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The mutation Lys234His yields a class A β-lactamase with a novel pH-dependence

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The lysine-234 residue is highly conserved in β-lactamases and in nearly all active-site-serine penicillin-recognizing enzymes. Its replacement by a histidine residue in the *Streptomyces albus* G class A β-lactamase yielded an enzyme the pH-dependence of which was characterized by the appearance of a novel pK, which could be attributed to the newly introduced residue. At low pH, the $k_{cat.}$ value for benzylpenicillin was as high as 50% of that of the wild-type enzyme, demonstrating that an efficient active site was maintained. Both $k_{cat.}$ and $k_{cat.}/K_m$ dramatically decreased above pH 6 but the decrease in $k_{cat.}/K_m$ could not be attributed to larger K_m values. Thus a positive charge on the side chain of residue 234 appears to be more essential for transition-state stabilization than for initial recognition of the substrate ground state.

INTRODUCTION

In class A β-lactamases the lysine-234 residue [the ABL numbering of class A β-lactamases is used throughout this paper (Ambler *et al.*, 1991)] is conserved in 19 out of the 20 known sequences, the only exception being the PSE-4 β-lactamase, where an arginine residue is found (Ambler *et al.*, 1991). It is the second residue of a DKT(S)G tetrad where the threonine or serine residue occurs in the third position. The tetrad is situated on the last strand of the β-pleated sheet and borders the active site, itself located at the hinge between the all α- and the α/β-domains. The side chain of K234 points into the active site and has been assumed to participate in binding of the substrate through the formation of a salt-bridge with the free carboxylate on C-3 of penicillins or C-4 of cephalosporins (Herzberg & Moult, 1987). In class C β-lactamases, a conserved KTG triad is found in a similar position, as shown by X-ray diffraction data (Oefner *et al.*, 1990). Class D β-lactamases and nearly all the penicillin-binding proteins of which the primary structures are known also contain a similar KTG triad 60–100 residues from the *C*-terminus of the protein (Joris *et al.*, 1988). It is widely believed that these residues are also situated near the active-site serine side chain in the three-dimensional structure, forming one of the walls of the active-site cavity. The sole exception is the *Streptomyces* R61 DD-peptidase, where an HTG sequence exists in a similar position (Joris *et al.*, 1988). This protein is the only penicillin-sensitive DD-peptidase for which a three-dimensional structure is at present available (Kelly *et al.*, 1989).

The spatial arrangements of several secondary-structure elements are quite comparable between the R61 DD-peptidase and the β-lactamases of classes A and C and, if the active-site serine residues are superimposed, the HTG triad of the former also superimposes nicely on to the KTG of the latter.

By site-directed mutagenesis, Ellerby *et al.* (1990) have replaced the lysine residue of the *Bacillus licheniformis* class A β-lactamase by alanine and glutamic acid, which resulted in a drastic decrease in the $k_{cat.}/K_m$ values of the enzyme towards its usual good substrates. Surprisingly, the most affected parameter was $k_{cat.}$, which indicated that lysine-234 contributed more to transition-state stabilization than to the initial binding of the substrate.

In the present work, we have replaced the corresponding lysine residue in the class A β-lactamase of *Streptomyces albus* G by a histidine residue. Our goals were to evaluate if this replacement would confer a characteristic pH-dependence to the catalytic parameters of the mutant enzyme and increase its catalytic efficiency as a transpeptidase, making it more similar to the R61 DD-peptidase. Indeed, like some other β-lactamases, the *S. albus* G β-lactamase behaves as a poor carboxypeptidase and an even poorer transpeptidase when suitable depsipeptide substrates (esters and thioesters) are used as carbonyl donors and D-amino acids as acceptors (Adam *et al.*, 1990).

MATERIALS AND METHODS

β-Lactam compounds

Benzylpenicillin was from Rhône–Poulenc (Paris, France), 6-aminopenicillanic acid and ampicillin were from Bristol Benelux (Brussels, Belgium), β-iodopenicillanic acid was from Pfizer Central Research (Sandwich, Kent, U.K.) and cephaloridine and cephalothin were from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). These antibiotics were kindly given by the respective companies. Nitrocefin was purchased from Oxoid (Basingstoke, Hants., U.K.).

