Probing the determinants of protein stability: comparison of class A β -lactamases

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Five class A β -lactamases produced by various mesophilic bacterial species have been compared. Although closely related in primary and overall structures, these enzymes exhibit very different stabilities. In order to investigate the factors responsible for these differences, several features deduced from the amino acid composition and three-dimensional structures were studied for the five proteins. This analysis revealed that higher stability

appeared to correlate with increased numbers of intramolecular hydrogen bonds and of salt bridges. By contrast, the global hydrophobicity of the protein seemed to play a relatively minor role. A strongly unfavourable balance between charged residues and the presence of a *cis*-peptide bond preceding a non-proline residue might also contribute to the particularly low stability of two of the enzymes.

INTRODUCTION

Owing to a delicate balance between stabilizing and destabilizing interactions, folded proteins are only marginally stable. The net difference between the free energies of the folded and unfolded states is small, typically 5–20 kcal/mol (1 cal = 4.184 J). Nevertheless, proteins from organisms living in extreme environments can acquire higher intrinsic stability without changing their overall structures. The investigation of the fundamental processes by which higher stability is achieved is of major importance to our understanding of the mechanism(s) of protein folding.

The comparison between homologous proteins from mesophilic and thermophilic organisms has provided some insight into the molecular factors responsible for the very different stabilities of related proteins performing the same function and exhibiting similar structures. Although there is no general principle for increasing protein stability, two experimental findings deserve mention: (1) one single amino acid substitution can enhance protein stability, although this effect is generally small [1,2]; and (2) the stabilization caused by substitutions may be additive [3–5]. This means that significant stabilization can be achieved by multiple residue substitutions.

In this paper, in order to investigate the relationship between protein stability and structural properties, five class A β -lactamases from various bacterial species have been compared for their stabilities, amino acid compositions and three-dimensional structures. β -lactamases are bacterial enzymes which catalyse the hydrolysis of the β -lactam amide bond in penicillins and related antibiotics. Most of them are active-site serine enzymes. On the basis of their primary structures, three classes of serine β -lactamases (A, C and D) have been identified. Preliminary observations revealed that class A β -lactamases can have very different thermal stabilities, although they exhibit important similarities both in primary and tertiary structures [6].

The five proteins studied here are the TEM-1 β -lactamase and the enzymes produced by *Staphylococcus aureus* PC1, *Streptomyces albus* G, *Bacillus licheniformis* 749/C and *Actinomadura*

R39 (A.R39). The three-dimensional structures of the first four enzymes have been solved at high resolution by X-ray diffraction analysis [7–12]. Although X-ray data for the A.R39 β -lactamase are not available, this enzyme was included in the study because it is much more thermosensitive than the *B. licheniformis* enzyme, although, on the basis of their amino acid sequences, these two proteins are the most closely related of the group.

MATERIALS AND METHODS

Chemicals and enzymes

Ultrapure guanidine hydrochloride (GdmCl) was purchased from ICN Biomedicals Inc. (Aurora, OH, U.S.A.) and used without further purification. All other chemicals were reagent grade.

The enzymes produced by *B. licheniformis* and *Strep. albus* G were purified as described by Matagne et al. [6] and the *Actinomadura* R39 β -lactamase according to Piron-Fraipont et al. [13]. The TEM-1 β -lactamase was obtained as described by Dubus et al. [14] and the *Staph. aureus* enzyme was provided by Dr. R. Virden (University of Newcastle, U.K.).

Fluorescence measurements

Fluorescence measurements were performed on a Perkin-Elmer LS 50 luminescence spectrometer. Protein unfolding was followed using excitation and emission wavelengths of 280 and 340 nm respectively. Protein concentrations were about 2.5 μ M for the *Staph. aureus* enzyme and between 0.2 and 0.7 μ M for the other β -lactamases.

Experimental determination of protein stability

All the experiments described in this section were performed in 50 mM sodium phosphate buffer, pH 7.0.

(1) Thermal-denaturation melting curves. Thermal unfolding curves were obtained by monitoring the intrinsic fluorescence of the protein as the temperature increased. For the TEM-1 and the *B. licheniformis* β -lactamases, the cuvette containing the sample

Abbreviations used: GdmCl, guanidine hydrochloride; S.a., *Staphylococcus aureus*; A.R39, *Actinomadura* R39; S.a.G, *Streptomyces albus* G; B.l., *Bacillus licheniformis*.

