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Site-directed mutagenesis of the *Actinomadura* R39 DD-peptidase

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The role of various residues in the conserved structural elements of the *Actinomadura* R39 penicillin-sensitive DD-peptidase has been studied by site-directed mutagenesis. Replacement of Ser-298 of the 'SDN loop' by Ala or Gly significantly decreased the k_{cat}/K_m value for the peptide substrate, but only by a factor of 15 and had little effect on the other catalytic properties. Mutations of Asn-300 of the same loop and of Lys-410 of the KTG triad yielded very unstable proteins. However, the N300S mutant could be purified as a fusion protein with thioredoxin that exhibited decreased rates of acylation by the peptide substrate and various cephalosporins. Similar fusion proteins obtained with the N300A, K410H and K410N mutants were unstable and their catalytic and penicillin-binding properties were very strongly affected. In transpeptidation reactions, the presence of the acceptor influenced the k_{cat}/K_m values, which suggested a catalytic pathway more complex than a simple partition of the acyl-enzyme between hydrolysis and aminolysis. These results are compared with those obtained with two other penicillin-sensitive enzymes, the *Streptomyces* R61 DD-peptidase and *Escherichia coli* penicillin-binding protein (PBP) 5.

Abbreviations : 7-ACA, 7-aminocephalosporanic acid ; 5'-Flu-Gly-6-APA, 5'-fluoresceylglycyl-6-aminopenicillanic acid ; PBP, penicillin-binding protein.

INTRODUCTION

Bacterial DD-transpeptidases are the targets of /Mactam antibiotics [1]. Although they have been recognized as active-site serine enzymes for many years [2,3], their detailed catalytic mechanism remains mysterious. The roles of various residues in the *Streptomyces* R61 DD-peptidase [4-7] and *Escherichia coli* penicillin-binding protein 5 (PBP5) [8] have been probed by site-directed mutagenesis of residues chosen on the basis of the three-dimensional structure of the former [9] and of the presence, in all DD-transpeptidases and PBPs of conserved structural elements revealed by sequence alignments [10] and that are found in similar positions in the active sites of the three PBPs of known three-dimensional structures and of the related active-site serine β-lactamases [9-13]. In element 1 (S*XXK, where S* is the active-site serine residue), the two residues whose side chains point into the active site (S* and K) seem to be universally conserved. Similarly, a basic residue is consistently found in the first position of element 3 (KTG in nearly all PBPs, HTG in the R61 DD-peptidase and K(R)T(S)G in β-lactamases). In contrast, two subfamilies of enzymes can be distinguished by the first residue of element 2 (YXN or SXN) [13]. Indeed, although the hydroxy group of the Tyr residue in the R61 DD-peptidase and class C β-lactamases superimposes nicely on that of the Ser residue of class A β-lactamases, it is difficult to assume that their roles are identical. In the former enzymes the phenolate ion is suspected to act as a general base [7,14], a role that would be totally exceptional for a Ser residue. In the *Actinomadura* R39 DD-peptidase (EMBL Databank accession number X64790), this residue is Ser as in the overwhelming majority of PBPs [15]. This soluble enzyme, which exhibits a very specific transpeptidase activity and a high sensitivity to most β-lactam compounds [16,17] is a close relative of *E. coli* PBP4 [15]. In the present study, the importance of the first and third residues of element 2 and of the first residue of element 3 has been investigated by site-directed mutagenesis of the *Actinomadura* R39 DD-peptidase.

Kinetic models

The interaction between the enzyme and both /Mactam antibiotics and its donor substrates can be described by

$$E + C \overset{K}{\rightleftharpoons} EC \overset{k_2}{\rightarrow} EC^* \overset{k_3}{\rightarrow} E + P \qquad (1)$$

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With the antibiotics, k_3 is usually in the range of 10^{-5} to 10^{-6} s^{-1} and the spontaneous decay of the acyl-enzyme, EC*, can be neglected [17]. In the presence of both donor (C) and acceptor (A) substrates, the simplest pathway involves the formation of a ternary EC*A complex, which yields the free enzyme and the transpeptidation product. The enzyme indifferently utilizes peptides, esters and *S*-esters (thiolesters) as donor substrates [18,19]. For one *S*-ester it has been shown that k_2 was much larger than k_3 [19]. The relative values of k_2 and k_3 are not known for the peptide.

