Communication

Formation, Crystallization, and Preliminary Crystallographic Data of the Ternary Complex of \( \alpha \)-Chymotrypsin, \( \beta \)-Trypsin, and the Bowman-Birk Inhibitor*

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The ternary complex of the Bowman-Birk inhibitor with \( \alpha \)-chymotrypsin and \( \beta \)-trypsin has been crystallized and preliminary crystallographic information describing the crystals has been obtained. The crystals are triodinic with unit cell dimensions: \( a = 51.96 \) Å, \( b = 56.34 \) Å, \( c = 46.70 \) Å, \( \alpha = 98.83^\circ \), \( \beta = 97.29^\circ \), and \( \gamma = 97.15^\circ \). There is 1 ternary complex/unit cell and the diffraction pattern extends to 2.2 Å resolution. The search for heavy atom derivatives based on known derivatives of chymotrypsin and trypsin is underway.

In 1946, D. E. Bowman reported a new trypsin inhibitor found in soy beans (1). About 15 years later, Y. Birk reported that this inhibitor also inhibits chymotrypsin (2); Birk, Gertler, and Khalef later showed that the inhibitor was in fact divalent and could inactivate both enzymes simultaneously (3). Soon after, the inhibitor became known as the Bowman-Birk inhibitor.

The molecular weight and amino acid composition of BBI (4, 5) showed that it was a protein of low molecular weight (7975) which is extensively cross-linked with seven disulfides (6). BBI is a competitive inhibitor of trypsin and chymotrypsin with high binding constants for either enzyme or both (TRYP, \( K_i = 2.8 \times 10^{-7} \) M; CHT \( K_i = 2.9 \times 10^{-7} \)) (7). The specificity regions of BBI have been located in the sequence (8-10) and these occur in very similar nonapeptide loops. When these peptides were synthesized, they were themselves inhibitors (11). Moreover, a large number of closely homologous inhibitors have also been characterized from other legumes (12). Preliminary x-ray crystallographic data of BBI have recently appeared on a crystal form with 2 BBI/asymmetric unit (13). In this communication, we report similar results but on the ternary complex of \( \alpha \)-CHT, \( \beta \)-TRYP, and BBI.

The structures of three serine protease-inhibitor complexes have been determined thus far: basic pancreatic trypsin inhibitor-trypsin (14), soybean trypsin inhibitor (Kunitz)-trypsin (15) and Streptomyces subtilisin inhibitor-subtilisin (16), and the crystallization of pancreatic secretory trypsin inhibitor (Kazal)-trypsinogen has been described (17). However, none of the foregoing complexes shows the unique divalent inhibitory characteristics of the BBI ternary complex class.

In addition, none of these inhibitors displays the complexity of primary structure manifested by BBI and its seven disulfides. Laskowski and Kato have categorized serine protease inhibitors into 10 fundamental groups according to the patterns of their disulfides (12). The BBI group is by far the most complex of these and the unusual stability toward heat and acid conditions of this class is probably a consequence of new structural folding features and/or principles.

Experimental Procedures and Results

BBI was isolated and purified by successive chromatography on CM- and DEAE-cellulose (18) followed by preparative isoelectric focusing in the range of pH 3.5-5.5 using an LKB 2117 Multiphor apparatus. The resulting preparation gave a single band with \( M_r = 8000 \) when examined by SDS gel electrophoresis. The \( \alpha \)-CHT and \( \beta \)-TRYP used to form the ternary complex were 3 times crystallized samples purchased from the United States Biochemical Co.

The ternary BBI complex was obtained by first forming a binary BBI-TRYP complex by adding an equimolar amount of TRYP to BBI. The solution was then allowed to stand for about 15 min to ensure complete reaction and a minimal amount of TRYP present before an equimolar amount of CHT was added. The total ternary complex concentration in the resulting solution was about 2%. The reaction and crystallization solution contained 0.1 M sodium acetate buffer, 10% PEG 4000 at pH 4.6. The solution also contained 0.02% sodium azide to inhibit bacterial growth. The solution was filtered prior to crystallization through a Metricel membrane with a pore size of 45 μm. Crystals usually appear in the supernatant within 4 to 5 days. Most of the crystals are prismatic but often some crystals of triangular morphology also appear (Fig. 1). X-ray diffraction experiments showed that both of these morphologies correspond to the same crystal form. Lastly, the crystals are stored under a similar solution except that the PEG concentration is raised to 25%. Under these conditions, the crystals appear to be stable for long periods of time and also appear to diffract x-rays somewhat better.

The ternary complex of the crystals was confirmed in an independent manner. The crystals were first washed 4 times with acetate-PEG solution and then were dissolved and treated with SDS and mercaptoethanol and subjected to disc gel electrophoresis (19). Gels were also made of each of the three components of the ternary complex for comparison purposes.

The electrophoretic migrations are shown in Fig. 2, from which it can be seen that the crystals are composed of CHT, TRYP, and BBI; moreover, amino acid analysis of the complex agreed with the theoretical amino acid composition expected for CHT-BBI-TRYP.

The x-ray diffraction studies of crystals of CHT-BBI-TRYP were carried out using precession and diffractometric method
Crystallization of the α-CHT-BBI-β-TRYP Complex

ods. The crystals were shown to belong to the triclinic system, $\alpha = 51.96 \pm 0.04$, $b = 56.34 \pm 0.06$, and $c = 46.70 \pm 0.04$ Å, $\alpha = 98.83 \pm 0.04$, $\beta = 97.29 \pm 0.06$, and $\gamma = 97.15 \pm 0.04^\circ$ with $V = 1.326 \times 10^5$ Å$^3$. Twenty-degree precession photographs of the (h0l) zone displayed diffraction at 2.2 Å spacings and this was confirmed with a FASCI diffractometer. The intensity distributions along three principal axes of the triclinic system are shown in Fig. 3.

Since CHT-BBI-TRYP crystals are stable in distilled water for relatively long periods (weeks), the salt-free density of the crystals was obtained by measuring the density of crystals soaked in distilled water. The density was found to be 1.17 g cm$^{-3}$ using a calibrated density gradient column made of xylene and iodobenzene. If the partial specific volume of the ternary complex is $\upsilon_p$, the molecular weight is given by

$$M_w = NV(d_0 - 1.0)/n(1 - \upsilon_p)$$

where $N$ is Avogadro’s number, $V$ is the volume of the unit cell, $d_0$ is the salt-free density, and $n$ is the number of complexes/unit cell. The cell constants of triclinic salt-free crystals are $a = 52.3$, $b = 55.7$, $c = 56.3$ Å, $\alpha = 99.9$, $\beta = 96.8$, $\gamma = 105.7^\circ$ with $V = 1.528 \times 10^5$ Å$^3$, so that if it is assumed that $\upsilon_p = 0.736$ cm g$^{-1}$, which is $\upsilon_p$ of CHT, the molecular weight calculated for the unit cell is 59,000, in excellent agreement with 57,550 expected for the CHT-BBI-TRYP complex. Thus, there is 1 ternary complex/unit cell. The density of crystals in 0.1 M acetate, 25% PEG 4000, pH 4.6, was also measured and found to be 1.26 g cm$^{-3}$. The latter implies that these crystals are about 58% protein and 42% solvent by weight.

Although a low resolution structure of the CHT-BBI-TRYP complex can probably be obtained using molecular replacement methods, a search for heavy atom derivatives is also in progress based on heavy atom derivative formation with CHT (20) and TRYP (21) alone. In this way, a detailed high resolution structure of this most interesting protease complex is also ensured.
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