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Deamidation in Proteins: The Crystal Structure of Bovine Pancreatic Ribonuclease with an Isoaspartyl Residue at Position 67

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The non-enzymatic deamidation of asparagine residues in proteins is a widely occurring reaction, both *in vivo* and *in vitro*. Although the importance of this process is commonly recognised, only little structural information is available on it. In order to evaluate the structural effects of this reaction in proteins, we have determined the crystal structure of a ribonuclease A derivative in which asparagine 67 has been replaced by an isoaspartyl residue, as a consequence of an *in vitro* deamidation reaction. The overall structure of the model, refined to a crystallographic *R*-factor of 0.159 at a resolution of 1.9 Å, is very similar to that of the native protein, but considerable deviations are observed in the region delimited by the disulphide bridge 65–72. In particular, the insertion of an extra methylene group in the main chain at residue 67 breaks up the hydrogen bond network that makes this region rather rigid in ribonuclease A. On the basis of the structure observed, some of the slightly but significantly different properties of this deamidated derivative, with respect to the native enzyme, can be explained.

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Keywords: deamidation; ribonuclease A; crystal structure; isoaspartyl residue

Non-enzymatic deamidation of asparagine residues represents one of the major chemical degradation pathways for peptides and proteins (Wright, 1991). Studies on proteins and model peptides (Geiger & Clarke, 1987; Patel & Borchardt, 1990a) have shown that, under neutral to basic pH conditions, the reaction proceeds *via* a succinimide derivative, which by spontaneous hydrolysis generates either an α-linked or a β-linked Asp (isoaspartyl) residue (Figure 1). The rate of deamidation is strongly influenced by the nature of the side-chain of the adjacent carboxyl residue, with Asn-Gly or Asn-Ser being the most susceptible sequences (Geiger & Clarke, 1987; Capasso *et al.*, 1989; Patel & Borchardt, 1990b; Tyler-Cross & Schirch, 1991). The approximate yield of the α to the β-linked product is 1:3 (Meinwald *et al.*, 1986) for synthetic oligopeptides and for proteins (Capasso *et al.*, 1991, 1995; Brennan & Clarke, 1993; Di Donato *et al.*, 1993). This suggests that the three-dimensional structure may not play a key role in determining the relative rate by which the proposed cyclic imide intermediate is opened. It is worth noting that the deamidation predominantly produces a species that presents a charged α-carboxyl group in place of the former Asn side-chain, and has an additional methylene group inserted into the backbone of the polypeptide chain. This latter modification, which is more drastic than other point mutations usually introduced in recombinant proteins, may produce appreciable changes in the tertiary structure and in the biological properties of the protein, depending on the specific asparagine residue subjected to the deamidation reaction (Di Donato *et al.*, 1986; Bischoff *et al.*, 1993; Friedman *et al.*, 1991; Artigues *et al.*, 1993).

Despite the numerous structural, chemical and biological studies on the deamidation reaction in proteins, no crystallographic evidence of the presence of a β-linked Asp residue in proteins has yet been presented. This is particularly surprising

Abbreviations used: RNase A, bovine pancreatic ribonuclease; (N67isoD)RNase A, RNase A with isoaspartyl residue replacing Asn at position 67; UpA, uridilyl (3'→5') adenosine; r.m.s., root-mean-square.

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considering that crystal growth often requires long incubation of the protein in buffers and high ionic strength conditions, which are known to promote the deamidation reaction (Capasso *et al*., 1991). However, it should be considered that in most cases only a small fraction of the protein is deamidated. Furthermore, labile Asn residues are normally located in flexible segments of the polypeptide chain with a relatively poor electron density, which may well have prevented detection of alternative conformations of the chain. The only structural information regarding a deamidated protein comes from a neutron diffraction study of aged trypsin crystal reported by Kossiakoff (1988), who presented a clear indication of three deamidated sites involving Asn-Ser sequences. All three Asn residues have similar conformations in the native enzyme and were found to deamidate only to α-linked Asp residues. This result can be explained assuming that in this case the deamidation reaction does not occur through the succinimide intermediate but through an alternative route catalysed by the adjacent Ser residue (Wright, 1991).

In order to study the structural effects of the formation of a β-linked Asp residue, we have carried out a crystallographic study of a derivative of bovine pancreatic ribonuclease (RNase A), which presents an isoaspartyl residue replacing Asn67 ((N67isoD)RNase A). The study was made possible taking advantage of the fact that RNase A can be selectively deamidated under mild conditions, and that both reaction products, i.e. (N67D)RNase A and (N67isoD)RNase A, can be isolated in pure form (Di Donato *et al*., 1993).

