ence of a relatively small hot-spot on the neutron-star surface.

In summary, the data suggest that a significant fraction of the X-rays are due to pulsed thermal emission which probably comes from a polar hot-spot. Compared with the other pulsars from which pulsed thermal emission has been detected (Vela, PSR0656+14, PSR1055−52 and Geminga)[25], PSRJ0437−4715 has a considerably larger characteristic age ($P/2\dot{P} \sim 2 \times 10^9$ yr). This strongly supports the idea that after neutrino and photon cooling, neutron stars are kept hot either by internal frictional heating[11-13] or by bombardment of the polar cap by energetic particles accelerated in the magnetosphere[14,15]. The possibility that the heating occurred during a past accretion phase seems unlikely because the cooling time of a neutron star is much shorter than the characteristic age of this pulsar. The ratio of pulsed X-ray luminosity to the spin-down power is $\sim 10^{-4}$, consistent with the models of reheating and polar cap activity. □

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Synthesis, structure and activity of artificial, rationally designed catalytic polypeptides

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BIOLOGICAL macromolecules with catalytic activity can be created artificially using two approaches. The first exploits a system that selects a few catalytically active biomolecules from a large pool of randomly generated (and largely inactive) molecules. Catalytic antibodies[1] and many catalytic RNA molecules[2] are obtained in this way. The second involves rational design of a biomolecule that folds in solution to present to the substrate an array of catalytic functional groups[3–8]. Here we report the synthesis of rationally designed polypeptides that catalyse the decarboxylation of oxaloacetate via an imine intermediate. We determine the secondary structures of the polypeptides by two-dimensional NMR spectroscopy. We are able to trap and identify intermediates in the catalytic cycle, and to explore the kinetics in detail. The formation of the imine by our artificial oxaloacetate decarboxylases is three to four orders of magnitude faster than can be achieved with simple amine catalysts: this performance rivals that of typical catalytic antibodies.

Decarboxylation of oxaloacetate is the final reaction in the Rozzell process for the industrial synthesis of phenylalanine[9]. This process achieves decarboxylation by means of a natural enzyme (an oxaloacetate decarboxylase) that requires a metal ion as a cofactor[10]. Because industrial processes may be incon-

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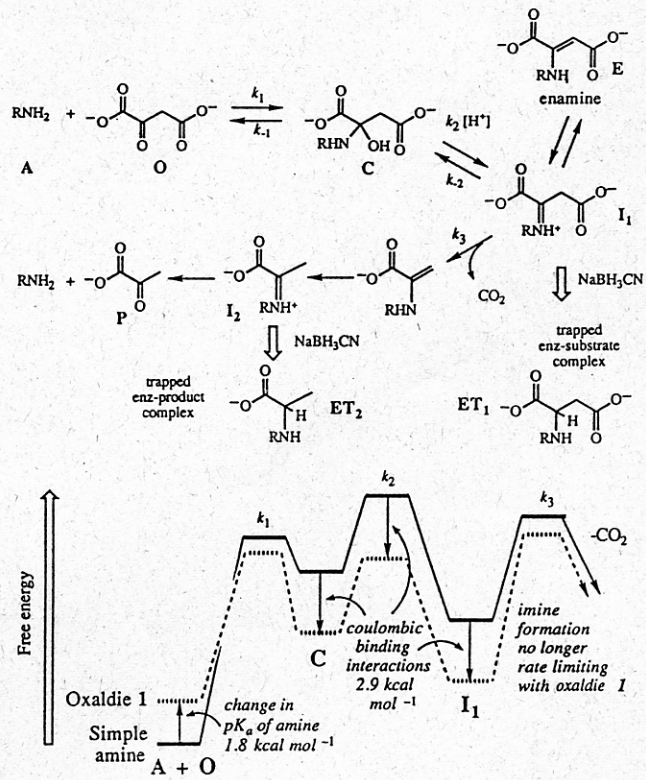


FIG. 1 Kinetic scheme and an approximate free-energy profile describing the decarboxylation of oxaloacetate catalysed by amines (R defined in Table 1). Open arrows indicate reactions used to trap intermediates in the catalytic cycle. The free-energy profile is for butyl amine (solid line) and oxaldie 1 (broken line), and shows the points where the designed peptide catalyst alters the free-energy profile to achieve rate acceleration. The relative free energies of bimolecular states are calculated using a 1 M standard state, corrected for amount of free amine. A, Amine; O, oxaloacetate; C, carbinolamine; P, pyruvate; I_1, imine intermediate between amine and oxaloacetate; I_2, imine intermediate between amine and pyruvate; ET_1, trapped imine with oxaloacetate; ET_2, trapped imine with pyruvate.

venienced by cofactors, a metal-free oxaloacetate decarboxylase that operates by a different mechanism would be desirable.