Constructs for expression in *Streptomyces*

The genetic construct for expression of the wild-type enzyme was that described by Dehottay *et al.* (1986). The mutated gene was obtained by the M13 double-primer method based on Zoller & Smith (1984), with the use of the mutL *Escherichia coli* strain BMH71.18 (Kramer *et al.*, 1984).

Oligonucleotide synthesis was performed on a 0.2 μM scale with an Applied Biosystems model 381A DNA synthesizer. The activated phosphoramidite monomers were from Cruachem Ltd. (Glasgow, U.K.). The 24-mer oligonucleotide dGGACCCTCGGCGACCACACCGGCG was purified on an oligonucleotide purification cartridge (Applied Biosystems).

Abbreviations used: 1 × SSC, 0.15 M-NaCl/0.015 M-sodium citrate, pH 7.2; pH*, pH-meter reading in ^2H_2O.

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The DNA sequence of the mutated gene was confirmed by using a battery of oligonucleotides priming at intervals along the gene. Sequencing reactions were carried out at 37 °C in the presence of deazaGTP to help resolve compressions due to the G+C-rich *Streptomyces* DNA sequences.

For expression, the *Kpn*I–*Pst*I fragment of the modified gene was cloned in the *Streptomyces* plasmid used by Jacob *et al.* (1990*a*). The presence of the mutation was confirmed by *Streptomyces* colony hybridization according to the following protocol (N. Davis, unpublished work). Inoculated plates were incubated overnight and overlayed with Whatman 541 filter paper previously soaked in thiostrepton (0.5 mg/ml, 1 ml per filter). Incubation was continued for 48 h. The filters were removed and prepared for hybridization while incubation of the plates was continued to provide a 'master' plate. The filters, processed batchwise, were boiled in 0.5 M-NaOH containing 1% (w/v) SDS for 5 min, washed twice with 0.5 M-Tris/HCl buffer, pH 7.5, for 5 min and once in 90% (v/v) ethanol, with 5–10 ml of each solution per filter, before air-drying. For hybridization itself, a standard procedure was used (Grunstein & Hogness, 1975; Woods, 1984) with an end-labelled oligonucleotide probe at a final concentration of 5 ng/ml in 6 × SSC at T_m −5 °C.

Enzyme purification

The modified enzyme was purified as described by Matagne *et al.* (1990) except that the last step (chromatofocusing on Mono P) was omitted. However, chromatofocusing on Mono P was performed on an analytical scale to determine the specific activity of the mutant enzyme. Preliminary tests indicated that the mutant enzyme was much more active below pH 6.0, so that all tests during purification were routinely carried out in 50 mM-acetic acid/sodium acetate buffer, pH 5.5.

Thermal inactivation

The pH-dependence of the thermal inactivation of the wild-type and mutant enzymes was determined in the same buffers as below by continuously monitoring the hydrolysis of a reporter substrate (benzylpenicillin) as described by De Meester *et al.* (1987).

pH-dependence of β-lactamase activity

The determination of the kinetic parameters was performed as described by Matagne *et al.* (1990).

The following buffers were used, all 50 mM: sodium acetate/acetic acid, pH 4–5.5, sodium cacodylate, pH 5–6.5, sodium phosphate, pH 6–7, Tris/HCl, pH 8–9, and sodium carbonate/sodium bicarbonate, pH 10. All buffers were adjusted to the desired pH by addition of HCl. The conductance (cell constant: 11.6 cm^{-1}) of the various buffers was increased to 600 μS (the measured conductance of 50 mM-phosphate buffer, pH 7, at 30 °C) by the addition of an adequate amount of NaCl.

Fitting of the experimental results to theoretical curves was performed by using a non-linear-regression method.