Selective deamidation of RNase A was carried out as described by Di Donato *et al*. (1993). The β-linked derivative was isolated by ion-exchange and hydrophobic HPLC. Crystals of (N67isoD)RNase A were obtained using free liquid diffusion methods. Crystallisation trials were successfully performed using the following recipe: 15 μl of a buffered aqueous solution (0.1 M acetic acid with ammonia added to pH 5.7) of lyophilised protein (20 mg/ml) were frozen in a capillary to avoid rapid mixing with the precipitating agent, isopropanol, that was successively layered above in equal volume. The capillary was then kept at the constant temperature of 20°C. Prismatic, well diffracting crystals, with maximum dimension up to 1 mm, were obtained after about one month. Cell dimensions, $a = 62.0$ Å, $b = 37.8$ Å, $c = 46.7$ Å, $\beta = 96.4°$ (space group $P2_1$), were different from those of the native protein despite the similarity of the crystallisation conditions. On the basis of a molecular mass of 13.7 kDa and two molecules per asymmetric unit, the volume per unit mass, V_M, is 1.98 Å^3/Da. This value compares well with those reported for various crystal forms of pancreatic ribonuclease and gives a solvent content of 38%. X-ray precession photographs of zero-layer reciprocal lattice sections showed the absence of low-order *h* odd reflections, indicating that the two molecules in the asymmetric unit are related by a translation of nearly one half along the *a* axis.

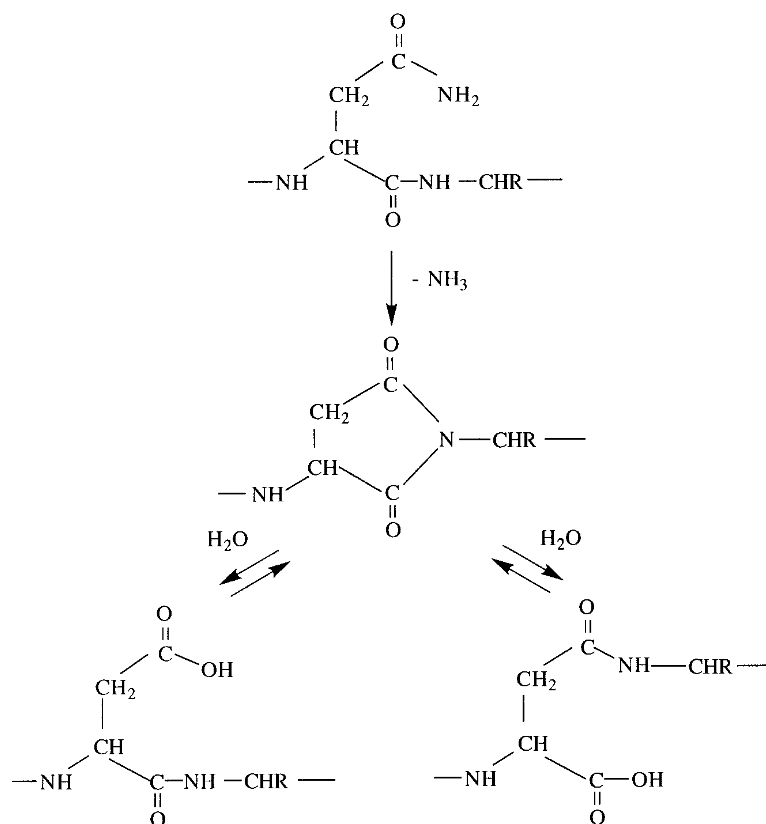

Figure 1. Deamidation reaction pathway *via* a cyclic succinimide derivative yielding either an α-linked or a β-linked Asp (isoaspartyl) residue.

The good diffracting quality of the crystals and their stability in the X-ray beam allowed the collection of data up to 1.9 Å resolution on a CAD4 Enraf-Nonius diffractometer. A total of 23,907 observations, with 17,221 unique reflections, were collected giving an R_{symm} on I of 5.1%.

The structure was solved by molecular replacement using the program XPLOR (Brünger, 1992). In all, 1791 reflections ($F_o > 2\sigma(F_o)$) from 10 to 4.0 Å and the coordinates (Protein Data Bank code 5RSA) from the ribonuclease A structure (Wlodawer & Sjölin, 1983) were used to calculate the Patterson maps. The self-rotation function yielded no outstanding peak other than the origin one, and the cross-rotation function presented only one prominent peak at 9.0σ (the second highest peak was at 4.0σ), thus confirming that the two molecules in the asymmetric unit are in the same orientation. Taking into account the non-crystallographic symmetry, a dimer was built for the translation search, which yielded a unique solution at 8.0σ. The model derived from the molecular replacement had a starting R-factor of 43%, which dropped to 39% after rigid-body refinement.

