Biophys. Struct. Mechanism 1, 189—201 (1975)

The Structure of the Complex Formed by Bovine Trypsin and Bovine Pancreatic Trypsin Inhibitor

III. Structure of the Anhydro-Trypsin-Inhibitor Complex *

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Received February 2, 1975

Abstract. The structure of the complex between anhydro-trypsin and pancreatic trypsin inhibitor has been determined by difference Fourier techniques using phases obtained from the native complex (Huber *et al.*, 1974). It was refined independently by constrained crystallographic refinement at 1.9 Å resolution. The anhydro-complex has Ser 195 converted to dehydro-alanine. There were no other significant structural changes. In particular, the high degree of pyramidalization of the C atom of Lys 15 (I) of the inhibitor component observed in the native complex is maintained in the anhydro-species.

Key words: Trypsin — Inhibitor — Protein-Structure — X-Ray Analysis — Enzyme Substrate Interaction.

The structure of the complex between bovine trypsin and bovine pancreatic trypsin inhibitor (PTI) has been determined and crystallographically refined at 1.9 Å resolution (Huber *et al.*, 1974), to gain detailed insight into the structure and the mechanism of interaction of these protein molecules. Structure analyses of chemical variants in either the enzyme or inhibitor, which would prevent the association from proceeding to completion, would be of particular interest. Here, stable intermediate states of the reaction could be observed which would be too short-lived to be seen with the un-modified components. Anhydro-trypsin is a species which has the catalytic site residue Ser 195 converted to a dehydro-alanine residue. In a complex of this modified enzyme with the inhibitor, one might hope to observe an intermediate state of the reaction before the nucleophilic addition event. Anhydro-trypsin has been synthetized and chemically characterized by Ako, Foster and Ryan (1974). It is enzymatically inert but binds natural inhibitors with nearly the same strength as native trypsin (Vincent *et al.*, 1974; Ako *et al.*, 1974). It is clear, however, that a chemical characterisation may not be regarded as entirely sufficient in view of the very drastic conditions of base-elimination required to convert trypsin to anhydro-trypsin.

* Publication II of this series is: Huber *et al.*, 1974.

Anhydro-trypsin was prepared by the method of Ako by affinity chromatography with immobilized soybean trypsin inhibitor. Before final chromatography the residual trypsin activity was reduced to less than 1% by weight by the addition of diisopropyl fluorophosphate. The final lyophilized anhydro-trypsin exhibited less than 3% residual activity as judged by assays with benzoyl-L-arginine methyl ester as a substrate.

Anhydro-trypsin PTI complex was prepared from the components and crystallized as the native complex (Rühlmann *et al.*, 1973). A few single crystals were dissolved and separated on Sephadex G 75 at pH 2 into the components. The trypsin component showed very low enzymatic activity as low as the material used for complex preparation (less than 3% of β-trypsin activity). X-ray intensities were determined photographically by screen-less precession and rotation techniques using the method and programmes described by Schwager *et al.* (1975). 76600 reflexions were measured and merged to yield a set of 22060 unique reflexions to 1.9 Å resolution. R_{merge} was 0.069. It is defined as $(\sum_i (I_i - \bar{I})^2 / \sum_i^2)^{1/2}$ with I_i, the individually measured intensity, $\bar{I}$, the averaged value and the summation running over all measurements. The merging procedure assigns individual scaling and temperature factors to each photograph (Steigemann, 1974). This set of data was scaled to the data set of the native complex using a scale- and temperature factor.

A difference Fourier map was calculated with coefficients $(|F_A| - |F_C|) \exp i\,\phi$, where $|F_A|$, $|F_C|$ and ϕ are the structure factor amplitudes of anhydro-trypsin-PTI complex, native complex and the phases of the native complex respectively. Several sections of the three-dimensional difference map near the catalytic site are shown in Fig. 1 with the atomic positions plotted into it.