MATERIALS AND METHODS

Substrates and *β-lactams*

The peptide substrate N^{α}-acetyl-L-Lys-D-Ala-D-Ala was a gift from UCB Bioproducts (Braine-l'Alleud, Belgium). The *S*-esters benzoyl-Gly-thioglycollate and benzoyl-D-Ala-thioglycollate were prepared as described previously [20]. Benzylpenicillin and cefotaxime were gifts from Roussel Uclaf Pharmaceuticals (Romainville, France), cephalosporin C and cephalothin were from Eli Lilly (Indianapolis, IN, U.S.A.) and 7-aminocephalosporanic acid (7-ACA) was purchased from Janssen (Beerse, Belgium).

Enzymic activity measurements

Unless otherwise stated, all experiments were performed in 10 mM sodium phosphate buffer, pH 7.2, at 37 °C. With the peptide, initial rates were determined by incubating the enzyme with various substrate concentrations and stopping the reaction by the addition of benzylpenicillin to a final concentration of 0.5 mM. The D-alanine released was estimated by the D-amino acid oxidase method [21]. The kinetic parameters were deduced by fitting the initial rate values to the Henri-Michaelis equation or to its linearized form as described by Hanes ([S]/v against [S]). With the *S*-ester, complete time courses were recorded at 250 nm and the results analysed as before [22]. The transpeptidation/ hydrolysis ratios were determined by HPLC as described by Jamin *et al.* [23].

With cephalosporins, the formation of the acyl-enzyme was followed by monitoring the time-dependent quenching of fluorescence [17]; pseudo-first-order rate constants (k_a) were deduced with the help of the Enzfitter program (Elsevier Biosoft, Cambridge, U.K.). The second-order rate constant for enzyme acylation (k_2/K, see [6]) was obtained from the slope of the plot of k_a against cephalosporin concentration. Excitation was at 280 nm (slit width 5 nm) and emission at 344 nm (slit width 15 nm). The enzyme concentrations were 0.09 μM (wild-type), 0.12 μM (S298G and N300S) or 0.06 μM (S298G). The antibiotic concentrations were in the ranges 0.4-0.5 mM (7-ACA), 21-42 μM (cefotaxime), 0.5-1.0 μM (cephalosporin C but 2-4 μM for N300S) and 0.5-1.0 μM (cephalothin, but 10-20 μM for N300S). The results were obtained at 20 °C (7-ACA) or 25 °C (all other compounds).

Production and purification of the modified enzymes

Only the S298A and S298G mutants could be produced by transformation of *Streptomyces lividans* TK24 and purified as previously for the wild-type *Actinomadura* R39 DD-peptidase [24]. The other mutants were produced in *E. coli* GI724 as thioredoxin-fused proteins. When assayed on the *S*-ester substrate, the fused thioredoxin-wild-type R39 protein exhibited the same k_{cat}/K_m value as the original enzyme secreted by *Actinomadura* R39, suggesting that a cleavage of the fused proteins by enterokinase was not necessary for kinetic studies. The thioredoxin-fused N300S mutant was purified by DEAE-cellulose chromatography to more than 80 % homogeneity as quantified by its ability to form a covalent, stable complex with 5'-fluoresceylglycyl-6-aminopenicillanic acid (5'-Flu-Gly-6-APA) (see below). Attempts to purify the other thioredoxin-fused mutant proteins failed owing to the proteins' instability. Enzymic tests were then performed with crude cytoplasmic extracts after dialysis to eliminate the free D-alanine; the amount of enzyme was estimated by enzyme-linked immunoassays, because the sensitivity to the fluorescent penicillin was too low to allow a reliable quantification by titration.

Details on the numerous plasmids constructed to obtain the various proteins, on the conditions used for enzyme production and on the difficulties encountered during these studies will be published elsewhere (details can be readily obtained from the authors).

