X-Ray Crystallography and the Amino Acid Sequence of Lysozyme

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Certain parts of the amino acid sequence of hen's-egg lysozyme were reinvestigated. The parts in question are those in which X-ray-crystallographic data were used to decide between the two reported versions of the sequence that were based on chemical data. We conclude that the X-ray evidence, in the main, led to the correct choice between the sequences.

The complete amino acid sequence of hen's-egg lysozyme (mucopeptide *N*-acetylmuramylhydrolase, EC 3.2.1.17) was determined independently by two groups (Jollès *et al.*, 1963; Canfield, 1963). There was a large measure of agreement, but, as is not uncommon in work of this complexity, there were differences at a few sites (Fig. 1).

A three-dimensional structure has been obtained by X-ray methods (Blake *et al.*, 1965). These authors relied heavily on the sequence results, but felt able, purely by inspection of electron-density contours, to suggest sequences in the disputed regions. These agreed with the upper line of the comparison given in Fig. 1 in positions 40-42, 92-93 and (a change in their original view) 58-59 (Blake *et al.*, 1967).

Electron-density maps cannot be used directly to assign amide groups, since the difference in density between the CO₂H and the CONH₂ groups is insufficient. However, Blake *et al.* (1967) reported that the environment and mode of hydrogen-bonding of residue 66 was somewhat more compatible with its being asparagine than aspartic acid. The crystallographers accepted Canfield's (1963) assignments at positions 46 and 48 without discussion.

We were asked to redetermine the relevant sequences. We have used chemical methods other than those used by either of the original laboratories. More recently Jollès's group (Hermann & Jollès, 1970) have revised some of their sequences to agree with those of Canfield (1963). This revision did not, however, include any direct experimental data, but was based on homology with the sequences of lysozymes from other animals.

Materials and Methods

Lysozyme (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was reduced and carboxymethylated as described for human prealbumin by Gonzalez & Offord (1971). Separate portions of protein were oxidized by performic acid for 2 h at 0°C [other conditions were as described by Hirs (1956)]. The digestion of the protein derivative by trypsin, the separation of fragments and their subsequent treatment employed the general methods and materials described by Gonzalez & Offord (1971) and Snell & Offord (1972). The extraction of the venom of the giant waterbug *Lethocerus cordofanus* Mayr and its use in proteolysis was as described by Rees & Offord (1969).

Results

To produce evidence of as independent a nature as possible, we have relied almost exclusively on the compositions and end groups of peptide fragments and very little on any of the techniques that involve sequential degradation by exopeptidases or chemical agents. Table 1 gives the amino acid compositions of the fragments. Many others were obtained in the course of this work that did not have any bearing on the regions of interest. These are not shown. Table 2 gives the mobilities at pH 6.5 and the net charge obtained from them (Offord, 1966), together with information on end groups and the various colours obtained with Barrolier's cadmium-ninhydrin reagent used as described by Offord (1969).

Residues from Canfield (1963)	...	Thr	Gln	Ala	Asn	Asp	Ile	Asn	Asn	Asp	Val	Asn
Residue no.	...	40	41	42	46	48	58	59	65	66	92	93
Residues from Jollès <i>et al.</i> (1963)	...	Gln	Ala	Thr	Asp	Asn	Asn	Ile	Asp	Asn	Asn	Val

Fig. 1. Comparison of relevant parts of the sequence of lysozyme

Table 1. *Amino acid compositions of the peptides after hydrolysis for 24h*

Peptides with names beginning with V were from a venom digest of the protein. After digestion the freeze-dried peptides were separated on Whatman no. 3 paper by high-voltage electrophoresis at pH 6.5. The numbering of the separated components was as follows: V1, V2, V3, V4: zones of progressively decreasing anodic migration; V5: neutral zone; V6, V7: zones of increasing cathodic migration. Each band was then further purified by either electrophoresis at pH 3.5 or paper chromatography, or a combination of both. Letters and numbers appearing after V1-V7 refer to relative positions of peptides in these separation schemes. Peptides with names beginning with T are the products of further degradation of the appropriate tryptic peptide of lysozyme. The tryptic peptides used were those corresponding to residues 34-45, 46-61, 62-68, 74-96. The means of fragmentation are indicated by the subsequent letters: N indicates Pronase digestion, P indicates pepsin, θ indicates thermolysin, S indicates subtilisin, π indicates papain and E indicates a single cycle of the Edman (1956) reaction. The numbers refer, as before, to the order of migration in the various steps of the separation schemes employed in the purification of the peptides. The compositions of peptides TS3 and TS3E were obtained by visual inspection of electrophoretograms of the hydrolysates. Half-cysteine was determined as cysteic acid. The results relating to the region 65-66 were first obtained, in preliminary form, by Mr. C. Quine as part of an undergraduate project.