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(1.4 ml) was heated at about 1.3 °C/min using a thermostatically regulated circulating-water bath. The temperature of the sample was directly measured with a differential thermometer. For the *Staphylococcus* enzyme, the temperature was increased by steps of 1 °C and the fluorescence (F_{mes}) was recorded at each temperature until it reached a constant value. The three enzymes unfolded reversibly in a simple co-operative fashion.

Thermal unfolding curves were analysed according to eqn. (1) with the help of the non-linear regression data analysis program ENZFITTER (Biosoft, Cambridge, U.K.). Eqn. (1) is based on the observation that van't Hoff plots are linear throughout the transition region, allowing an estimation of the unfolding enthalpy at the mid-point of the transition.

$$F_{mes} = \{(N + yT) + (U + zT) \exp a\} / \{1 + \exp a\}$$

$$\text{with } a = \Delta H_m [(T/T_m) - 1] / RT \quad (1)$$

where T_m is the mid-point of the transition and ΔH_m is the enthalpy of unfolding at T_m . The terms $(N + yT)$ and $(U + zT)$ were necessary to account for the linear decreases of the intrinsic fluorescence of the native and denatured states of the protein (N and U respectively) upon increasing the temperature.

(2) Determination of the activation thermodynamic parameters. Heat-induced protein denaturation was followed using fluorescence spectroscopy. The samples were incubated at various temperatures and the intrinsic fluorescence of the protein recorded as a function of time. The signal was analysed according to a single exponential equation and the activation enthalpy for the unfolding reaction, $\Delta H^\ddagger$, was calculated from Arrhenius plots.

The activation free energy of unfolding was obtained from GdmCl-induced unfolding kinetics: unfolding reactions were initiated by rapid mixing of the protein in solutions containing various concentrations of denaturant and changes in the intrinsic fluorescence of the protein were monitored. The temperature of the samples was maintained at 25 °C. The traces were fitted to a single exponential equation and the activation free energies associated with the unfolding reactions, $\Delta G^\ddagger$, were calculated by the Eyring equation: $\Delta G^\ddagger = -RT \ln(kh/k_B T)$, where k is the observed rate constant and h and k_B are the Planck and Boltzmann constants respectively. The observed linear relations between $\Delta G^\ddagger$ and GdmCl concentrations obeyed eqn. (2):

$$\Delta G^\ddagger = \Delta G^\ddagger(\text{H}_2\text{O}) + m[\text{GdmCl}] \quad (2)$$

where $\Delta G^\ddagger(\text{H}_2\text{O})$ is the extrapolated $\Delta G^\ddagger$ at $[\text{GdmCl}] = 0$.

Analysis based on amino acid composition

The five β -lactamases were compared on the basis of the following structural parameters calculated from their global amino acid compositions.

(1) The average hydrophobicity, $H\Phi$, based on Tanford's free energy of transfer of amino acid side chains from an organic to an aqueous environment [15]:

$$H\Phi = \sum n_i F_{t_i} / \sum n_i \quad (3)$$

where n_i is the number of residues i , and F_{t_i} the free energy of transfer of amino acid side chain i from ethanol to water [16].

(2) Fisher's parameter, p , representing the ratio of the volume occupied by polar residues to that occupied by non-polar residues [15,17].

$$p = V_{\text{polar}} / V_{\text{non-polar}} \quad (4)$$

(3) Waugh's parameter, NPS (larger non-polar side-chains), based on the frequency of non-polar residues [18]:

$$NPS = (nW + nI + nY + nF + nP + nL + nV) / \sum n_i \quad (5)$$

where n_i is the total number of residues.

(4) The aliphatic index, $I.A.$, which accounts for the proportion of aliphatic residues [19]:

$$I.A. = x_A + ax_v + b(x_i + x_L) \quad (6)$$

where $a = 2.9$, $b = 3.9$ and x_i is the proportion of residue i in the polypeptide chain.

Parameters $H\Phi$, p , NPS and $I.A.$ are related to the global hydrophobicity of the protein: $H\Phi$, NPS , $I.A.$ increase and p decreases when the global hydrophobicity increases.