Rate of thermal denaturation

The enzyme preparations were incubated at 50 °C in 10 mM sodium phosphate buffer, pH 7.2. The time-dependent decrease in protein fluorescence (excitation at 280 nm, emission at 344 nm) was monitored with a

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Perkin-Elmer LS50 luminescence spectrometer. The enzyme concentrations were: wild-type, 0.115 μM ; wild-type-thioredoxin, 0.075 μM; N300S, 0.07 μM; S298A and S298G, 0.02 μM. For residual activity measurements, samples were taken after various periods and assayed at 37 °C with 2 mM N^{α}-acetyl-L-Lys-D-Ala-D-Ala as substrate. The enzyme concentrations were: wild-type, 0.016 μM; wild-type-thioredoxin, 0.011 μM; N300S, S298G and S298A mutants, 0.043 μM.

Penicillin-binding assay

The fluorescein-labelled penicillin [25,26] 5'-Flu-Gly-6-APA (2.5 μM) was used to acylate the R39 wild-type or mutated proteins in 100 mM Tris/HCl, pH 7.7. After 15 min of incubation at 37 °C, the acylated proteins were separated from the excess of 5'-Flu-Gly-6-APA by SDS/PAGE [10 % (w/v) gel] and revealed on a UV transilluminator (λ_{max} 312 nm). Alternatively, the thioredoxin-fused proteins were loaded on an SDS/8 % (w/v) polyacrylamide gel in an A.L.F. DNA sequencer. To each sample an identical amount (30 fmol) of the wild-type R39 DD-peptidase, previously labelled with 5'-Flu-Gly-6-APA, was added, thus providing an internal standard.

Immunological screening

Plates were coated with a mouse monoclonal anti-thioredoxin antibody purchased from Invitrogen (Leek, The Netherlands) and incubated for 1 h at 37 °C with crude cytoplasmic extracts. Thereafter, rabbit anti-(*Actinomadura* R39 DD-peptidase) anti-serum obtained from Gamma S.A. (Liège, Belgium) was added and the complex was then recognized by a pig anti-rabbit antibody coupled to peroxidase. The cytoplasmic fraction of *E. coli* transformed with pTrxFus (Invitrogen) without insert and a known concentration of purified thioredoxin-fused wild-typeR39 enzyme served as negative and positive controls respectively.

RESULTS

Stability

Incubation at 50 °C resulted in the quite rapid and irreversible denaturation of the protein, which could be followed by monitoring the time-dependent decrease in protein fluorescence or enzymic activity. Both methods revealed first-order phenomena that seemed to be concomitant. The rate constants (means ± S.D.) ranged from $(6.7 \pm 0.04) \times 10^{-3}$ s^{-1} (wild-type) to $(2.1 \pm 0.4) \times 10^{-2}$ s^{-1} (S298G). Interestingly, the wild-type-thioredoxin hybrid exhibited the same stability as the wild-type enzyme and the instability of the most sensitive mutant (S298G) was increased only 3-fold compared with the wild-type enzyme.

Kinetic parameters

Table 1 shows the results obtained with the peptide and *S*-ester substrates. In some cases, the ionic strength and pH were increased in an attempt to obtain higher enzymic activities. With the K410H mutants, tests were performed at both pH 5.5 and pH 8.0 to estimate the effects of a possible protonation of the His side chain at the lower pH.

Table 1 Kinetic parameters of the Actinomadura R39 wild-type and mutant DD-peptidases for the hydrolysis of N^{α}-acetyl-L-Lys-D-Ala-D-Ala peptide and benzoyl-D-Ala-thioglycollate S-ester substrates
Data are shown as means ± S.D. Abbreviations: AcKAA, N^{α}-acetyk-Lys-D-Ala-D-Ala; n.d. not determined.