Alternative mechanisms are conceivable: for example, oxaloacetate can be decarboxylated by amines that react to form an imine, which then loses carbon dioxide (Fig. 1)[11 14]. But no natural oxaloacetate decarboxylase is known to use the imine mechanism, presumably because the metal-dependent mechanism is evolutionarily optimal for this particular substrate[15,16]. Design would seem to be the only way to obtain a metal-free oxaloacetate decarboxylase that operates by an imine mechanism.

To design a new peptide to catalyse the decarboxylation of oxaloacetate via an imine (we call the peptide an 'oxaldie'), the mechanisms of amine-catalysed decarboxylation in general must be understood. Six features relevant to the design emerged by combining published information[11-14,17] with details of the kinetics of the decarboxylation of oxaloacetate catalysed by five model amines[18]. (1) Decarboxylation can proceed via an imine intermediate (Fig. 1)[11 14]. The new peptide catalyst should therefore carry an active-site amino group capable of forming an imine with oxaloacetate. When constrained to use only those amino acids encoded naturally by messenger RNA, this must be either the amino-terminal amine of the polypeptide itself (strategy (a)) or the ε-amino group of a constituent lysine (strategy (b)). (2) Imine formation is largely rate-determining for decarboxylation above pH 4 (refs 18, 41). Speeding imine formation therefore becomes the focus of the design. (3) The rate-determining step in imine formation between pH 4 and 7 is the specific acid-catalysed elimination of water from carbinolamine C ($k_2[H^+]$ in Fig. 1)[17-20]. This implies that general acids need not be built into the active site to speed imine formation. (4) Carbinolamine C is the high-energy intermediate along the reaction coordinate. Thus the rate of imine formation can be accelerated by introducing stabilizing binding interactions between the peptide and carbinolamine C (and therefore, according to the Hammond postulate, the transition state that follows)[18,21,41]. (5) The catalytically most effective amine has a pK_a equal to the ambient pH. For each log unit decrease in its pK_a, the intrinsic catalytic power of an imine decreases by half a log unit (a Brønsted β of 0.5)[18,22], but the concentration of free amine (the reactive species) increases by a full log unit. This implies that the catalytic effectiveness increases by half a log unit until the pK_a of the amine reaches the ambient pH. (6) The partition ratio of the imine intermediate towards product and back to substrate (k_3/k_{-2} in Fig. 1) is independent of both the pH and the pK_a of amine[18]. Thus, perturbing the pK_a of the amine will not influence the partitioning unfavourably for catalysis.

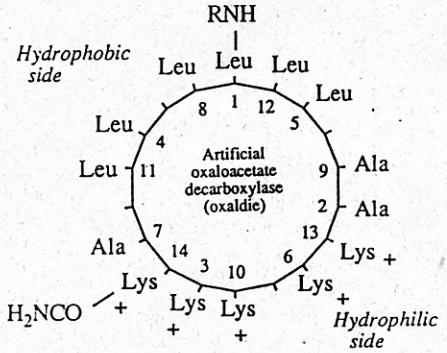
The scaffolding for holding a reactive amine is an amphiphilic α-helix[23-28] capable of building helix bundles in aqueous media (Fig. 2)[29]. Positively charged residues were attached to the helices to bind the dianionic carbinolamine C (Fig. 1) by coulombic interactions. Two strategies were explored to lower the pK_a of the amino group. In strategy (a) (yielding the peptide oxaldie 1), the terminal amino group of the polypeptide chain is left free to serve as the reactive amine. Its pK_a is depressed by interactions with the 'helix dipole'[30 32] when a helix is formed. In strategy (b) (yielding oxaldie 2), the amino-terminal amine is blocked; the reactive amino group must therefore come from a lysine side chain whose pK_a is depressed by coulombic interaction with neighbouring protonated lysine side chains when the helix is formed.

The peptides were synthesized by solid-phase methods (Fig. 2). Circular dichroism spectra indicate that oxaldies 1 and 2 are, respectively, 18 and 33% α-helical in aqueous buffer at infinite dilution; the different helical content relates to their relative stabilities as predicted from the helix dipole, which interacts unfavourably with the free amino-terminal amine in oxaldie 1 (refs 30–32). Helicity is a strong function of peptide concentration, however, suggesting that the α-helices are stabilized by aggregation[24 29]. The size of the aggregates formed was estimated by concentration studies[24 28] and size-exclusion chromatography. Oxaldie 1 seems to aggregate to form a four-helix bundle first, whereas oxaldie 2 forms larger aggregates.