(5) The αI and βI parameters, which reflect $P\alpha$ and $P\beta$, the probability factors of Chou and Fasman [20] for the participation of residue i in α -helices or in β -sheets, and which should be related to protein stability [21].

$$\alpha I = \sum (n_i P_{\alpha_i}) / \sum n_i \quad (7)$$

$$\beta I = (n_i P_{\beta_i}) / \sum n_i \quad (8)$$

Protein sequence comparison

Amino acid sequences were aligned using the Lineup, Pileup and Pretty programs from GCG (Genetic Computer Group, Madison, WI, U.S.A.).

Building of a three-dimensional model for the *Actinomadura* R39 β -lactamase

The structures of four of the five proteins studied here have been solved at high resolution by X-ray diffraction analysis [7–12]. Starting from these available data, a model for the structure of the *Actinomadura* R39 enzyme was built using the homology modelling program COMPOSER [22,23]. This program utilizes the following general strategy.

(1) In the first step, the most conserved regions in the known structures and in the modelled sequence are detected and defined as the framework.

(2) This framework is used to align fragments of the known protein structures of high sequence identity with the unknown, and this alignment provides a basis to build the desired tertiary structure.

(3) Each structurally conserved region of the unknown is then constructed using the most similar, known tertiary structure.

(4) Connecting regions, to which most of the changes in sequences and structures of homologous proteins are confined, are derived by a fully automated procedure that searches a database for suitable loops and efficiently connects the selected loops to the framework. In this particular case, and in order to minimize the number of connecting regions, the *Actinomadura* R39 model was built on the basis of a consensus protein obtained by selecting in each of the other sequences, the peptides exhibiting the highest degree of identity with its own sequence.

Computation of salt bridges and hydrogen bonds

The energies of the three-dimensional structures of the five enzymes were pre-minimized (100 cycles of optimization) with the AMBER molecular mechanics V3 framework [24] as described by Lamotte-Brasseur et al. [25]. The resulting structures were then used to identify the hydrogen bonds between heavy atoms separated by not more than 3.5 Å (0.35 nm). These calculations were achieved with the help of the ANAL program of AMBER.

Informatics

COMPOSER is run on a Silicon Graphics 4D/30 computer and AMBER on a VAX 4000-200 computer. The geometric features

of the enzymes were analysed with the Biosym Insight/Discover version 2.1 on the Silicon Graphics workstation.

RESULTS AND DISCUSSION

Experimental investigation of protein stability

A typical example of a thermal unfolding curve is given in Figure 1 and the results obtained from this analysis are summarized in Table 1. The *B. licheniformis* enzyme appeared as rather stable whereas the *Staph. aureus* β -lactamase was the least stable, even when compared with the TEM-1 enzyme.

Thermal unfolding curves could be established for only three β -lactamases, since the unfolding of the *Strep. albus* G and the *Actinomadura* R39 enzymes was not fully reversible. In order to include the two latter enzymes in the analysis, the activation thermodynamic parameters for the unfolding reaction were measured (Table 1). Although these data are not directly related to the intrinsic stability of the protein, but rather reflect kinetic parameters, the results presented here indicate that activation and equilibrium thermodynamic parameters correlate well with each other, and thus, both may be used in order to allow a comparison of the stabilities of the five enzymes.

The activation free energies of unfolding for the *Staph. aureus* and the *Actinomadura* R39 enzymes were found to be lower by at least 2 kcal/mol (1 cal = 4.184 J) than those of the *B. licheni-*

Figure 1 Thermal unfolding curve of the *B. licheniformis* β -lactamase as followed by fluorescence spectroscopy

The excitation and emission wavelengths were 280 and 340 nm respectively. The experiment was performed in 50 mM sodium phosphate, pH 7. The sample (1.4 ml) was heated at a rate of about 1.3 °C/min. The theoretical curve was obtained by fitting eqn. (1) to the experimental data.

Figure 2 Sequence comparisons

(a) Alignment of the five sequences. The secondary structure elements are also indicated (H = α -helix; S = β -strand; Ω = omega loop region). R39 = *Actinomadura* R39; B.1 = *B. licheniformis*; S.a = *Staph. aureus*; SaG = *Strep. albus* G; Tem = TEM-1. (b) Alignment of the *Actinomadura* R39 β -lactamase sequence (R39) with that of the hybrid β -lactamase constructed with the indicated pieces of each individual protein (Blac).