	AcKAA				Benzoyl-D-Ala-thioglycollate			
Enzyme	E_0 (μM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	E_0 (μM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
Wild-type	0.06	18 ± 0.08	0.28 ± 0.04	63000 ± 1000	0.19	5.6 ± 0.1	15.3 ± 0.4	366000 ± 11000
S298A	0.21	5.1± 0.3	1.3 ± 0.1	4000 ± 200	0.13	1.1 ± 0.1	15.5 ± 0.9	70000 ± 5000
S298G	0.38	7.7 ± 0.2	1.2 ± 0.1	6700 ± 120	0.08	1.5 ± 0.1	14 ± 1	107000 ± 5000
N300S	0.11	2.8 ± 0.2	2.8 ± 0.1	1000 ± 100	0.07	2.1 ± 0.2	14 ± 0.7	150000 ± 4000
N300A	7.2	$< 10^{-3}$	1.5 ± 0.15	0.13 ± 0.03*	2.2	‡	‡	‡
K410H	10.2	$< 10^{-3}$	1.4 ± 0.1	0.36 ± 0.04†	3.0	n.d.	n.d.	14 ± 5§
K410H	10.2	$<10^{-3}$	1.5 ± 0.2	0.33 ± 0.13*	3.0	n.d.	n.d.	11 ± 1†§
K410N	6.2	$<10^{-3}$	1.5 ± 0.1	0.55 ± 0.06*	1.9	<0.1	>500	45 ± 5

* In 0.1 M Tris/HCl, pH 8.0, containing 0.2 M NaCl and 50 mM $MgCl_2$.
† In 0.1 M sodium cacodylate/HCl, pH 5.5.
‡ Not detectable; there was no significant variation in absorbance at 250 nm after 1 h.
§ Calculated from initial rate measurements.

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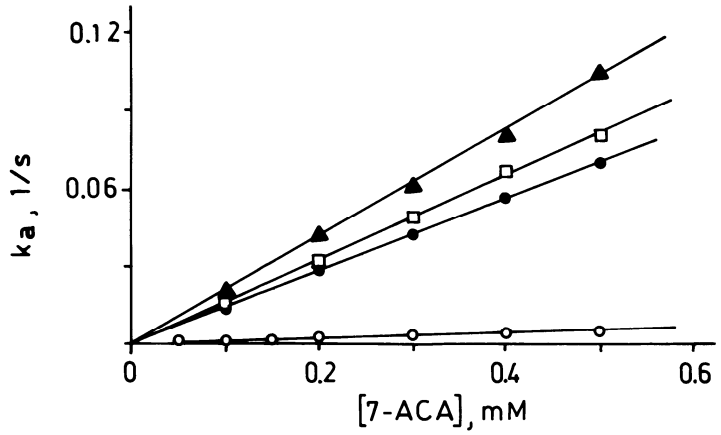
Transpeptidation reaction

With the *S*-ester and the wild-type enzyme, the deacylation step is rate-limiting [19] so that an increase in both k_{cat} and K_m was expected in the presence of D-alanine, a good acceptor. This was indeed observed but, in contrast with what occurred with the R61 DD-peptidase [19,23], a significant increase in the k_{cat}/K_m ratio was also observed. This trend was even more apparent with the three active mutants, S298A, S298G and N300S (Table 2), particularly with the latter. Accordingly the transpeptidation/ hydrolysis ratios measured with two good acceptors also increased for the three modified proteins. The behaviour of the N300S mutant in the presence of increasing concentrations of D-alanine was studied in more detail. Between 0 and 100 mM D-alanine the k_{cat} value increased in a nearly linear manner, from 2.1 ± 0.2 to 445 ± 40 s^{-1} and the K_m value increased similarly, although less markedly, from 14 ± 0.7 to 240 ± 30 μM. In consequence, k_{cat}/K_m increased from 0.15 to 1.9 $\mu M^{-1} \cdot s^{-1}$. Under the same conditions, the k_{cat}/K_m value of the wild-type protein increased only from 0.37 ± 0.01 to 0.49 ± 0.03 $\mu M^{-1} \cdot s^{-1}$. With the K410 mutants, the low activity restricted the analysis to initial rate measurements. Nevertheless in the presence of 0.1 mM benzoyl-Gly-thioglycollate the addition of 100 mM D-alanine increased the initial rates approx. 20-fold, compared with 5-fold with the wild-type protein.

Table 2 Effects of the acceptors on the k_{cat}/K_m parameters and the transpeptidation/hydrolysis ratios
The values in column two are the ratios of the k_{cat}/K_m values observed in the presence and the absence of 100 mM D-alanine respectively. The k_{cat}/K_m values were deduced from the analysis of complete time courses. The transpeptidation/hydrolysis ratios were measured under conditions where less than 50% or 30% of the donor substrate was utilized by the wild-type or mutant enzymes respectively. Data are shown as means ± S.D.