Peptide	Relates to region	Amino acid content (mol/mol of peptide)												
		Lys	Arg	Asp	Thr	Ser	Glu	Gly	Ala	Cys	Val	Ile	Leu	Tyr
V4Cl	40-43			1.86	1.0	1.06	1.68							
V7a	40-43		0.97	1.0	0.93				1.34					
TN3i	40-43			1.0	0.87		1.0							
TS2	40-43			1.16	0.90	0.3	1.0							
TN3iii2	40-43				0.95		1.0							0.69
TN4	40-43		0.97	1.35	1.1				1.0					
TN5	40-43		1.1	1.0										
V3a3	46-48			2.6	2.0	1.0		1.0						
V4d4	46-48			1.0		0.70		1.1						
TS6	58-59		1.2			1.0								0.75
TP5	58-59		0.89	1.10		1.01	1.01					1.00		
TP5 θ 5	58-59		0.93	1.19		1.07						1.09		
TP π 5	65-66		1.0	1.8				1.2						
T π 4	65-66		0.83	1.03				1.0						
TS3	65-66		1	1				1						
TS3E	65-66		1											
V4e2	92-93			1.10	0.84	2.1			1.0		0.83	0.80		
TN5iii1	92-93			1.0							0.88			
TP4i	92-93	1.13		1.0		1.0			1.0	0.97	0.91			
TN7	92-93	1.0							1.2					

Table 2. Further characterization of the peptides

Mobilities were measured and interpreted as described by Offord (1966). The colours were recorded when the intensities had reached a maximum.

Peptide	Mobility at pH6.5 (Asp = -1)	Net charge at pH6.5	Dansyl end group	Colour with ninhydrin-cadmium
V4Cl	-0.37	-1	Ala	Red
V7a	0.48	+1		Red
TN3i	0	0		Orange
TS2	0	0		Red
TN3iii2	0	0		Yellow
TN4	0.47	+1	Asp or Asn	Orange
TN5	0.58	+1		Orange
V3a3	-0.58	-2		Yellow-orange
V4d4	-0.45	-1		Red
TS6	0.61	+1		Orange
TP5	0.42	+1	Glu or Gln	Red
TP5θ5		+1	Ile	Red (slow)
Tπ5	0	0	Asp or Asn	Yellow
Tπ4	0	0	Asp or Asn	Red
TS3	0	0	Val	Red
TS3E	0.62	+1		Yellow
V4e2	-0.38	-1		Orange
TN5iii1	0	0		Red (slow)
TP4i	0	0		Red
TN7	0.64	+1		Red

Fig. 2. Proposed sequences of peptides from lysozyme based on results in Tables 1 and 2

For clarity, residues 34-45, 46-61 and 62-68 are shown in full although, as will be seen from the peptides indicated, there are regions (none of them in dispute) for which evidence is not presented here. These regions are taken from the sequences already referred to (Canfield, 1963; Jollès *et al.*, 1963). — indicates a peptide for which an end group was determined.

Discussion

These results lead us to propose the sequences shown in Fig. 2. There seems little ambiguity in the assignments. There is also no reason to suspect the

venom protease of inducing transpeptidation with greater facility than any other protease.

It will be noted that we propose that residue 46 is asparagine, rather than aspartic acid. This is

based on the colour that is given with the cadmium-ninhydrin reagent by the tryptic fragments corresponding to residues 46–61 and on the similar colour given by peptide V3a3 (Table 2). As far as we are aware the rule that states that peptides beginning with aspartic acid give red colours, whereas those beginning with asparagine give yellow–orange colours, has not been called into question (except that peptides that possess a lysine residue in addition to *N*-terminal asparagine will eventually turn orange-red: peptide 46–61 has no lysine). We believe that this rule has been established for sufficiently long to be regarded as all but conclusive. Consideration of the mobility of peptide 46–61 ($m = -0.25$) and of the other peptides shows that if residue 46 is asparagine then residue 48 must be aspartic acid. Turning to region 65–66, some of the difficulties may arise from the fact that peptide 62–68 ($m = -0.25$) loses its amide group on handling (m of new form = -0.46).

We conclude that, as other examples have shown, electron-density maps may be quite effective in distinguishing between certain side chains. It is, clearly, not always easy to assign amide groups by bonding and environment.

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