Thioesterase activity

Hydrolysis of the thioester substrate hippuryl thioacetate (Adam *et al.*, 1990) was performed with both the wild-type and the K234H enzymes at pH 5.5, 7 and 8.5. The buffers were respectively 50 mM-sodium acetate, pH 5.5, 50 mM-sodium phosphate, pH 7, and 50 mM-Tris/HCl, pH 8.5. The ionic strength of the buffers was adjusted as above. Hydrolysis was monitored spectrophotometrically at 250 nm by using absorption coefficient variations of $-2000\ M^{-1}\cdot cm^{-1}$ (pH 5.5), $-2200\ M^{-1}\cdot cm^{-1}$ (pH 7) and $-2300\ M^{-1}\cdot cm^{-1}$ (pH 8.5). For the wild-type enzyme complete time courses were recorded, and for the K234H mutant initial rates were measured, from which $k_{cat.}/K_m$ values were deduced.

Transpeptidation assays

Transpeptidation experiments were performed at pH 7.0 and 8.5 with the wild-type and the K234H β-lactamases with 1 mM-thioester as a donor and 20 mM-D-alanine as an acceptor. The reaction mixtures were incubated until at least 25% of the donor was utilized. Samples were injected on to a C_{18} reverse-phase column and elution was performed with 10% (v/v) acetonitrile in 10 mM-acetic acid, pH 3. The quantities of the residual thioester, hippuric acid and hippuryl-D-alanine were determined by integration of the corresponding peaks.

N.m.r. spectra

These were recorded on a Bruker AM400 spectrometer. The enzyme solution was extensively dialysed against 50 mM-sodium phosphate buffer, pH 4.5, in ^2H_2O and concentrated to about 3 mg/ml. At each pH* value 200 scans were accumulated after pre-saturation of water. The pH* was progressively increased by addition of 0.01 M-NaO^2H and measured with a glass electrode conditioned in water.

RESULTS

Expression and production in *Streptomyces*

The enzyme was produced in 3 litres of modified YEME medium as described by Erpicum *et al.* (1990). On the basis of the specific activity of the purified mutant, production could be estimated at about 60 mg/l.

Purification was performed as explained by Matagne *et al.* (1990) for the wild-type protein. After the Q-Sepharose column, the preparation yielded a single band upon SDS/PAGE. At that stage, the specific activity of the enzyme was 390 μmol of benzylpenicillin hydrolysed/min per mg of enzyme at pH 7.0 and 1700 μmol/min per mg at pH 5.5. Chromatofocusing of this preparation yielded three active peaks eluted between pH 4.7 and 4.4, a pattern that was very similar to that obtained with the wild-type protein (Matagne *et al.*, 1991).

The specific activities, at pH 7.0, of the enzyme contained in the three peaks were respectively 210, 640 and 620 units/mg of protein. Since the first peak was relatively much less important, this indicated that the preparation after the Q-Sepharose column eluate contained about 60% of fully active enzyme molecules. The values given below were accordingly corrected.

Table 1. pH-dependence of the rate of thermal inactivation

First-order rate constants were determined from the rate of decrease in enzymic activity. At pH 5, the experiments were performed in both acetate and cacodylate buffers, and at pH 6 in cacodylate and phosphate. The results were not significantly modified by the nature of the buffer. At 45 °C and pH 7, the k_{in} value for the wild-type enzyme was $0.21 \times 10^{-3}\ s^{-1}$. Abbreviation: N.D., not determined.

	$10^3 \times k_{in}$ (s^{-1})	
pH	Wild-type (51 °C)	Lys234His (45 °C)
4.0	16.8±0.3	84±6
5.0	1.6±0.2	9.2±0.6
6.0	2.3±0.3	11±1.0
7.0	2.45±0.15	17±1.0
7.5	N.D.	34±4.0
8.0	9.5±2	> 100
9.0	68±8	

Table 2. Substrate profiles of the wild-type and Lys234His enzymes

When a minimum value is given for K_m, the time course was first-order at the mentioned concentration. Individual $k_{cat.}$ and K_m values were derived from complete time courses and, in that case, the $k_{cat.}/K_m$ ratio was computed from those values. Abbreviations: N.D., not determined.