The model was refined with XPLOR and PROLSQ (Hendrickson, 1985), using ideal parameters defined by Engh & Huber (1991). In the course of the refinement, the loop 65–72 of the two molecules was rebuilt, taking into account the presence of the iso-Asp residue at position 67, and the conformation of several side-chains was manually fitted in the electron density. Two sulphate anions, in the active site regions, and 115 ordered water molecules were added to the model. In the last refinement run, a bulk solvent model, with mask parameters $k = 0.39$ e/Å^{-3} and $B = 87$ Å^2, was used and low-resolution terms were included. The final model gave a crystallographic R-factor of 0.159, computed on 16,618 reflections with $F_o > \sigma(F_o)$, for data extending from 20.0 to 1.9 Å. The electron density is continuous and well defined for all residues, except for very few polar side-chains on the surface of the two molecules. The overall B-value is 13.3 Å^2 (10.8 Å^2 and 14.0 Å^2 for main-chain and side-chain protein atoms, respectively); no water molecule has a B-value greater than 50 Å^2. The r.m.s. deviations from standard geometry values are 0.017 Å for bond distances and 2.7° for bond angles. Refinement statistics are presented in Table 1. The geometry of the main-chain and side-chains of the two molecules has been analysed using the program PROCHECK (Laskowski *et al.*, 1993).

Table 1. Refinement parameters and statistics for (N67isoD)ribonuclease A

Molecules in the asymmetric unit	2
Resolution range (Å)	20.0–1.9
No. of unique reflections (with $F_o > \sigma(F_o)$)	16,618
Completeness (%)	99.4
R-value (%)	15.9
Non-H protein atoms	1902
Water molecules	115
Sulphate anions	2
r.m.s. deviation of geometry from ideal values:	
1–2 distances (Å)	0.017
1–3 distances (Å)	0.033
1–4 distances (Å)	0.043
Planar group distances (Å)	0.009
Chiral volumes (Å^3)	0.147

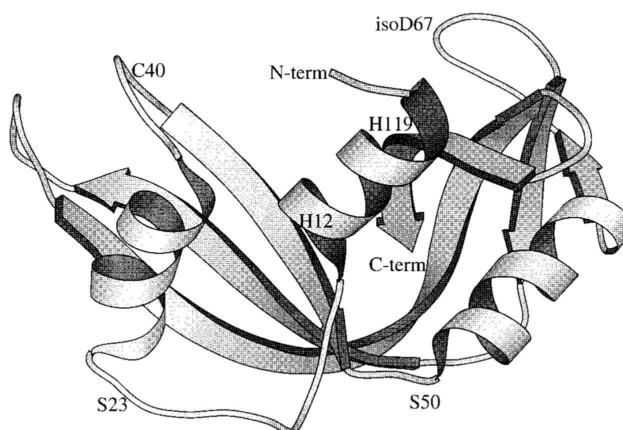

Figure 2. Schematic representation of (N67isoD)RNase A drawn using MOLSCRIPT (Kraulis, 1991). IsoD67 is the isoaspartyl residue.

The structures of the two molecules in the asymmetric unit are very similar, as shown by a r.m.s. deviation of 0.25 Å on the backbone atoms (N, C$^\alpha$, C) of all residues. This value is only slightly greater than the average error in the coordinates (in the range 0.12 to 0.18 Å), as determined by the method of Luzzati (1952). Furthermore, the overall structure (Figure 2) of each molecule of (N67isoD)RNase A is very similar to that of the native enzyme (r.m.s. deviation less than 0.50 Å on backbone atoms), with small differences mostly located in regions with different packing environments.

Even from the beginning of the refinement, the interpretation of the electron density around residue 67 was unambiguous. In particular, a reduced side-chain with respect to the original Asn residue and the presence of an additional methylene group in the main-chain were evident. In Figure 3 is shown the electron density map of the deamidation site, derived from the refined model. In the crystal, the structure of the loop is stabilised by several intermolecular hydrogen bonds, either direct or mediated by ordered water molecules, mainly with residues 39, 40, 86 and 92 to 94 of an adjacent symmetry-related molecule.

A detailed comparison of this structure with that of the wild-type enzyme was performed to evaluate the structural differences occurring in the loop region upon deamidation. Both structures of this region, after a preliminary superposition of the backbone atoms of the remaining parts of the molecules (residues 1 to 64 and 73 to 124), are reported in Figure 4. Evident is a large rearrangement of the loop structure upon the deamidation process. Although larger deviations with respect to the average differences are observed from residue