Table 1 Activation and equilibrium thermodynamic parameters for the unfolding reaction

The activation parameters are given for T = 25 °C. $\Delta G^*(H_2O)$ and ΔH^* were obtained by extrapolation to zero concentration of GdmCl and from Arrhenius plots respectively (1 cal = 4.184 J). ΔS^* was calculated from $[\Delta H^* - \Delta G^*(H_2O)]/T$. S.a. = *Staph. aureus*; A.R39 = *Actinomadura* R39; S.a.G = *Strep. albus* G; B.1. = *B. licheniformis*.

	T_m (°C)	ΔH_m (kcal/mol)	$\Delta G^*(H_2O)$ (kcal/mol)	ΔH^* (kcal/mol)	ΔS^* (cal/mol K)
S.a.	41.8 ± 0.3	100 ± 6	22.7 ± 0.1	49 ± 2	88 ± 5
A.R39	—	—	23.2 ± 0.1	61 ± 1	127 ± 4
TEM-1	50.1 ± 0.2	112 ± 7	—	—	—
S.a.G	—	—	24.9 ± 0.2	77 ± 2	175 ± 5
B.1.	63.2 ± 0.6	164 ± 9	25.5 ± 0.1	—	—

Table 2 Percentages of identities observed between the various sequences as aligned in Figure 2(a)

S.a. = *Staph. aureus*; A.R39 = *Actinomadura* R39; S.a.G = *Strep. albus* G; B.I. = *B. licheniformis*.

	S.a.	A.R39	TEM-1	S.a.G	B.I.
S.a.	—	37	33	31	43
A.R39	37	—	33	39	52
TEM-1	33	33	—	37	37
S.a.G	31	39	37	—	39
B.I.	43	52	37	39	—

formis and the *Strep. albus* G β -lactamases. The TEM-1 β -lactamase was not included in this comparison since the plot of ΔG^* versus [GdmCl] was not linear and did not allow accurate estimation of the $\Delta G^*(H_2O)$ value.

Arrhenius plots could be derived for only three enzymes, since the unfolding reactions at temperatures resulting in complete denaturation were too fast to be measured accurately for the TEM-1 and the *B. licheniformis* β -lactamases. Nevertheless, it is clear from Table 1 that increases in activation enthalpy were in general counterbalanced by increases in the activation entropy, so that the global activation free energies were rather similar.

To summarize, the thermodynamic stabilities of the enzymes studied here decrease as follows: *B. licheniformis* > TEM-1 > *Staph. aureus*. Taking the kinetic data into consideration, it is reasonable to conclude that the *B. licheniformis* and the *Strep. albus* G enzymes on the one hand, and the *Staph. aureus* and the *Actinomadura* R39 enzymes on the other, are similar in terms of stability.

Comparisons based on amino acid composition

The alignment of the primary structures and an analysis of the sequence similarities of the five class A β -lactamases are presented in Figure 2(a) and Table 2 respectively. The *B. licheniformis* and *Actinomadura* R39 enzymes exhibit the highest number of identical residues (52%). If this pair is chosen as a reference, the sequences diverge in the following order: *Staph. aureus*, *Strep. albus* G and finally TEM-1. These results are in good agreement with those of Joris et al. [26].

The comparison of the amino acid compositions given in Table 3 indicates that the ratio of positively to negatively charged residues was the only major feature deduced from the primary structures which could be directly related to protein stability. Indeed, the *Actinomadura* R39 and the *Staph. aureus* β -lactamases, which are the less stable enzymes, exhibit strongly biased balances between charged residues, and the electrostatic repulsion due to a large excess of positively or negatively charged groups at the surface of the protein might significantly decrease their stabilities. Indeed, proteins are generally most stable near their isoelectric points, where the net charge of the protein is low [27]. Furthermore, unfolding induced by extreme pH values is believed to be induced mainly through electrostatic repulsions.

Increased protein stability has been tentatively related to a higher Arg/(Arg+Lys) ratio [21,28] and/or to an increased prolyl content [29,30]. In the present case however, no such correlations were found.