The difference map shows one dominant high minimum (> 0.38 e/Å^3) centred at Ser 195 O$^\gamma$, in accord with the absence of the O$^\gamma$ in anhydro-trypsin. C$^\beta$ has moved into the plane formed by 195 N, C$^\alpha$, C in accord with a trigonal C$^\alpha$ in anhydro-trypsin. The positive peaks on sections 26 and 27 adjacent to the dominant minimum indicate the new position of C$^\beta$. As it is only about 1 Å from the O$^\gamma$ position, it is partially screened by the minimum. The average value of the difference map outside the molecular boundaries is 0.02 e/Å^3, providing an indication of the error level. A rough inspection shows that there are several positive and negative peaks up to 0.075 to 0.1 e/Å^3 all over the complex molecule. These indicate small structural alterations throughout the molecule. We did not attempt to analyze these in detail except at the residues forming the inhibitor-enzyme contact.

The residues at the contact were analyzed as follows: Phases were calculated from the complex coordinate set omitting Ser 195 O$^\gamma$ and shifting C$^\beta$ into the plane formed by N, C$^\alpha$ C. With these phases and the observed structure factor amplitudes a constrained crystallographic refinement (Deisenhofer and Steigemann, 1974; Huber *et al.*, 1974) was initiated. A part of the final difference map calculated with coefficients $(|F_A| - |F_{AC}|) \exp i\,\phi_A$ ($|F_A|$ and $|F_{AC}|$, observed and calculated structure factor amplitudes of the anhydro-complex, ϕ_A, calculated phases) is shown in Fig. 2. It is without interpretable features. The density left at the original position of Ser 195 O$^\gamma$ might be due to residual active trypsin in

