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## De kristalstructuur van papaïne

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## SUMMARY

The first stages are described of the determination of the crystal structure of papain (papain C, space group  $P2_12_12_1$ ). This plant proteolytic enzyme of MW 22,000 was extracted from dried papaya latex and crystallized from a mixture of 65% methanol and 35% water. The interpretation of the X-ray photographs of papain and three isomorphous heavy-atom derivatives resulted in a three-dimensional Fourier map at  $4.5 \text{ \AA}$  resolution.

In this map the protein molecules can easily be distinguished which must be partly due to the low electron density of the medium. The heavy-atom positions are found at the surface of the molecule. In spite of the almost complete knowledge of the amino acid sequence [87] it was not possible to trace the polypeptide chain in the molecule. There are many intersections and only three of these can represent disulphide bridges. The others are probably due to the 30 aromatic side chains which are not resolved at  $4.5 \text{ \AA}$  resolution. Two regions of high electron density are probably right handed helices of about three turns each (arrows in fig. 7.1 (a) and 7.3 (d)). The helix content thus seems to be low which is in agreement with ORD measurements [125] and statistical predictions [113] based on the amino acid sequence. There is an indication in the Fourier map for the position of one of the ends of the polypeptide chain.

The -SH group in the active centre is blocked in crystalline papain if no special precautions are made. It has been located by B. G. Wolthers (unpublished results). The -SH group lies at the surface of the molecule in a shallow depression which extends from the side to the bottom as seen in figs. 7.1 (a) and 7.3 (b), (c), (d). This location offers the possibility for various contacts between protein and substrate.

Although papain is not active in 65% methanol-water solution [126], there are reasons to believe that its conformation in this medium, and in the crystals, is essentially the "native" conformation [125].

In Chapter 1 an outline is given of the history of protein crystallography with special emphasis on the results, obtained by Perutz and Kendrew et al. for haemoglobin and myoglobin. Also attention has been paid to the structure determinations of lysozyme, ribonuclease and chymotrypsin, which have led to the complete knowledge of the conformation of the polypeptide chain, and in the case of lysozyme to a detailed picture of the mechanism of the reactions performed by this

enzyme. Table 1.1 summarizes the progress made in the X-ray studies of other globular proteins.

Chapter 2 deals with the isolation and crystallization of papain. From 70% alcohol monoclinic crystals, space group  $P2_1$ , have been obtained. This crystal form, referred to by us as papain A, did not grow to dimensions suitable for a detailed investigation. From 65% methanol large orthorhombic single crystals were obtained, space group  $P2_1 2_1 2_1$ , 1 molecule per asymmetric unit. The reflexion pattern extended to  $d = 2.2 \text{ \AA}$ . This crystal form, papain C, was chosen for further investigation.

The determination of the absolute scale of intensities of papain C is described in Chapter 3. This has been done by comparing on precession photographs integrated intensities of reflexions in the  $[010]$  zone of papain with those in the  $[1\bar{1}0]$  zone of anthracene and basic beryllium acetate crystals. The intensities of the papain reflexions on the anthracene scale appeared to be about 25% higher than on the basic beryllium acetate scale. Low et al. found a difference of 20% between the two scales in their determination of the absolute scale of insulin citrate crystals (personal communication). We used the mean of the two scales.

The theory of refinement of heavy-atom parameters and phase determination in the isomorphous replacement method is dealt with in Chapter 4. Much attention is paid to the phase probability method, devised by Blow & Crick [13] and first used by Dickerson et al. [19], as well as to the use of anomalous scattering in phase determination [46, 110] and in earlier stages of protein crystal structure determination [106-111].

In Chapter 5 the preparation of heavy-atom derivatives is described. Binding was achieved at 5 different sites on the papain molecule (Table 5.1). The value of the heavy-atom derivatives was tested by difference Patterson and Fourier summations in the centrosymmetric  $[001]$  and  $[100]$  zones, followed by least squares refinement of the heavy-atom positions found. Three derivatives were ultimately chosen for use in the phasing: papain-PCMB, papain- $\text{Na}_2\text{PtCl}_6$  and papain- $\text{HgCl}_2$ . Fig. 5.1 shows the  $[001]$  and  $[100]$  difference Fourier projections obtained with these derivatives and "best" protein signs. The agreement between the signs predicted by each derivative separately and the "best" signs is shown in Table 5.3. In fig. 5.2 the  $[100]$  and  $[001]$  Fourier projections, at  $4.5 \text{ \AA}$  resolution, of the protein are given. From these the approximate centres of the molecules can be deduced, as was done before at  $5 \text{ \AA}$  resolution [99].

The three-dimensional structure determination is described

in Chapter 6: Nearly all reflexions within one half of the reciprocal  $4.5 \text{ \AA}$  sphere were recorded on 20 layers parallel to  $b^*$  (see fig. 6.1), and the zero, first and second layer perpendicular to this axis, for the native protein and four derivatives. The intensities were measured with an automatic integrating densitometer [123], and processed on a Telefunken TR4 computer with a series of computer programmes especially written for this purpose. The scaling of the 23 layers was performed according to Hamilton, Rollett & Sparks [122]. Ultimately a series of about 1550 independent reflexions was obtained for the protein and each derivative, and, in addition, separate intensities for the Bijvoet pairs of some 1100 non-centric reflexions from the heavy-atom derivatives.

The first refinement of the heavy-atom parameters was made with a full-matrix least squares programme according to Rossmann [18]. Applying this to the 400 centric reflexions, we obtained better results than with the use of all reflexions. The fourth derivative, with "Baker mercurial", did not give consistent results in the two refinement procedures (see Table 6.1) and was abandoned. Phases, calculated using the remaining derivatives, were used as a starting point in a "lack of closure error" refinement [19], first without (II) and afterwards with (III) the use of anomalous scattering. In Table 6.2 the results of the various phase calculations are given in terms of the "mean figure of merit" and  $\langle \Delta\rho \rangle^2$ . The results of the refinements II and III are listed in Table 6.3.

The absolute configuration of the papain molecule has been derived unambiguously by comparing the observed anomalous scattering differences with those predicted from the protein and heavy-atom structure factors. The improvement of the protein phases during the refinement process can be seen in Table 6.4 from the sign agreements between  $(|F_H^+| - |F_H^-|)$  en  $(|F_C^+| - |F_C^-|)$ .

The "best" fourier, calculated with the phases III in Table 6.2 (mean  $m = 0,91$ ) ultimately gave the results which are discussed in Chapter 7.