2). The mean residue ellipticity at 222 nm, $[\theta]_{r,222\,nm}$, of Apoxaldie-1 was measured to be $-11\,500 \pm 1000$ deg cm^2 dmol^{-1} per residue. From this value, the α-helical content was estimated to be ~35%; this suggests the formation of approximately two turns of an α-helix. The addition of 5% 2,2,2-trifluoroethanol led to a shift of the minimum from 205 nm to 208 nm (data not shown). The $[\theta]_{r,222nm}$ value increased slightly to $-15\,000$ deg cm^2 dmol^{-1} per residue by the cosolvent (equating to ~48% helicity). The α-helical content of Apoxaldie-1 was therefore lower than would have been expected from the NMR structure[20,29] of apamin (~44%). This suggested that the C-terminal end of Apoxaldie-1 might have been frayed. This interpretation was supported by molecular dynamics (MD) simulations, which indicated an unravelling of the three N-terminal residues of Apoxaldie-1. The structure of apamin, on the other hand, remained largely stable during comparable MD simulations (C. H. Cureton, unpublished work). The presence of the three neighbouring positively charged lysine residues on the solvent-exposed face of the helix, might therefore have been the cause of the apparently reduced α-helicity. An alternative explanation for the reduced $[\theta]_{r,222\,nm}$ intensity measured for Apoxaldie-1 might be the formation of a 3_{10}-helix rather than an α-helix due to electrostatic repulsions between neighbouring lysines. Indeed it has been suggested that 3_{10}-helices possess CD spectra in which the $[\theta]_{r,222\,nm}$:$[\theta]_{r,208\,nm}$ ratio is distinctly smaller than unity.[30] The ratio was measured to be 0.69 for Apoxaldie-1 and 0.82 for apamin; this suggests that while apamin had a largely α-helical CD signal consistent with its NMR structure,[20,24,29] the CD spectrum of Apoxaldie-1 was more representative of a 3_{10}-helix. It must be noted, however, that the "correct" ratio of the values for $[\theta]_{r,222\,nm}$ and $[\theta]_{r,208\,nm}$ alone is not sufficient to prove the presence of a 3_{10}-helix.[31]

The extent of helicity observed for Apoxaldie-1 was independent of the concentration of peptide throughout the whole concentration range studied (1–21 μM; Figure 2); this suggests that Apoxaldie-1 remained folded at concentrations as low as 1 μM, as was expected for a monomeric peptide. Addition of the reducing agent tris(2-carboxyethyl) phosphine (TCEP) to a solution of oxidised Apoxaldie-1 led to a significant change in the CD spectrum (Figure 2). $[\theta]_{r,222\,nm}$ was measured as $-4\,150$ deg cm^2 dmol^{-1} per residue, which indicates that the amount of helicity was reduced to ~13%. The minimum at 205 nm observed for oxidised Apoxaldie-1 was shifted to 200 nm (i.e. a "random-coil" CD spectrum). It appeared, therefore, that the structure of oxidised Apoxaldie-1 was dependent on the disulfide bonds; in their absence the peptide was largely "unfolded".

Apoxaldie-1 displays high thermal and chemical stability

The structures of all designed oxaloacetate decarboxylases reported previously were of rather limited stability. Oxaldies-1 and -2 formed poorly defined α-helical bundles.[9,26] Oxaldie-3, which relied for stability on the avian pancreatic polypeptide fold, formed a molten globule-like structure of only limited stability. The unfolding process of Oxaldie-3, induced by both temperature and chemical denaturants, showed no cooperativity.[4] The stability of Oxaldie-4, the design of which was based on bovine pancreatic polypeptide, was slightly higher with a melting point of 50 °C and a midpoint of the chemical denaturation of approximately 4 M urea.[6]

Apoxaldie-1, on the other hand, was highly resistant to denaturation. Thermal unfolding experiments revealed that it maintained its secondary structure at temperatures in excess of 75 °C. Up to this value, the CD spectrum of Apoxaldie-1 was independent of temperature. It was only at temperatures above 75 °C that the CD spectrum of the peptide changed (Figure 3). This change was very gradual, however, and might