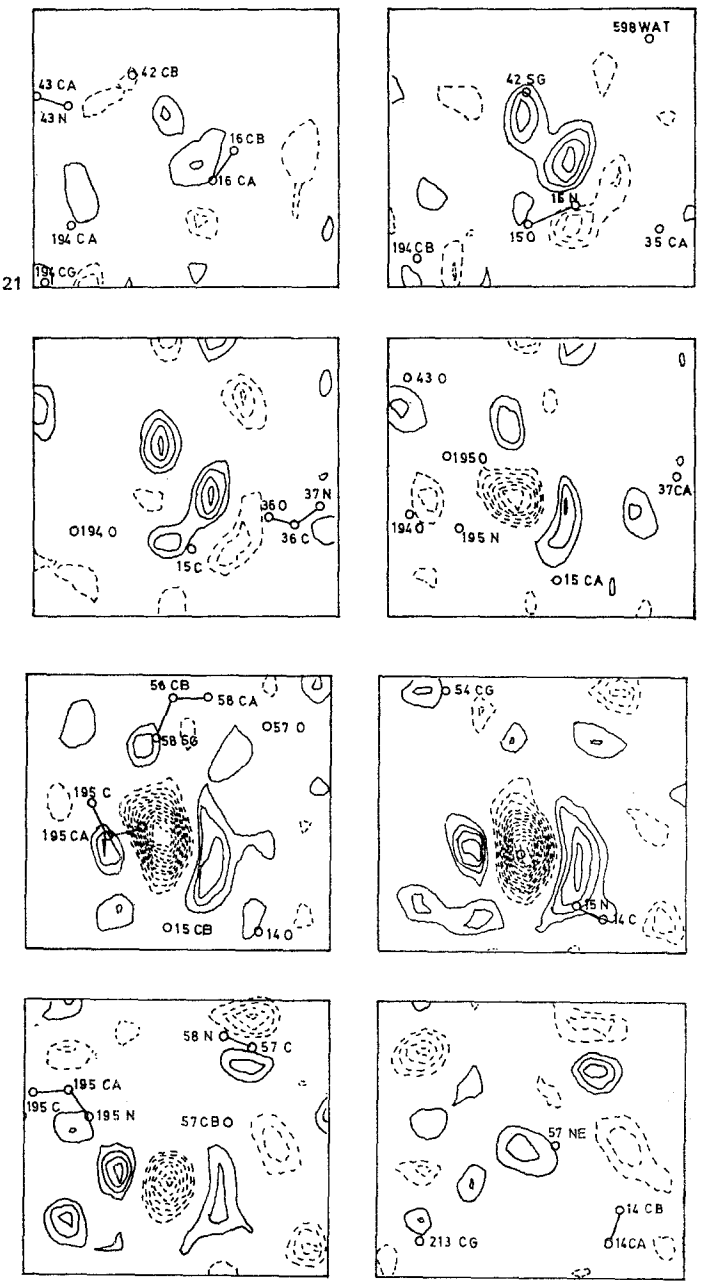
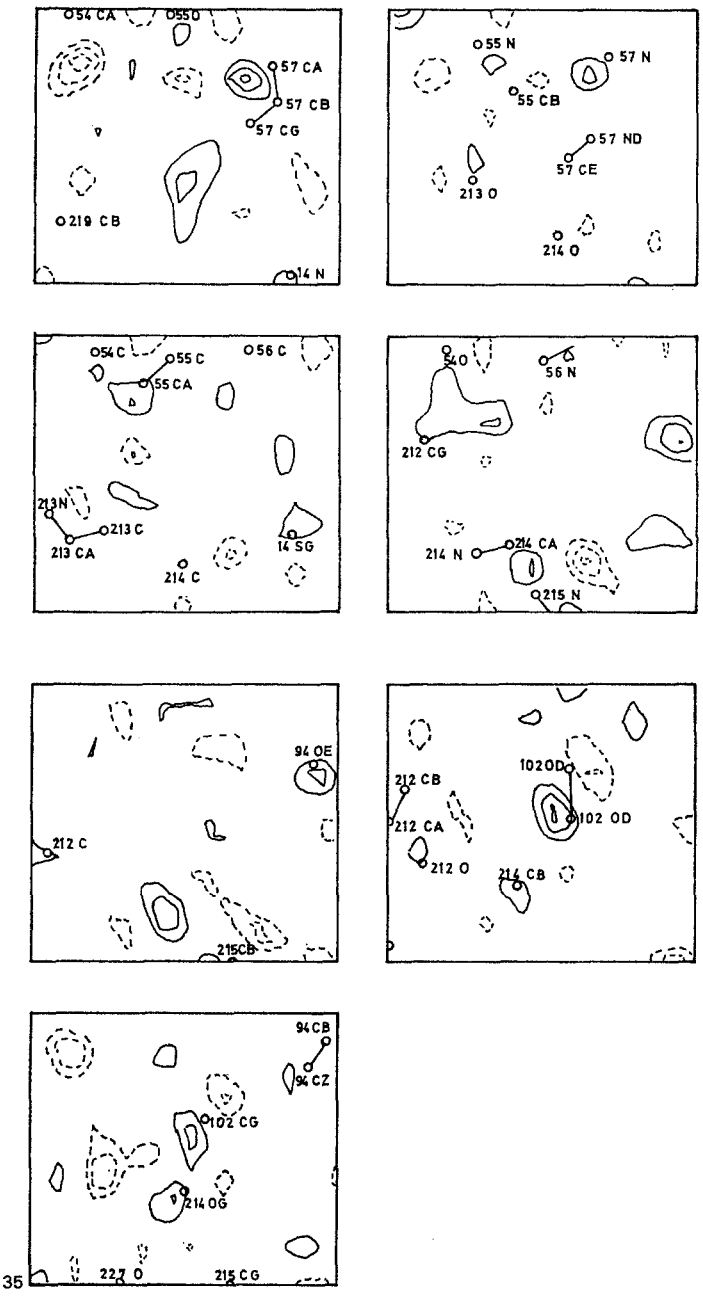


Fig. 1. Sections 21 to 35 of the difference Fourier map anhydro-complex versus native complex of segments near the contact site. Contours from 0.05 e/Å^3 in 0.025 e/Å^3 steps. Solid lines indicate positive, broken lines negative residual density. Residue numbers 14, 15, 16, 35, 36, 37 indicate constituent parts of the inhibitor. All others are constituent parts of trypsin. The non-indicated atomic positions on sections 25 and 26 are Ser 195 C^{β} and O^{γ}, respectively