Thermophilic and mesophilic proteins might also differ by their respective amino acid composition [15,21]. Four parameters, $H\Phi$, p , NPS and $I.A.$ (see Materials and methods section), linked to the global hydrophobicity of the protein were calculated for

Table 3 Main features deriving from the amino acid compositions of the proteins

The one-letter code is used for residues. * n_T , total number of residues; ** theoretical charge at pH 7; *** pI , theoretical isoelectric point; † ratio of positively to negatively charged residues (the H residues have not been counted as positive). The meaning of $H\Phi$, p , NPS , $I.A.$, αI and βI is explained in the text. S.a. = *Staph. aureus*; A.R39 = *Actinomadura* R39; S.a.G = *Strep. albus* G; B.I. = *B. licheniformis*.

	n_T^*	D + E	K + R	Charge**	pI^{***}	+ / - †	R/(R + K)
S.a.	257	32	47	15	10.4	1.50	0.09
A.R39	271	64	16	-48	3.8	0.25	0.88
TEM-1	263	36	29	-7	5.5	0.81	0.62
S.a.G	275	38	33	-5	5.0	0.89	0.88
B.I.	265	44	39	-5	5.1	0.89	0.38
	P	H	A + G	S + T	N + Q	I + L + V + M	F + W + Y
S.a.	9	2	32	30	25	60	20
A.R39	13	5	51	35	11	61	15
TEM-1	12	6	46	35	16	68	13
S.a.G	12	1	61	41	15	56	15
B.I.	11	1	41	32	20	61	16
	$H\Phi$	p	NPS	$I.A.$	αI	βI	
S.a.	1190	1.45	0.346	0.873	1.013	1.068	
A.R39	910	1.05	0.310	0.813	1.072	1.119	
TEM-1	1070	0.97	0.319	0.921	1.042	1.091	
S.a.G	950	1.07	0.295	0.820	1.003	1.039	
B.I.	1090	1.21	0.313	0.866	1.045	1.094	

the five β -lactamases, but no significant correlation with the observed stabilities was found (Table 3). Similarly, the parameters αI and βI (see Materials and methods section) are unrelated to the experimental results (Table 3).

Comparison based on three-dimensional structure

The polypeptide chain of class A β -lactamases is folded into two domains, one all α and one α/β . The overall size of the molecules is about $51 \times 42 \times 38 \text{ \AA}$ ($1 \text{ \AA} = 0.1 \text{ nm}$).

Figure 2(b) shows the alignment of the *Actinomadura* R39 enzyme sequence with that of the consensus protein (see Materials and methods section). The high number of identities (62% without any gap) readily allowed a three-dimensional model to be built. A large proportion of the side-chains were well oriented in the initial model and the minimization process did not result in significant modifications.

The three-dimensional structures of the five enzymes were then compared on the basis of several features. This analysis is summarized in Table 4.

The only major structural difference between these enzymes is the presence of a disulphide bond which connects residues 77 and 123 in the TEM-1 β -lactamase [7,8]. Disulphide bonds are believed to stabilize proteins mainly by reducing the entropy of the denatured state, although enthalpic and native-state effects cannot be neglected [31]. In the present case however, the TEM-1 enzyme exhibited an intermediate stability when compared with the other class A β -lactamases and it is reasonable to assume that the disulphide bridge contributes only marginally to the intrinsic stability of the protein.

By contrast, Table 4 shows that the number of salt bridges and the total number of hydrogen bonds, including those involving the backbone, correlate with the observed protein stabilities, and that this observation is even more convincing when the

Table 4 Main features deriving from the three-dimensional structures of the proteins

* S-S, number of disulphide bonds; ** total number of intramolecular hydrogen bonds per 100 residues; *** number of intramolecular hydrogen bonds per 100 residues when hydrogen bonds within secondary structure elements are not taken into account; **** overall temperature factor ($\text{\AA}^2$); † number of *cis* peptide bonds preceding a non-prolyl residue. S.a. = *Staph. aureus*; A.R39 = *Actinomadura* R39; S.a.G = *Strep. albus* G; B.l. = *B. licheniformis*.

	S-S*	Hydrogen bonds/100 residues**	Hydrogen bonds/100 residues***	Salt bridges	B factor****	<i>cis</i> non-Pro†
S.a.	0	109	63	10	20.7	1
A.R39	0	116	72	11	-	1
TEM-1	1	122	77	14	14.5	0
S.a.G	0	131	82	17	10.6	0
B.l.	0	126	81	18	10.4	0
Number of charged residues in the first and last helix turns						
	N-cap (+/-)	C-cap (+/-)	Favourable	Unfavourable		
S.a.	9/4	7/5	11	14		
A.R39	4/8	3/7	11	11		
TEM-1	6/4	7/3	11	9		
S.a.G	5/7	4/4	11	9		
B.l.	6/8	7/2	15	8

Figure 3 Temperature factors of the α -carbon atoms in the four known crystallographic structures