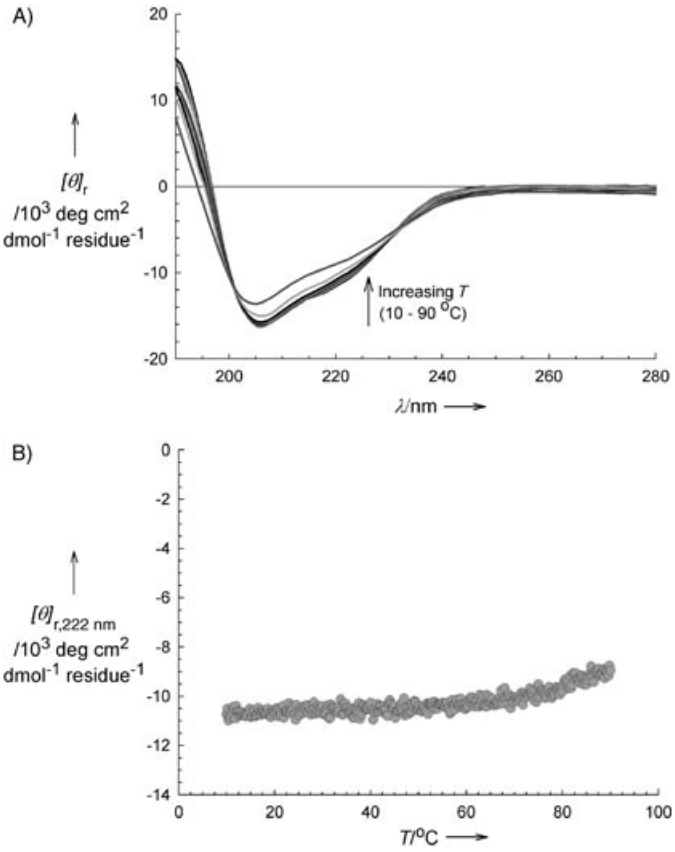


Figure 3. *Temperature dependence of the CD spectrum of Apoxaldie-1. A) Changes in the CD spectrum of Apoxaldie-1 from 10–90 °C (10 °C increments were used). B) The temperature dependence of the measured ellipticity at 222 nm, $[\theta]_{r,222\,nm}$, of Apoxaldie-1 between 10–95 °C. The buffer was 10 mM potassium phosphate, pH 7.0 and the concentration of Apoxaldie-1 was 15 μM.*

have reflected a conformational change of Apoxaldie-1 as a whole rather than a loss of helicity per se. Apamin underwent a similar conformational change (data not shown); this indicated that the substitution of residues on the solvent- exposed face of the C-terminal helix did not affect the thermal stability of the peptide to any great extent.

Apoxaldie-1 also showed significant resistance to chemical denaturants. Only a slight change in the CD spectrum was observed upon the addition of 1–6 M Gdn·HCl (Figure 4). Apamin itself was resistant to denaturation by Gdn·HCl, reiterating the

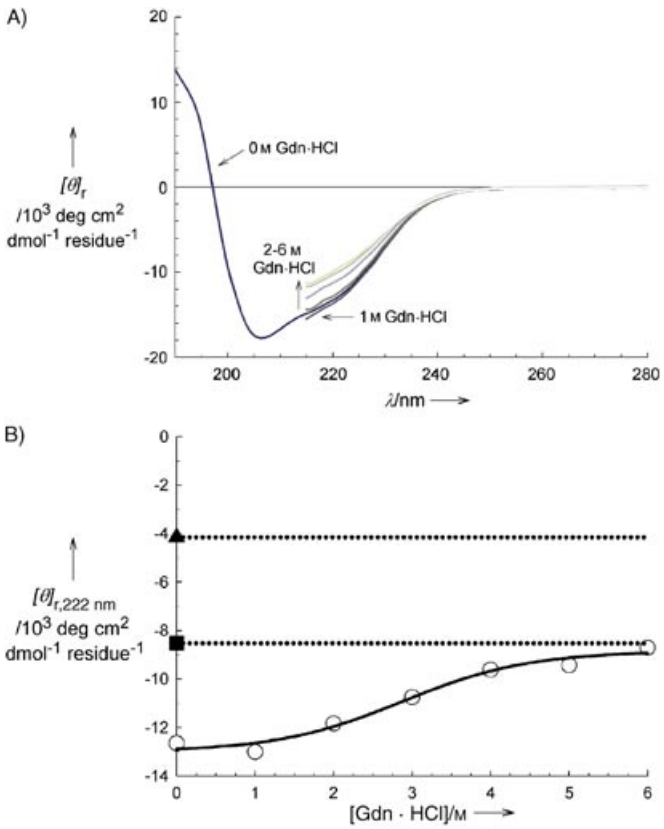
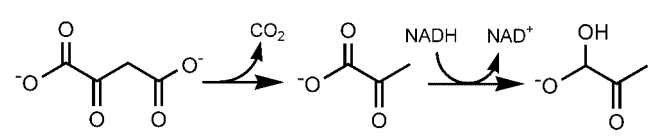


