The Role of the Imidazolyl Nitrogen Atoms of Histidine-12 in Ribonuclease S

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S-peptide (residues 1–14) analogues in which the active histidine-12 residue is replaced by *N*^π-methyl-L-histidine, *N*^τ-methyl-L-histidine and β-(pyrid-3-yl)-L-alanine were synthesized and tested for their capacity to bind to S-protein and to activate it. The results show that both imidazolyl nitrogen atoms are required for optimal catalytic functioning, *N*^τ being essential to the catalytic reaction itself, *N*^π playing a role in keeping the imidazole ring in the correct position.

In previous communications (van Batenburg *et al.*, 1976*a,b*) we reported the synthesis and the S-protein-activating capacity of two S-peptide analogues in which the directly active histidine-12 residue is replaced by the homohistidine (Hhi) or the imidazolylglycine (Nhi) residue. The intriguing results (both analogues give highly active complexes on combination with S-protein) stimulated further exploration along this line of approach. To obtain more information about the specific role of each of the two imidazolyl nitrogen atoms we synthesized and investigated two analogues in which histidine-12 is replaced by *N*^π-methyl-L-histidine [His(*π*Me)] and by *N*^τ-methyl-L-histidine [His(*τ*Me)]. In these analogues only the non-methylated nitrogen atom is readily protonated at approx. pH 7. Also the analogue with β-(pyrid-3-yl)-L-alanine at position 12 {abbreviated as [(3-Pyd)Ala¹²]S-peptide} has been studied to check whether the presence of two nitrogen functions is essential to the role of the residue at position 12. All above-mentioned amino acids have, or are estimated to have, side-chain *pK_a* values such as to enable the side chains to act efficiently as acid–base catalysts in the ribonuclease S' complexes. Whether they actually will do so would depend on the steric factors in each case.

Materials and Methods

All S-peptide (residues 1–14) analogues were prepared by solid-phase synthesis (Merrifield, 1969) as described earlier (van Batenburg *et al.*, 1976*a*), except for [(3-Pyd)Ala¹²]S-peptide (1–14), which was obtained by coupling the protected peptide fragments Boc-Lys(Boc)-Glu(OtBu)-Thr(tBu)-Ala-Ala-

Ala-NHNH₂* (I) and H-Lys(Boc)-Phe-Glu(OtBu)-Arg(HBr)-Gln-(3-Pyd)Ala-Met-Asp(OtBu)-OtBu (II) by the method of Honzl & Rudinger (1961). Fragment (I) was synthesized as described earlier (Visser *et al.*, 1968). Fragment (II) was prepared in essentially the same way as described for the synthesis of rat S-peptide (Voskuyl-Holtkamp *et al.*, 1976, 1977). The α-amino group of β-(pyrid-3-yl)-L-alanine was protected with the Bmv group (Southard *et al.*, 1971). *N*^π- and *N*^τ-Methyl-L-histidine were from Calbiochem A.G. (Lucerne, Switzerland) and checked for purity by t.l.c. and amino acid analysis. The α-amino function of these amino acids was protected by the Boc group as described previously (van Batenburg & Kerling, 1976). β-(Pyrid-3-yl)-L-alanine was obtained by asymmetric enzymic hydrolysis of *N*^π-benzoyl-β-(pyrid-3-yl)-DL-alanine methyl ester (Griffith & Harwood, 1964) by using chymotrypsin.

Enzymic studies were performed at pH 5.0 by the method of Berger & Levit (1973) with yeast RNA (BDH Chemicals, Poole, Dorset, U.K.) as a substrate. The binding capacity of the synthesized S-peptide analogues towards S-protein in the absence of substrate was studied by u.v. difference spectroscopy (Woodfin & Massey, 1968) by using split-cell cuvettes.

Results and Discussion

Table 1 summarizes the results of our measurements of the activity of the synthetic ribonuclease S'

* Abbreviations, from CBN Recommendations [*Biochem. J.* (1972) 126, 773–780]: Boc, t-butoxycarbonyl; tBu, t-butyl; Cm, carboxymethyl; Bmv, 2-benzoyl-1-methylvinyl.

Table 1. *S*-protein-activating ability (A_{max}) and binding capacity of some ribonuclease *S*-peptide (1–14) analogues in which the active-site histidine-12 is replaced by other residues with yeast RNA as a substrate at pH 5.0

A_{max} is expressed relative to *S*-peptide (1–14) (100%). Binding capacity in the presence of substrate was determined directly from activity assays (Berger & Levit, 1973); values are expressed relative to *S*-peptide (1–14)+substrate = 1000, which corresponds to a binding constant of $5 \times 10^6 \text{ M}^{-1}$. That in the absence of substrate was determined by u.v. difference spectroscopy; values are expressed relative to *S*-peptide (1–14)+substrate = 1000.

Residue 12	A_{max} (%)	Binding capacity	
		In the presence of substrate	In the absence of substrate
His	100	1000	1
Hhi*	80	10	0.01
Nhi*	80	0.01	Immeasurably low
His(π Me)	20	0.1	0.01
His(τ Me)	<1	100†	0.1
(3-Pyd)Ala	0	300†	1

* See van Batenburg *et al.* (1976*a,b*).