	Wild-type at pH 7.0*			Lys234His mutant				
				At pH 5.5				At pH 7.0
Substrate	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)	Relative $k_{cat.}/K_m$†	$k_{cat.}/K_m$ ($mM^{-1} \cdot s^{-1}$)
6-Aminopenicillanic acid	720	0.2	3700	> 80	> 2	40±4	90	N.D.
Benzylpenicillin	2800	1	2800	1400±150	5.5±0.5	250±40	11	63±1
Ampicillin	3900	0.65	6100	> 275‡	> 1	275±15	22	48±2
Nitrocefin	2500	1.1	2400	600±100	1.6±0.3	400±50	6	50±7
Cephaloridine	200	0.32	620	> 8	> 1	8±0.1	78	1.4±0.05
Cephalothin	260	0.72	370	> 0.4	> 0.6	0.7±0.02	530	N.D.

* Data from Matagne *et al.* (1990).
† $(k_{cat.}/K_m)_{(wild\text{-}type)}/(k_{cat.}/K_m)_{(mutant)}$.
‡ When 10 mM-ampicillin was used, the parameters could not be determined because of the presence of a detectable lag in the hydrolysis time-course.

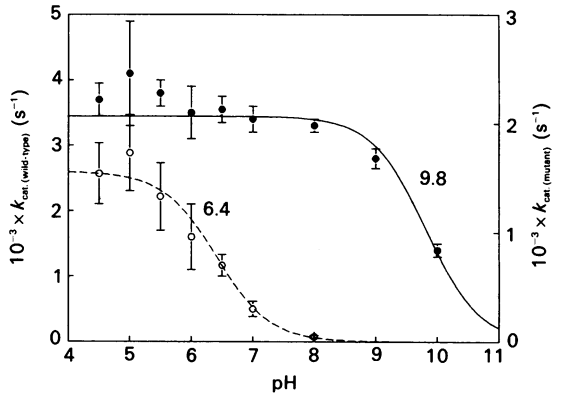

Fig. 1. Comparison of the pH-dependence of the $k_{cat.}$ values of the wild-type (●) and Lys234His (○) β-lactamases with benzylpenicillin

The data were fitted to eqn. (1):

$$y = \frac{y_{max.}}{1 + \frac{K_2}{[H^+]}} \quad (1)$$

which yielded the continuous curves and values of 9.8±0.05 and 6.4±0.05 for the pK_2 of the wild-type and mutant proteins respectively. The $k_{cat.}$ values were derived from complete time courses with an initial penicillin concentration of 10 mM with the mutant and 2–10 mM with the wild-type enzyme.

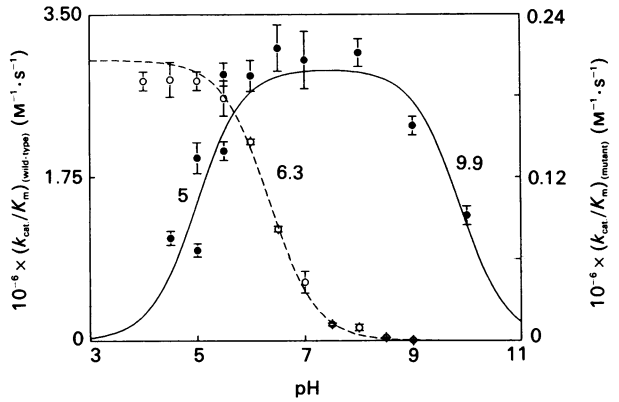

Fig. 2. pH-dependence of the $k_{cat.}/K_m$ values of the wild-type (●) and Lys234His (○) β-lactamases with benzylpenicillin

The data for the wild-type enzyme were fitted to eqn. (2):

$$y = \frac{y_{max.}}{\frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H^+]}} \quad (2)$$

which yielded the continuous line and values of 9.9±0.2 and 5±0.2 for pK_2 and pK_1 respectively. At pH 5.0 and 5.5, the lower values are those observed in cacodylate buffer. The data for the mutant were fitted to eqn. (1), yielding $pK_2 = 6.3 \pm 0.06$. The values were derived from complete time courses with initial penicillin concentrations of 0.5–1.5 mM. With the mutant, the values were confirmed under initial-rate conditions at 0.5 mM-substrate. This explains the 30% difference from the value given in Table 2, which was derived from the ratio of the individual $k_{cat.}$ and K_m values as obtained under intermediate-order conditions.