Figure 4. *Dependence of the CD spectrum of Apoxaldie-1 on the concentration of guanidinium-hydrochloride. A) Effect of 0–6 M Gdn·HCl addition to solutions of Apoxaldie-1 (30 μM) in 1 M increments on the CD spectrum of the peptide. B) Changes in the $[\theta]_{r,222nm}$ signal with increasing concentrations of Gdn·HCl. For comparison, the $[\theta]_{r,222nm}$ for Apoxaldie-1 at 95 °C (■) or in the presence of 0.5 mM TCEP (▲) are indicated at 0 M Gdn·HCl. These two data points are extrapolated across the range of Gdn·HCl concentrations studied. The concentration of Apoxaldie-1 was 30 μM.*

slightly reduced stability of Apoxaldie-1. It is interesting to note, however, that, like apamin,[18] Apoxaldie-1 retained its structure to pH values as low as 2.2 (data not shown).

Apoxaldie-1 is an efficient catalyst of the decarboxylation of oxaloacetate

The peptide-catalysed decarboxylation of oxaloacetate was studied by UV spectroscopy in an enzyme-coupled assay (Scheme 1).[4,6,9] The assay revealed that Apoxaldie-1 was not only a highly stable, but also a very efficient peptide catalyst. For the oxidised form of Apoxaldie-1, the reaction followed saturation kinetics with a k_{cat} of 0.0660 ± 0.02 s^{-1} and a Michaelis constant, K_M, of 29.8 ± 5 mM (Figure 5 and Table 1). The kinetic efficiency k_{cat}/K_M of Apoxaldie-1 was therefore 2.22 M^{-1}s^{-1}. Due to its monomeric structure, the folded state



Scheme 1. *The assay of decarboxylation of oxaloacetate to form pyruvate and carbon dioxide was based on monitoring the conversion of NADH to NAD$^+$ during the reduction of pyruvate.*

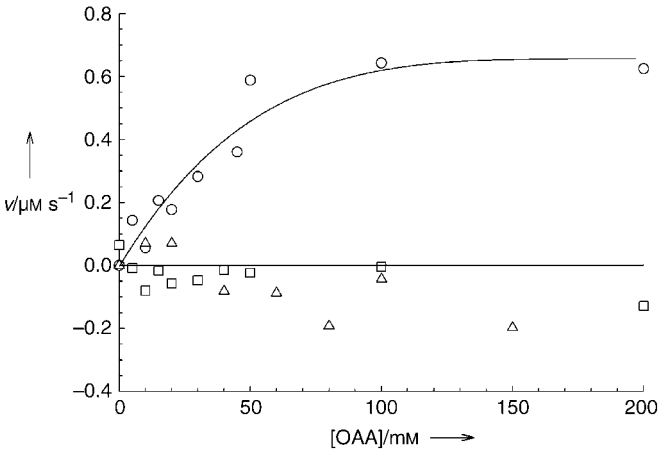


Figure 5. *Rate of the catalysed decarboxylation of oxaloacetate as a function of substrate concentration. Typical example of a rate profile for a reaction carried out at 293 K in* N,N-*bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (50 mM, pH 7) containing 10 mM NaCl in the presence of oxidised Apoxaldie-1 (○), reduced Apoxaldie-1 (□) and wild-type apamin (△).*