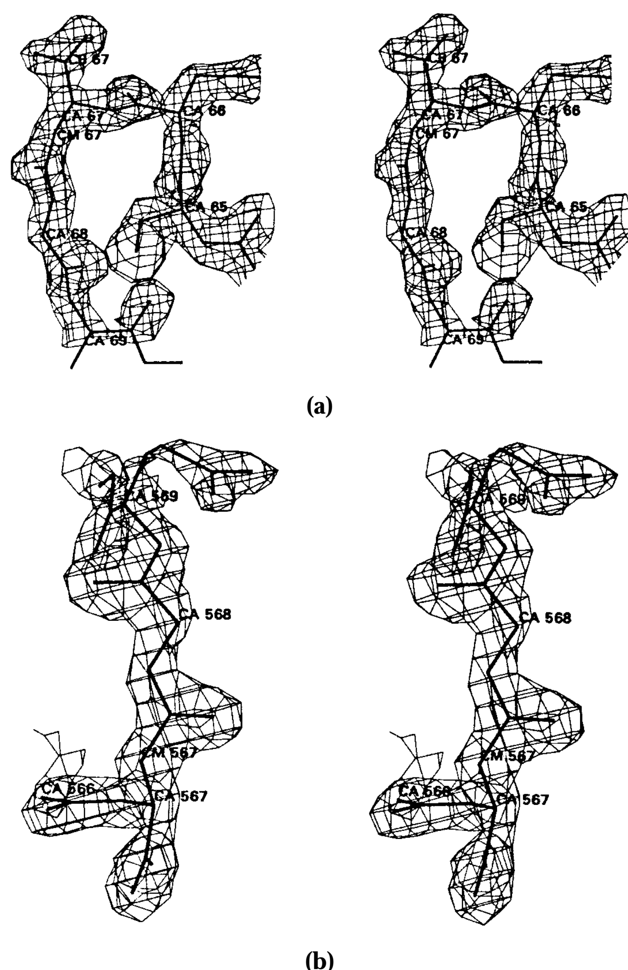

Figure 3. Stereoviews of the omit electron density maps of the deamidation region of (a) the first and (b) the second molecule in the asymmetric unit, drawn using FRODO (Jones, 1978). The coefficients $2F_o - F_c$ and calculated phases have been used. The maps have been contoured at the 1.5σ level. The carbon atom of the extra methylene group of the main-chain of the isoaspartyl residue has been labelled CM and the residue numbers of the second molecule have been augmented by 500.

66 to residue 72, the highest structural differences are located at the level of residues 67 and 68 (the distance of the corresponding C^{α} atoms is 4.0 and 5.5 Å, respectively). As a consequence, two important hydrogen bonds (between O^{δ} of Asn67 and N of Gln69, and between O of Cys65 and N of Gly68) of two β-turns of RNase A are no longer present in (N67isoD)RNase A. On the other hand, the interaction between O of Gln69 and N of Cys65 is conserved (Figure 4). The loss of this hydrogen bond network and the consequent increased flexibility of the studied region of the deamidated protein may well be related to its reduced rate of refolding (Di Donato *et al*., 1993). In fact, it has been hypothesised that the rather rigid structure of this region in pancreatic RNase A can be important for the correct pairing of cysteine residues 65 and 72 in the early stages of the folding of RNase A (Montelione & Scheraga, 1989).

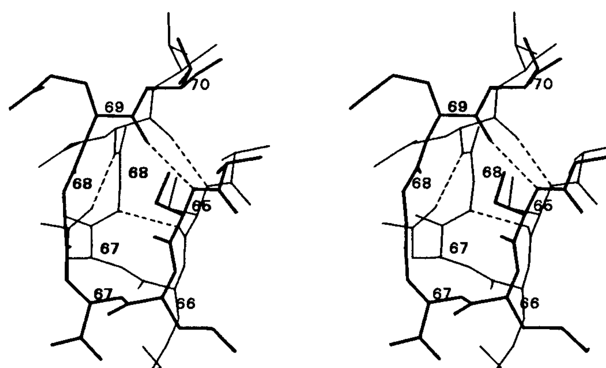

Figure 4. Comparison of the 65–71 region of RNase A (thicker lines) and (N67isoD)RNase A (thinner lines). Broken lines represent hydrogen bonds.

Another clear structural effect is a large shift of the loop containing residue 67 toward the main body of the molecule in the region of His119 (Figure 2), with the consequence of reducing the space available to the purine base in the B2 binding site; however, substrate analogues can still be modelled in the active site with minor overlaps with atoms of the loop 65–72. Even though the observed conformation of the loop might be influenced by the packing contacts mentioned, it is worth noting that it can be correlated to the kinetic data reported for (N67isoD)RNase A (Di Donato *et al*., 1993). In fact, a threefold increase in the K_m value has been reported for the binding of uridilyl (3' → 5') adenosine (UpA) dinucleotide to deamidated ribonuclease with respect to native RNase A. These data may be explained by the shrinking of the B2 site available for the purine base in the deamidated protein.

In conclusion, we report the first crystallographic evidence of the presence in a protein structure of an isoaspartyl residue, which is the main product of the deamidation reaction. Comparison with the native protein shows that the occurrence of a deamidation process can produce significant alterations in the local structure of the protein, and that variations of its functional properties are therefore not surprising.

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