The absence of contamination by the wild-type protein was demonstrated at pH 7.0 and in the presence of 1 M-NaCl by incubating a portion with 4–7% of the stoichiometric amount of β-iodopenicillanate. Under these conditions the presence of a detectable proportion of wild-type enzyme would have resulted in a disproportionate decrease in the total activity. No such phenomenon was observed.

pH-dependence of thermal inactivation

Table 1 shows that the mutant was significantly less stable than the wild-type enzyme throughout the investigated pH range.

Substrate and pH–activity profiles

The results are reported in Table 2. The individual values of $k_{cat.}$ and K_m were only obtained for benzylpenicillin and nitrocefin with the Lys234His mutant. A 4–8-fold decrease was observed for the $k_{cat.}/K_m$ values when the pH was increased from 5.5 to 7.0. A more detailed analysis of the pH-dependence of the kinetic parameters was performed with benzylpenicillin and ampicillin as substrates (Figs. 1–3). With the former substrate, the individual values of $k_{cat.}$ and K_m were obtained. With the wild-type enzyme, the K_m values (not shown) were stable between pH 10 and 6.0 (1 mM) and sharply increased under more acidic conditions. With the mutant, the K_m values decreased monotonously from 9 mM at pH 4.0 to 4 mM at pH 8.0.

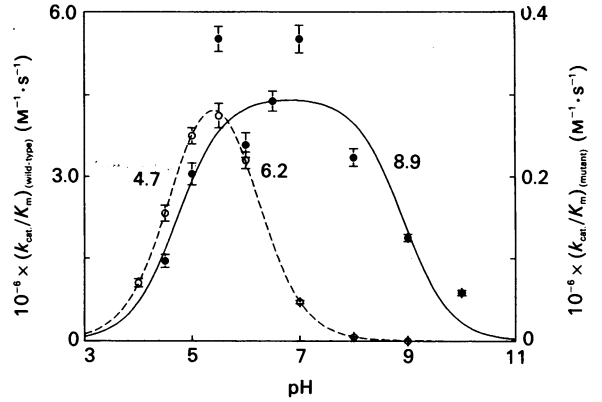

Fig. 3. pH-dependence of the $k_{cat.}/K_m$ values of the wild-type (●) and Lys234His (○) enzymes for ampicillin

The data were fitted to eqn. (2), yielding $pK_1 = 4.7 \pm 0.3$ and $pK_2 = 8.90 \pm 0.20$ for the wild-type and $pK_1 = 4.66 \pm 0.03$ and $pK_2 = 6.15 \pm 0.02$ for the mutant.

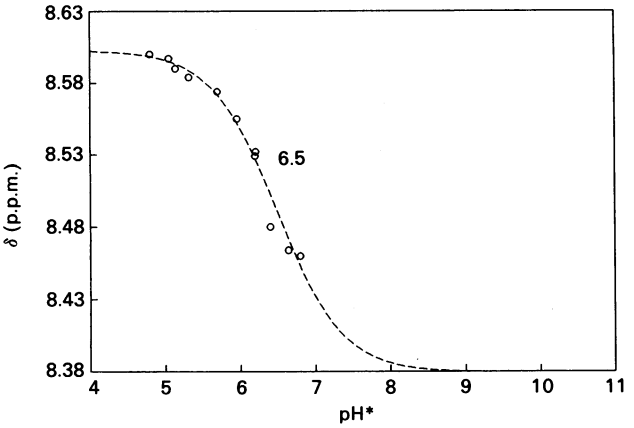

Fig. 4. Titration of the histidine residue of the Lys234His mutant by ^1H n.m.r.

The chemical shift of the C-2 proton is plotted against pH. The curve was drawn by fitting the experimental points to equation:

$$y = y_{min.} + \frac{y_{max.} - y_{min.}}{1 + \frac{K}{[H^+]}}$$

where $y_{min.}$ and $y_{max.}$ are respectively the limit values of the chemical shift at high and low pH. The values deduced from the fitting procedure were $pK = 6.44 \pm 0.13$, $y_{max.} = 8.60 \pm 0.006$ and $y_{min.} = 8.38 \pm 0.027$. Above pH 6.8, the signal-to-noise ratio became too poor to allow an accurate determination of the chemical shift.