and hence catalytic efficiency of Apoxaldie-1 were not dependent on the concentration of the peptide (Table 1). Following reduction of Apoxaldie-1 with TCEP, the peptide no longer displayed saturation kinetics (Figure 5). Neither the reduced form of Apoxaldie-1 nor wild-type apamin speeded the decarboxylation reaction to any significant extent when compared to the uncatalysed reaction (Figure 5). This indicated that both the disulfide-stabilised folded structure and the presence of the three lysines within the α-helix of oxidised Apoxaldie-1 were central to the catalytic activity of Apoxaldie-1. The absence of comparable catalytic activity of apamin indicated that the activity of oxidised Apoxaldie-1 was not due to the amino terminal amine or Lys4, which is also solvent-exposed but not flanked by additional lysine side chains. Because of its central position between Lys9 and Lys16 (Figure 1), Lys13 could be expected to be the most nucleophilic of the amines. However, it is worth noting that all lysine residues of Oxaldie-2 showed similar reactivity.[26]

Support for the involvement of an imine intermediate in the decarboxylation reaction was obtained from reductive alkylation experiments.[9] Oxidised Apoxaldie-1 was incubated with oxaloacetate in the presence of $NaCNBH_3$. Analysis of the reaction products by MALDI-TOF mass spectrometry revealed a mass increase in the peptide of 72 Da (data not shown); this indicated the reductive alkylation of Apoxaldie-1 by one molecule of pyruvate. This result suggested that decarboxylation of the imine formed with oxaloacetate occurred more rapidly than its reduction by $NaCNBH_3$.

The small size of Apoxaldie-1 was clearly not a limitation for its catalytic performance; its catalytic efficiency was similar to that measured for Oxaldie-3 and -4 (Table 1). While the catalytically active form of Oxaldie-4 was a dimer of 62 amino acids,

Apoxaldie-1 acted as a monomer of only 18 amino acid residues. On a molecular weight basis, Apoxaldie-1 was therefore more than twice as efficient as Oxaldie-4. Naturally occurring oxaloacetate decarboxylases speed the decarboxylation reaction by approximately eight orders of magnitude relative to simple amines, such as butylamine. Despite the absence of a designed binding pocket, Apoxaldie-1 enhanced the rate of decarboxylation by almost four orders of magnitude. Even relative to a catalyst such as phenylalanine ethyl ester, which for catalysis at physiological pH has the nearly optimal pK_a of 7.2, Apoxaldie-1 speeded the reaction almost 400-fold (Table 1).[26] Its catalytic performance was equal to that of the catalytic antibody 38C2, which, in addition to efficiently catalysing aldol, retroaldol and deuterium-exchange reactions, also decarboxylated 2-(3′-(4′′-acetamidophenyl)propyl)acetoacetic acid ($k_{cat}/K_M = 2.9\ M^{-1}s^{-1}$).[32] The catalytic performance of Apoxaldie-1, however, was achieved by a peptide that is 100 times smaller than naturally occurring enzymes and abzymes.

In summary, Apoxaldie-1 is an 18 amino acid peptide of significant stability that, despite its small size, catalyses the decarboxylation of oxaloacetate with an efficiency rivalling that of the best, designed peptide catalysts. However, its remarkable thermal stability and high resistance to chemical denaturation suggest that apamin-based catalysts might be useful for the generation of peptides that retain their catalytic activity under nonphysiological conditions, such as high temperature or low pH.

Despite the replacement of six of the nine residues of the apamin α-helix and the close proximity of three charged lysine residues, Apoxaldie-1 displayed a high degree of resistance to denaturation. This study reinforces the concept that, due to its high stability, the apamin fold serves as a useful general scaffold for molecular-recognition processes based on α-helices.[17,23,25,33]

Experimental Section

Materials: All chemicals were purchased from Sigma unless stated otherwise. Apoxaldie-1 was synthesized by Jerini AG (Berlin, Germany). Peptide concentrations were determined by amino acid analysis conducted by Alta Biosciences (University of Birmingham, UK).