Secondary structures of both peptides at 8 mM were established by two-dimensional NMR spectroscopy. Helical segments were identified from nuclear Overhauser effects (NOEs) of the type d_{NN}, $d_{\alpha N}(i, i+3)$, and $d_{\alpha N}(i, i+4)$ (ref. 33). Such cross-peaks are observed for all but the first four residues of oxaldie 1 in 20% aqueous trifluoroethanol, a structure-forming solvent[34]. For oxaldie 2, NOEs indicative of α-helical secondary structure were observed for the entire length of the peptide without trifluoroethanol, again indicating greater stability in the terminally acetylated helix. Titration identified a single amino group in oxaldie 1 with a pK_a of 7.2, assigned as the terminal amine by trapping with acetic anhydride[35,36]. This pK_a is depressed 0.6 units by helix formation (relative to Leu-Ala-NH_2)[18]. The lowest pK_a in oxaldie 2 is 8.9 (1.6 units below that of a typical lysine).

Both oxaldies 1 and 2 catalyse the decarboxylation of oxaloacetate (Table 1) with Michaelis–Menten saturation kinetics ($k_{cat} = 0.4\ min^{-1}$ and $0.5\ min^{-1}$; $K_m = 14$ mM and 48 mM, respectively). By coincidence, the first k_{cat} is the same as that reported for the first catalytic antibody[37]. Imines I_1 and I_2 (Fig. 1) with oxaldie 1, proposed as intermediates in the catalytic cycle, were trapped with $NaBH_3CN$. Rates were not increased by external general acids or bases. Monoanionic β-ketoacids (for example, acetoacetate) were not substrates, and acetone dicarboxylate, acetate and malate were not inhibitors. Pyruvate, the product of the decarboxylation, weakly inhibited oxaldie 1, ($K_I \approx 70$ mM).

When both the amount of trifluoroethanol[34] and the concentration of the peptides are varied, catalytic activity correlates with the fraction of helix formed. This suggests that the catalytically active forms of the oxaldies are the helices. To confirm this, the helical structure of oxaldie 2 was disrupted by mutating leucine residues at positions 4, 8 and 11 to Gly, Pro and Gly, respectively. Unlike oxaldie 1 and 2, the mutant peptide was found by circular dichroism to adopt random-coil conformation at all concentrations; its catalytic activity is decreased by an



RHN-Leu-Ala-Lys-Leu-Leu-Lys-Ala-Leu-Ala-Lys-Leu-Leu-Lys-Lys-$CONH_2$
1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 2 Sequences of two designed artificial oxaloacetate decarboxylases (oxaldie 1 and oxaldie 2; R for oxaldie 1 is hydrogen, and for oxaldie 2 is an acetyl group together with a representation of these structures on a helical wheel to illustrate the amphiphilic nature of the helices that they build. Five amino groups from the side chains of lysine protrude on one face of the helix. In oxaldie 1, the amino-terminal amine is the principal active site. In oxaldie 2, the ε-lysine amino groups provide the active site. Both peptides were synthesized using standard *t*-Boc chemistry[40] on polystyrene/1%'divinylbenzene resin with the *p*-methylbenzhydrylamine linker. Deprotection of the peptide and release from the support were achieved by treatment with trifluoromethylsulphonic acid in trifluoroacetic acid in the presence of ethanedithiol and thioanisole. Peptides were purified by reversed-phase C_{18} HPLC.

TABLE 1 Comparison of kinetic parameters for decarboxylation of oxaloacetate with oxaldie and some model amines*

Catalyst	pK_a (lowest)	Trifluoro-ethanol (v/v)	k_{cat}/K_m or k_{obs} (s^{-1} M^{-1})	log relative k_{cat}/K_m (or k_{obs})	$k_{cat} \times 10^3$ (s^{-1})	K_m (mM)	k_{im} (s^{-1} M^{-1})	log relative k_{im}
Oxaldie 1	7.2	5%	0.63	2.8	5.5	8.7	2.6	3.4
Oxaldie 1	7.2	—	0.47	2.6	6.7	14.2	—	
Oxaldie 2	8.9	5%	0.21	2.3	6.8	33	—	
Oxaldie 2	8.9	—	0.15	2.1	7.5	48	—	
Mutant	9.9	5%	0.028	1.4	—	—	—	
Mutant	9.9	—	0.022	1.3	—	—	—	
PheOEt	7.2	5%	0.013	1.1	—	—	—	
PheOEt	7.2	—	0.012	1.0	—	—	0.024†	1.3
Butylamine‡	10.6	—	0.0011	0.0	—	—	0.0011	0.0
Spontaneous	—	—	—	—	0.013	—	—	

All parameters determined at pH 7, 25 °C, ionic strength = 0.15 (NaCl) and [peptide] = 0.2 mM. Rates were measured by following either the loss of absorbance at 285 nm arising from the enol of oxaloacetate (oxaldie 2, mutant, and simple amines) or the rate of formation of pyruvate using lactate dehydrogenase (oxaldie 1, oxaldie 2) in aliquots treated first with malate dehydrogenase (necessary because of the residual catalytic activity of lactate dehydrogenase with malate).