Table 3. pH-dependence of $k_{cat.}/K_m$ values for the hydrolysis of the thioester

Enzyme	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)		
	At pH 5.5	At pH 7.0	At pH 8.5
Wild-type	16000±1000	22000±2400	16500±2500
Lys234His	600±100	100±10	< 10

With the wild-type, but surprisingly not with the mutant enzymes, discontinuities were observed in the plots of $k_{cat.}/K_m$ versus pH when the nature of the buffer was modified. With benzylpenicillin and ampicillin, that phenomenon only concerned the K_m values and had a double origin. Firstly, the K_m value increased with increasing ionic strength. Secondly, at a given ionic strength, some buffers appeared to specifically increase the K_m value: at pH 6.4, for example, and an ionic strength of 0.2, the K_m value was twice as large in phosphate as in cacodylate. With the former buffer, this specific effect only became significant at pH values lower than 7.0. When cacodylate and acetate were compared at pH 5.5 and at an ionic strength lower than 0.1, no strong differences were observed. Since the buffer did not appear to influence $k_{cat.}$, the data of Fig. 1 represent averages of all the measured values. $k_{cat.}/K_m$ values obtained in phosphate at pH below 7.0 with benzylpenicillin were not included in Fig. 2. By contrast with ampicillin, experiments were only performed in acetate and phosphate (Fig. 3). The discontinuity between pH 5.5 (acetate) and 6.0 (phosphate) is clearly visible.

Hydrolysis of the thioester

Table 3 summarizes the results obtained with the thioester and indicates that the pH-dependence of the mutant with this substrate was similar to that observed with benzylpenicillin. Transpeptidation was only observed with the wild-type enzyme at pH 8.5 (about 5% of the hydrolysis). At pH 7.0 and with both mutant and wild-type enzymes, the transpeptidation product was barely detectable. At pH 8.5, the mutant activity was too low to allow meaningful quantification of hydrolysis, let alone transpeptidation.

Titration of the histidine residue

The major forms of the wild-type enzyme do not contain any histidine and the modified residue is thus the sole histidine residue of the mutant. Fig. 4 shows the titration of this residue as performed by n.m.r. The results fit a theoretical curve indicating a pK of 6.44 ± 0.13 for the titrated residue, in excellent agreement with the pK values determined on the basis of interaction with the substrate.

DISCUSSION

The introduction of a histidine side chain into the active site of an enzyme presents the advantage that the involvement of that residue in catalysis should result in a characteristic pH-dependence of some of the kinetic parameters of the enzyme. If the pK of the imidazole group is not grossly perturbed by the environment, easily detectable effects should be recorded in a pH range where one can expect few difficulties arising from secondary phenomena such as enzyme stability. The results obtained in our study clearly demonstrate the validity of this strategy.

First, it was necessary to determine the pH-dependence of the kinetic parameters of the wild-type *S. albus* G enzyme. With benzylpenicillin, the pH-dependence of $k_{cat.}/K_m$ indicated pK_1 and pK_2 values of about 5.0 and 9.9 respectively. Surprisingly, for the $k_{cat.}$ values, the first pK was not detected, possibly indicating a value below 4.0. The second pK was similar to that observed with $k_{cat.}/K_m$. These results can be compared with those obtained by Waley (1975) and Ellerby *et al.* (1990) with two other class A β-lactamases, from *Bacillus cereus* and *B. licheniformis* respectively. These authors reported pK values of 4.85–5.0 and 8.6 for $k_{cat.}/K_m$ and 4.5–4.85 and 8.1–8.6 for $k_{cat.}$ when very similar substrates (benzylpenicillin and phenoxymethylpenicillin) were studied. Our results thus indicate that the *S. albus* G β-lactamase appears to have an expanded 'optimum' pH range. The K_m increase at low pH values also characterizes our enzyme. With ampicillin, the differences are less pronounced but also indicate a lower pK_1 (4.7 versus 5.4) and a higher pK_2 (8.9 versus 8.6) than those of the *B. cereus* β-lactamase I (Waley, 1975). It is

noteworthy that the sequences of the two *Bacillus* enzymes also exhibit a high degree of isology, so that one could also expect them to resemble each other more closely than the *S. albus* G β-lactamase for other properties.