Enzyme	$(k_{cat}/K_m)_{100}/(k_{cat}/K_m)_0$ acceptor D-alanine	Transpeptidation/hydrolysis ratio with	
		5 mM D-Ala	2 mM D-GIU
Wild-type	1.34 ± 0.12	1.1 ± 0.05	2.6 ± 0.1
S298A	2.40 ± 0.22	1.5 ± 0.1	3.0 ± 0.2
S298G	1.8 ± 0.16	1.5 ± 0.3	3.4 ± 0.1
N300S	12.5 ± 0.7	24.6 ± 1.0	20 ± 0.5

Figure 1 Variation in the pseudo-first-order rate constant (k_a) for the acylation of the wild-type and mutant R39 peptidases by increasing concentrations of 7-ACA at 20 °C
SD values are 6% at most (n = 3 or 4). For conditions, see the text.



Interaction with *β*-lactam antibiotics

Cephalosporins, and not penicillins, induce a decrease in the fluorescence of the *Actinomadura* R39 enzyme owing to the formation of the acyl-enzyme [17]. For this reason the characteristic rate constants were measured with 7-ACA, cefotaxime, cephalosporin C and cephalothin. As stated above, the three 'active' mutants also remained sensitive to benzylpenicillin, which allowed the titration of their active site by the latter compound. With all compounds tested, the k_a values increased linearly with the inactivator concentration (see, for example,

Figure 1), indicating that the highest cephalosporin concentration remained well below *K*. With the S298 mutants, the second-order acylation rate constants, k_2/K, were not significantly different from those of the wild-type enzyme, i.e. 140 ± 10 (7-ACA), 960 ± 40 (cefotaxime), 33 000 ± 1600 (cephalosporin C) and 59 000 ± 3000 $M^{-1} \cdot s^{-1}$ (cephalothin) with the exception of the 7-ACA-S298G value, which was slightly increased (210 ± 10 $M^{-1} \cdot s^{-1}$). In contrast, the rates of acylation of the N300S mutant by 7-ACA (9.2 ± 0.3 $M^{-1} \cdot s^{-1}$), cephalosporin C (1100 ± 200 $M^{-1} \cdot s^{-1}$) and cephalothin (1300 ± 200 $M^{-1} \cdot s^{-1}$) were significantly decreased but, surprisingly, with cefotaxime only a very slight decrease (590 ± 80 $M^{-1} \cdot s^{-1}$) was observed, a result that remains unexplained but might be related to the bulky side chain of this third-generation cephalosporin. As stated above, the three other mutants did not seem to bind the fluorescent penicillin and in consequence were not studied further.

DISCUSSION

Stability of the mutants

Owing to the irreversibility of the thermal denaturation process, it was not possible to deduce thermodynamic parameters for this reaction. However, the rate of the phenomenon, measured by monitoring both the fluorescence intensity and the enzyme activity, was very similar in all cases. In particular, the presence of the fused thioredoxin moiety did not significantly influence the stability of the wild-type enzyme. The largest increase in thermal sensitivity was recorded with the S298G mutant. This factor of 3 corresponds to a 0.66 kcal (2.76 kJ) decrease in the activation energy of the denaturation reaction. It could correspond to the disappearance of a very poor hydrogen bond and it is reasonable to assume that the mutations do not induce major structural modifications.

Kinetic properties of the mutants

The *Actinomadura* R39 DD-peptidase is the third penicillin-sensitive enzyme to be studied in detail by site-directed muta-genesis. On the basis of the structure of the second conserved element (SNN), it is expected to be more similar to *E. coli* PBP5 than to the R61 DD-peptidase, which are respectively 'SXN' and 'YXN' PBPs. Note that the R61 enzyme also exhibits an 'HTG' third element, whereas all other known PBPs contain Lys as the first residue of this element.