The horizontal line gives the overall average value. (a) = *Staph. aureus*; (b) = TEM-1; (c) = *Strep. albus* G; (d) = *B. licheniformis*.

hydrogen bonds involved in secondary structure elements are not included. It is interesting to note that similar findings were reported from the comparison of ferredoxins from mesophile and thermophile bacteria [32]. The results presented here are in agreement with recent analyses which suggest intramolecular hydrogen bonding as a dominant feature in stabilizing the folded states of proteins, even if the polar groups form hydrogen bonds with water in the unfolded state [33]. Furthermore, mutational studies have indicated that electrostatic interactions could significantly contribute to the stability of at least some proteins [34–36]. Interestingly, K73/E166 is the only salt-bridge conserved in the 26 known class A β -lactamase amino acid sequences. Five other salt-bridges R164/D179 (24 out of 26), R222/D233 (23 out of 26), R61/E64 (22 out of 26), R61/E37 (20 out of 26) and R43/E64 (20 out of 26) are conserved in at least 75% of the known class A β -lactamases [37,38].

The low stability of the *Staph. aureus* enzyme also correlates

with a significantly higher overall temperature factor [$21 \text{ \AA}^2$ versus $15 \text{ \AA}^2$ for the TEM-1 and $11 \text{ \AA}^2$ for the most stable *Strep. albus* G and *B. licheniformis* enzymes (see Table 4)]. By contrast, some of the loops of both the *B. licheniformis* and the *Staph. aureus* enzymes exhibit a higher mobility than those of the TEM-1 and the *Strep. albus* G enzymes (Figure 3), indicating that the flexibility of the whole molecule rather than the mobility of the loops can be related to the intrinsic stability of the protein.

In class A β -lactamases, the peptide bond preceding residue 167 is in the *cis* conformation. This peptide bond is located on a loop near the active site and the *cis* conformation is necessary to allow the correct orientation of residues involved in the catalytic mechanism. In most class A β -lactamases, residue 167 is a proline [37,38] but it is a threonine and an isoleucine in the *Actinomadura* R39 and the *Staph. aureus* enzymes respectively. Since the *cis* conformation is strongly unfavourable for peptide bonds that do not involve proline residues [39], one can assume that the lower stability of the *Staph. aureus* and the *Actinomadura* R39 enzymes is, in part, due to the presence of this *cis* peptide bond in the structure.

An important aspect of helix stability derives from charged residues interacting with helix dipoles [40–42]. The results of a search of charged residues in the first and last turns of the eight main helices (Table 4) show that, for the unstable *Staph. aureus* enzyme, unfavourable interactions outnumber the favourable ones while the opposite situation prevails for the very stable *B. licheniformis* enzyme. For the three other proteins, this parameter did not appear to be of major importance.

Finally, Ramachandran plots of the main-chain dihedral angles revealed that most of the non-glycine residues conformed satisfactorily to the preferred regions defined by Ramakrishnan and Ramachandran [43] (results not shown). Among the few interesting exceptions found in all the enzymes are residue 69, which is adjacent to the active-site serine, and residues S130 and D179 which are in an α_1 conformation. The total number of residues exhibiting unusual (ϕ , ψ) angles was not correlated to the observed stabilities.

CONCLUSION

Although the compared proteins show extensive similarities in their amino acid sequences and in their overall folding topologies, they nevertheless exhibit very different stabilities. These dif-

ferences in stability do not seem to reflect an adaptation of the bacteria to a hostile environment. Indeed, the TEM-1 enzyme can be produced by various gram negative enterobacteria. *Staph. aureus* is a well-known organism sometimes responsible for generally mild human diseases and *Actinomadura* R39, *B. licheniformis* and *Strep. albus* G are bacteria that live in the soil.

The five β -lactamases were compared on the basis of parameters related to their amino acid compositions or three-dimensional structures. Several findings emerged from this analysis but the most significant was the strong correlation observed between the experimentally determined protein stability and the number of hydrogen bonds and salt bridges. By contrast, no relationship was found between stability and the global hydrophobicity of the proteins.

Moreover, two structural features could, at least in part, explain the lower stability of the *Staph. aureus* and the *Actinomadura* R39 β -lactamases: (1) these two enzymes exhibited a strongly unfavourable balance between charged residues and (2) they contain a *cis* peptide bond preceding a non-proline residue.

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