Ellerby *et al.* (1990) replaced the lysine-234 residue by alanine and glutamic acid in the *B. licheniformis* β-lactamase. In the pH range 4.0–8.5, this caused a dramatic decrease in the enzymic activity. The $k_{cat.}/K_m$ values never amounted to more than 0.4% of those of the wild-type enzyme, with $k_{cat.}$ being most affected at low pH and K_m at high pH. For the K234A mutant, the $k_{cat.}$ value at pH 8.5 was 12% of that of the wild-type, but the value of K_m increased 50-fold between pH 4.0 and 8.5. In contrast, the Lys234His mutant described here exhibited a $k_{cat.}$ value very close to that of the wild-type enzyme at pH $\leqslant$ 5.5, indicating that, under these conditions, the active site remained completely functional. The K_m of the mutant was slightly higher than that of the wild-type enzyme at all pH values, but decreased between pH 4.0 and 8.0. This behaviour was opposite to that expected if the protonated form of the histidine residue was mainly responsible for forming an ion pair with the substrate carboxylate.

All the presently available data indicate that the positive charge on residue 234 of class A β-lactamases plays a major role in catalysis. Indeed, the only natural variation at that position (lysine → arginine; see the Introduction) conserves the charge; the lysine → glutamic acid and lysine → alanine mutations (Ellerby *et al.*, 1990) yield strongly disabled enzymes and, most convincingly, the pH-dependence of the Lys234His mutant $k_{cat.}$ and $k_{cat.}/K_m$ parameters clearly reflects the titration of the newly introduced histidine residue. Surprisingly, this conclusion can probably not be extended to all other penicillin-recognizing enzymes. Firstly, the pH-dependence for the inactivation of the R61 DD-peptidase by penicillins does not reveal the participation of a group exhibiting a pK around 7.0. If the histidine residue of the HTG triad in this enzyme plays a role similar to that of the corresponding lysine residue in class A β-lactamases, its pK must be increased to 9.0 or more. Conversely, with the same DD-peptidase, a side chain with a pK value close to 7.0 appears to be involved in the transpeptidation but not in the hydrolysis pathways. Secondly, the lysine residue of the KTG triad of a class C β-lactamase has also been mutated to histidine and the effects were quite different from those described here (D. Monnaie, A. Dubus & J.-M. Frère, unpublished work).

The titration of the histidine residue in the free enzyme indicates a pK of about 6.4, which is quite close to those deduced from the pH-dependence of $k_{cat.}$ and $k_{cat.}/K_m$. This would suggest that the pK of the imidazole ring of histidine-234 is not grossly modified by either the enzyme environment or the binding of the substrate.

At the present stage, the individual values of the rate constants K', k_2 and k_3 have not been determined for the wild-type *S. albus* G β-lactamase. Our results, however, allow us to exclude an influence of pH on the sole k_3 value for the mutant. Indeed, in this case, the decrease in $k_{cat.}$ on deprotonation of the histidine residue should be accompanied by a proportional decrease in K_m, and the $k_{cat.}/K_m$ value should remain unchanged. The behaviour of the mutant can thus be explained by a similar influence of the protonated histidine side chain on both k_2 and k_3. An influence on the sole k_2 value might also account for our data if that constant was much smaller than k_3.