* Oxaldie 1 is H_2NLeuAlaLysLeuLeuLysAlaLeuAlaLysLeuLeuLysLysCONH$_2$; oxaldie 2 is AcNHLeuAlaLysLeuLeuLysAlaLeuAlaLysLeuLeuLysLysCONH$_2$; Mutant is CH_3CONHLeuAlaLysGlyLeuLysAlaProAlaLysGlyLeuLysLysCONH$_2$; PheOEt is phenylalanine ethyl ester; k_{im} is rate of imine formation. Michaelis–Menten kinetics were observed up to an oxaloacetate concentration of 30 mM. At higher concentrations, oxaldie 1 catalyses a Mannich reaction between two molecules of oxaloacetate. Rate data for oxaldie 1 are corrected for catalytic activity arising from the lysine side chains.

† Interpolated for an amine with a pK_a of 7.2, with correction for steric hindrance.

‡ Extrapolated from ionic strength of 1.

order of magnitude, and it no longer follows Michaelis–Menten saturation kenetics.

Upon mixing oxaldie 1 with oxaloacetate, a transient species was observed by ultraviolet spectroscopy (at 290 nm) in a pre-steady-state 'burst'. This was assigned to enzyme-bound enamine (*E* in Fig. 1)[38,39]. The formation of enamine is first-order in oxaloacetate and is negligibly catalysed by buffers, suggesting that imine formation limits the rate of enamine formation[13,39]. Analysis of the burst suggests that the rate of imine formation in oxaldie 1 is 3–4 orders of magnitude higher than for a simple amine with a pK_a of 10.6 (Table 1).

Natural enzymes catalyse reactions by using the conformational energy of their polypeptide fold to force functional groups in the polypeptide into environments where their reactivity is enhanced, and by exploiting binding between the enzyme and non-reacting portions of the substrates to lower the energy of a transition state relative to a state with free enzyme and substrate[21]. The oxaldies seem to exploit both mechanisms to achieve catalysis. First, placing the reacting amine near the end of a helix (in oxaldie 1) or adjacent to positively charged side chains (in oxaldie 2) lowers its pK_a and increases its reactivity. This contributes more to catalysis in oxaldie 2 ($\Delta pK_a = 1.6$) than in oxaldie 1 ($\Delta pK_a = 0.6$) (Table 1).

But, this alone does not account for catalysis, as imine I_1 is formed with oxaldie 1 100 times faster than expected for an amine with a pK_a of 7.2 (Table 1). The additional catalytic power is probably due to coulombic binding between the cationic peptide and the anionic transition state following carbinolamine C. The dissociation constant of substrate (in all forms) from oxaldie 1, estimated from saturation kinetics of the burst, offers an estimate of the magnitude of these interactions. The dissociation constant for oxaldie 1 ([oxaloacetate] [oxaldie 1]/[complexes]) is ~6 mM. This is four times lower than the K_s dissociation constant estimated (20 mM) for the complex between oxaloacetate and ethylenediamine[13] (where one positive charge on the amine contributes to binding), and far lower than analogous dissociation constants for simple amines (~1 M)[12] in which no positive charges stabilize the intermediate.

Together these factors yield a rate of formation of imine that is 3–4 orders of magnitude larger with oxaldie 1 than with simple amines. This is within the range of rate enhancements achieved by selection strategies that yield catalytic antibodies (10^3–10^6) (ref. 1), and is not insignificant when compared with the 10^8-fold rate enhancement in the natural (metal-dependent) oxaloacetate decarboxylases used in the Rozzell process[9]. Remarkably, an explicitly designed binding pocket is not necessary to achieve these rates. However, an understanding of the kinetics and mechanism of the process is helpful both for guiding design and for understanding what has transpired as a result of the design. From our analysis, imine formation seems not to be rate-determining in the catalytic cycle in oxaldie 1, so efforts to enhance the catalytic power of these peptides must focus on other steps in the reaction sequence. □

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