Second element: Ser-298

The role of the first residue of the second element seems to be much less crucial than that of the corresponding side chains in both *E. coli* PBP5 and the *Streptomyces* R61 DD-peptidase. Indeed, the hydrolysis rates of both the peptide and the *S*-ester remained higher than 5 % of those of the wild-type protein and the rate of acylation by 7-ACA was even slightly increased in one of the mutants where the hydroxy group of the side chain was eliminated. In a way this is reminiscent of the situation in class A /Mactamases, where the corresponding S130A and S130G mutations did not result in spectacular effects on the k_{cat}/K_m values for penicillins. However, in these cases the rate of acylation by cephalosporins was affected much more [27], a result in marked contrast with that observed here.

Second element: Asn-300

The N300A mutant was completely inactive, whereas the N300S mutation had mild to medium effects on the enzymic properties, results that highlight the crucial importance of the hydrogen-bonding properties of the Asn-300 side chain. With the N300S mutant, the rate of acylation by the peptide substrate was affected much more (67-fold) than that of the *S*-ester (2.6-fold). This is in agreement with a role of the Asn-300 side chain in the positioning of the substrate in the catalytic cavity, which needs to be more accurate for the peptide than for the more reactive *S*-ester. With the *β*-lactams, the rates of acylation of the N300S mutant decreased to 1/20 and 1/45 respectively with cephalosporin C and cephalothin, which would suggest a distortion of a potential hydrogen bond formed between the Asn-300 amino group and the carbonyl oxygen of the antibiotic exocyclic amide, as proposed for the R61 enzyme [9,13]. However, the decreased acylation rate by 7-ACA (15-fold) remains unexplained, as does the very small influence (1.6-fold) of the mutation on the interaction with cefotaxime, which was noted in the Results section.

Third element: Lys-410

Both mutants were nearly inactive. Moreover, a low pH did not markedly increase the activity of the K410H mutant, indicating that a protonated His side chain could not successfully replace the alkylammonium of the Lys

residue. This situation is similar to that found for PBP5 [8] and class C /Mactamases [28] but very different from that prevailing with the class A β-lactamase from *Streptomyces albus* G [29]. With the class A enzymes, it seems that a positive side chain in this position is sufficient to retain a large proportion of the activity [29,30]. In all cases the presence of an uncharged residue in this position is highly detrimental. However, a comparison with the R61 DD-peptidase is quite difficult because the wild-type enzyme contains a His residue in this position, of which the ionization status remains undetermined.

The transpeptidation reaction

The relative transpeptidation properties of the S298 mutants did not differ markedly from those of the wild-type enzyme. However, with the wild-type and active modified proteins, an increase in the k_{cat}/K_m value was observed in the presence of the acceptor substrate, the most spectacular effect being recorded with the N300S mutant. This is in complete contrast with the results previously obtained with the R61 DD-peptidase and the same acceptor, D-alanine [19,23], and suggests that, in the present case, the acceptor can bind to either the free enzyme or the noncovalent E-S complex or both. This alternative pathway would become predominant for the N300S mutant, which, at high acceptor concentrations becomes even more efficient than the wild-type enzyme (with 100 mM D-alanine, $k_{cat}/K_m = 1.9\ \mu M^{-1} \cdot s^{-1}$ compared with 0.49 for the wild-type enzyme). The transpeptidation mechanism of the R39 enzyme thus seems to be more complex than that proposed for its R61 counterpart [23] and is currently under further investigation. Another major difference concerns the role of the first residue of the second element. As stated above, the S298A and S298G replacements do not modify the transpeptidation/hydrolysis ratio for the R39 enzyme, whereas both the Y159F and Y159S mutations strongly decrease this ratio for the R61 DD-peptidase.

Comparison with PBP5 and conclusions

As stated in the Introduction section, among the compared PBPs the residues that constitute the conserved elements are most similar in PBP5 and the R39 enzyme. If, to a first approximation, it can be considered that the modifications of the Asn and Lys of the second and third elements respectively result in similar alterations of the properties of both enzymes, the situation is very different for the Ser residue of the second element. The peptidase activity of both the S298A and S298G mutants of the R39 enzyme are only slightly affected, whereas that of the corresponding PBP5 S110A mutant disappears completely. One can conclude that, in those PBPs that contain similar SXN second elements, the role and importance of the first residue might be rather different. In consequence, the elucidation of the structure of one PBP will certainly not be sufficient to understand the catalytic mechanisms of all of those exhibiting nearly identical conserved elements.

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