Ellerby *et al.* (1990) have calculated the contribution of the lysine-234 side chain to catalytic efficiency and found that the Lys234Glu and Lys234Ala mutations increased the free-energy barrier between free enzyme and benzylpenicillin and the transition state by about 22 kJ/mol (5 kcal/mol) at pH 7.0. With the K234H mutant and benzylpenicillin, this increase is only 5.2 kJ/mol (1.2 kcal/mol) at pH 4.5 but reaches 23 kJ/mol (5.5 kcal/mol) at pH 9.0 (Fig. 5), which allows us to estimate the contribution of the positive charge to be about 18 kJ/mol (4.3 kcal/mol). This charge might participate in the creation of a 'positive sink' at the surface of the enzyme, with the help of the lysine-73, arginine-220 and arginine-274 side chains and the dipole of the α_2-helix. This environment would play a very important role in decreasing the transition-state energy.

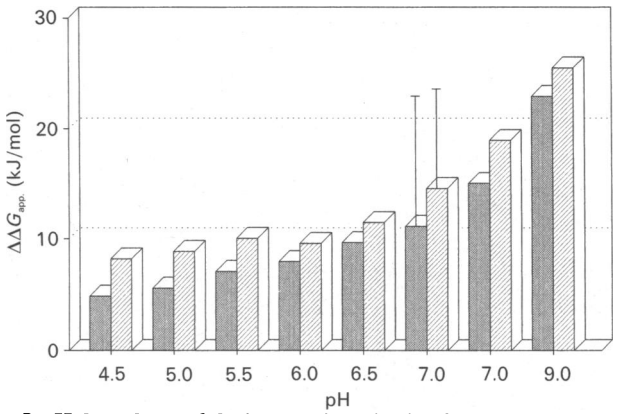

Fig. 5. pH-dependence of the increase in activation free energy due to the Lys → His mutation for the $k_{cat.}/K_m$ values, thus reflecting the rate of formation of the acyl-enzyme

▨, benzylpenicillin; ▨, ampicillin. The additional lines at pH 7.0 represent the values given by Ellerby *et al.* (1990) for the Lys → Ala mutation in the *B. licheniformis* β-lactamase.

The Lys234His mutation increased the sequence similarity between the β-lactamase and the DD-peptidase. However, it failed to improve the efficiency of transpeptidation catalysed by the former enzyme. In fact, the decrease in the total activity of the mutant above pH 7.0 was such that both hydrolysis and transpeptidation became barely detectable. As already mentioned above, this result sharply contrasted with those obtained with the DD-peptidase and might indicate different roles for the first residue of the HTG and KTG triads in the DD-peptidase and the class A β-lactamases respectively. In this respect, it will be interesting to study the effects of the lysine → histidine mutation in other DD-peptidases or penicillin-binding proteins.

Among the few substrates that were studied, the $k_{cat.}/K_m$ values for nitrocefin, benzylpenicillin and ampicillin were least affected whereas those for 6-aminopenicillanate, cephalothin and cephaloridine suffered a more marked decrease. This is in agreement with the results of other mutations that systematically seem to affect the hydrolysis of cephalosporins (with the possible exception of nitrocefin) and of 6-aminopenicillanate more strongly than that of 'normal' acylamidopenicillins (Jacob *et al.*, 1990*a*,*b*).

The Lys234His mutation significantly affected the rate of thermal inactivation of the β-lactamase, which also appeared to be irreversible. On those two counts, the behaviour of the *S. albus* G differed from that of the *B. licheniformis* enzyme, for which thermal inactivation seemed to be reversible and was little affected by replacement of lysine-234 by other side chains (Ellerby *et al.*, 1990). These differences might be due to the intrinsic properties of the enzymes or to differences in the methodologies, and we therefore need to compare the behaviours of the two wild-type enzymes under identical conditions, experiments that might supply interesting data about protein-unfolding phenomena.

Finally, it is interesting to underline the fact that the newly introduced histidine residue of the mutant is the sole histidine residue of the major form of that enzyme. Since it exhibits a

reasonable activity, the mutant protein thus becomes an excellent candidate for n.m.r. studies of the catalytic pathway.

In conclusion, we have shown that a positively charged histidine residue can successfully replace the native lysine residue in a class A β-lactamase. However, the pH-dependence of the kinetic parameters clearly demonstrates that the positive charge on residue 234 is extremely important for the stabilization of the transition state(s) in acylation and deacylation, much more so than for the complementarity between the enzyme and the substrate ground states, which is responsible for the initial binding.

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