† Determined by competitive-inhibition experiments by using ribonuclease *S'* as a standard.

analogues with substitutions at position 12. Investigations by Hofmann *et al.* (1970) and by Dunn *et al.* (1974) showed that replacement of the imidazolyl group ($pK_a \approx 6$) by the isosteric pyrazol-3-yl or 4-fluoroimidazolyl moiety with a much lower pK_a leaves unimpaired the capacity of binding to *S*-protein, but results in an inactive ribonuclease *S'* complex. The data on the peptides with Hhi-12 and Nhi-12 prove that steric changes in the residue at position 12 may leave the function intact though the binding to *S*-protein is affected. The fact that the N^τ -methylated compound is practically inactive (in the presence of yeast RNA at pH 5.0) and has reasonably good binding capacity, whereas the N^π -methyl isomer binds less, but gives a quite active complex, constitutes direct evidence for a mechanistic picture as presented

in Fig. 1 for the first reaction step. The imidazolyl group is held in the proper position among other things by hydrogen-bonding of its *pros*-NH group to the peptide carbonyl group of the threonine-45 residue, as earlier suggested by X-ray studies (Richards & Wyckoff, 1971, 1973) and nuclear-magnetic-resonance experiments (Patel *et al.*, 1975). The *tele*-N atom (provided it has the appropriate pK_a) functions as a catalysing general base, deprotonating the ribose 2'-hydroxyl group (Findlay *et al.*, 1962). The resulting strongly nucleophilic oxygen atom attacks at the phosphorus atom, yielding a bi-pyramidal intermediate from which in a subsequent step the cyclic phosphate is formed (Roberts *et al.*, 1969; Usher *et al.*, 1972). Possibly the protonated N^τ atom co-operates in stabilizing this intermediate or

Fig. 1. Schematic representation of the role of the imidazolyl nitrogen atoms of residue 12 in the first step of ribonuclease action

the transition state leading to its formation or collapse. The observations by Hofmann *et al.* (1970) of the properties of the [His(π Cm)¹²]S-peptide (1–14) can be considered to be in line with the above-mentioned evidence. The finding that the analogue containing β -(pyrid-3-yl)-L-alanine is inactive although it has an appropriate pK_a fits nicely into this picture. This analogue binds well to S-protein, probably by formation of a hydrogen-bridge through its nitrogen atom. Lack of a second nitrogen atom then makes it impossible to function as an acid–base catalyst.

To summarize, we present the following conclusions. (1) The structure (conformation) of ribonuclease S must be rather flexible. Activity is maintained even when substantial steric alterations are made. (2) There is now direct chemical evidence that the histidine-12 residue functions as a base (acid) catalyst through its *tele*-N atom and is hydrogen-bonded (to the

carbonyl group of threonine-45) by its *pros*-NH group.

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References

- Berger, A. & Levit, S. (1973) in *Peptides 1971* (Nesvadba, H., ed.), pp. 373–383, North-Holland, Amsterdam
- Dunn, B. M., DiBello, C., Kirk, K. L., Cohen, L. A. & Chaiken, I. (1974) *J. Biol. Chem.* **249**, 6295–6301
- Findlay, D., Herries, D. G., Mathias, A. P., Rabin, B. R. & Ross, C. A. (1962) *Biochem. J.* **85**, 152–153
- Griffith, R. K. & Harwood, H. J. (1964) *J. Org. Chem.* **29**, 2658–2662
- Hofmann, K., Visser, J. P. & Finn, F. M. (1970) *J. Am. Chem. Soc.* **92**, 2900–2909
- Honzl, J. & Rudinger, J. (1961) *Coll. Czech. Chem. Commun.* **26**, 2333–2344
- Merrifield, R. B. (1969) *Adv. Enzymol.* **32**, 221–296
- Patel, D. J., Canuel, L. L., Woodward, C. & Bovey, F. A. (1975) *Biopolymers* **14**, 959–974
- Richards, F. M. & Wyckoff, H. W. (1971) *Enzymes 3rd Ed.* **4**, 647–806
- Richards, F. M. & Wyckoff, H. W. (1973) *Atlas of Molecular Structures in Biology* (Phillips, D. C. & Richards, F. M., eds.), vol. 1, Clarendon Press, Oxford
- Roberts, G. C. K., Dennis, E. A., Meadows, D. H., Cohen, J. S. & Jardetzky, O. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 1151–1158
- Southard, G. L., Brooke, G. S. & Pettee, J. M. (1971) *Tetrahedron* **27**, 1359–1361
- Usher, D. A., Erenrich, E. S. & Eckstein, F. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 115–118
- van Batenburg, O. D. & Kerling, K. E. T. (1976) *Int. J. Pept. Protein Res.* **8**, 1–2
- van Batenburg, O. D., Raap, J., Kerling, K. E. T. & Havinga, E. (1976a) *Rec. Trav. Chim. Pays-Bas* **95**, 278–281
- van Batenburg, O. D., Kerling, K. E. T. & Havinga, E. (1976b) *FEBS Lett.* **68**, 228–230
- Visser, S., Roeloffs, J., Kerling, K. E. T. & Havinga, E. (1968) *Rec. Trav. Chim. Pays-Bas* **87**, 559–571
- Voskuyl-Holtkamp, I., Schattenkerk, C. & Havinga, E. (1976) *Int. J. Pept. Protein Res.* **8**, 455–458
- Voskuyl-Holtkamp, I. & Schattenkerk, C. (1977) *Int. J. Pept. Protein Res.* in the press
- Woodfin, B. M. & Massey, V. (1968) *J. Biol. Chem.* **243**, 889–892