Molecular modelling: Apoxaldie-1 was constructed on an apamin template based on recent NMR coordinates[29] and adjusted to incorporate likely interactions[20,34] with Thr9 and Asn2. The helix-stabilising cysteine spacing of apamin (*i*, *i*+2, *j*, *j*+4) was maintained, and Apoxaldie-1-specific residues were introduced by using the Sybyl 6.7 package. To assess the likely stability of Apoxaldie-1, MD simulations were undertaken. The structure was minimised by using AMBER 7.0, implementing the all-atom Amber force field and parm99 parameters.[35] The peptide was then solvated in an 8.0 Å periodic water box (WATBOX216) and neutralised with chloride counter ions. Subsequently, a 1 ns solvated MD simulation was performed at 300 K by using SHAKE to constrain hydrogen bonds to permit a 1 fs time step with an 8.0 Å nonbonded cut-off and PME to treat long range electrostatics.

Oxidation and purification of Apoxaldie-1: Samples (517 μM) of Apoxaldie-1 were reduced with TCEP (15 mM) under N_2 for 3 h with constant stirring. The sample was then allowed to air oxidise overnight at room temperature. The sample was applied to a Sephadex G-10 size-exclusion column (Amersham Biosciences) to remove excess reducing agent and allowed to further air oxidise overnight. The resulting solution was lyophilised, resuspended in a small volume of water and applied to a C18 reverse-phase HPLC column. The peptide eluted as a single peak at approximately 75% acetonitrile/water in 0.05% TFA. The mass of the peptide (1933 Da), as determined by MALDI-TOF and ESI mass spectrometry, corresponded exactly to that of Apoxaldie-1 with all four cysteine residues in their oxidised form. In addition, treatment of Apoxaldie-1 with Ellman's reagent[27] indicated that the peptide did not possess any detectable free thiol groups.

CD measurements: CD spectra were collected by using a JASCO-810 spectropolarimeter that was fitted with a Peltier temperature controller. Spectra were recorded between 190 and 280 nm with a data pitch of 0.2 nm. A stepped scan was employed with a 0.5 s response and a 2 nm bandwidth. The buffer used was potassium phosphate (10 mM, pH 7.0) in a 5 mm path-length cuvette. Buffer background signals were subtracted in all cases. For temperature scans, the temperature was increased at $40\,°C\,h^{-1}$. Reported measurements are averages of at least five separate scans. Mean residue ellipticity ($[\theta]_R$) was calculated by using Equation (1).

$$[\theta]_R = \frac{[\theta]}{10 \times (n-1) \times c \times l} \quad (1)$$

Here $[\theta]$ is the measured ellipticity in mdeg, n is the number of amide bonds, c is the molar concentration, and l is the pathlength in cm. The predicted $[\theta]_R$ for 100% helix was calculated according to Equation (2):[36]

$$[\theta]_{R,\,100\%\,helix} = \frac{-40\,000 \times (n-4)}{n} \quad (2)$$

Determination of catalytic activity: The catalytic activities of oxidised and reduced Apoxaldie-1 (7–14 μM) and of wild-type apamin (28 μM) were monitored by using the enzyme-coupled assay described previously (Scheme 1).[6] Kinetic experiments were carried out by using a Shimadzu UV-2401PC UV/Vis spectrophotometer equipped with a Shimadzu Peltier temperature controller. The rate of formation of pyruvate from oxaloacetate was measured at 298 K in *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (50 mM, pH 7) and NaCl (10 mM). Lactate dehydrogenase was employed to follow the decrease of NADH (0.2 mM) absorbance at 340 nm ($\varepsilon = 6.23 \times 10^3\ M^{-1}cm^{-1}$). Concentrations of oxaloacetate were between 0 and 200 mM. Peptide concentrations were determined by quantitative amino acid analysis. Kinetic parameters were determined with the program SigmaPlot. All kinetic values reported were corrected for the background rate observed in the absence of catalyst.

To detect imine formation, Apoxaldie-1 (1 mM) was incubated at 293 K with oxaloacetate (10 mM) in the presence of $NaCNBH_3$ (10 mM) in *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid (50 mM, pH 7) and NaCl (10 mM). The reaction products were purified by reverse-phase chromatography in a Zip tip (Millipore) according to the manufacturer's protocol and analysed by MALDI-TOF mass spectrometry.

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