054 CA
055 O
57 CA
57 CB
57 CG
219 CB
14 N
055 N
57 N
55 CB
57 ND
57 CE
213 O
214 O
054 C
055 C
056 C
55 CA
213 N
213 C
213 CA
14 SG
214 C
540
56 N
212 CG
214 N
214 CA
215 N
94 OE
212 C
215 CB
102 OD
212 CB
102 OD
212 CA
212 O
214 CB
94 CB
94 CZ
102 CG
214 OG
227 O
215 CG

35

Fig. 1

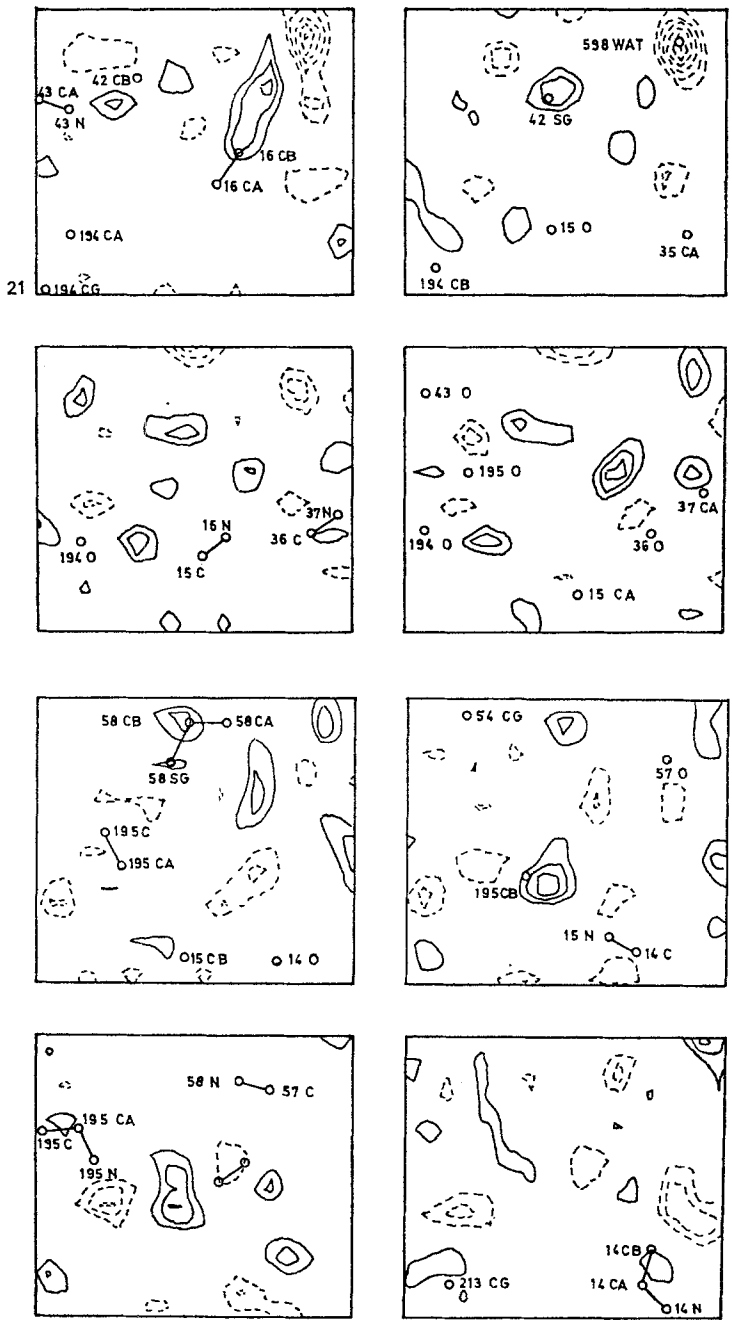
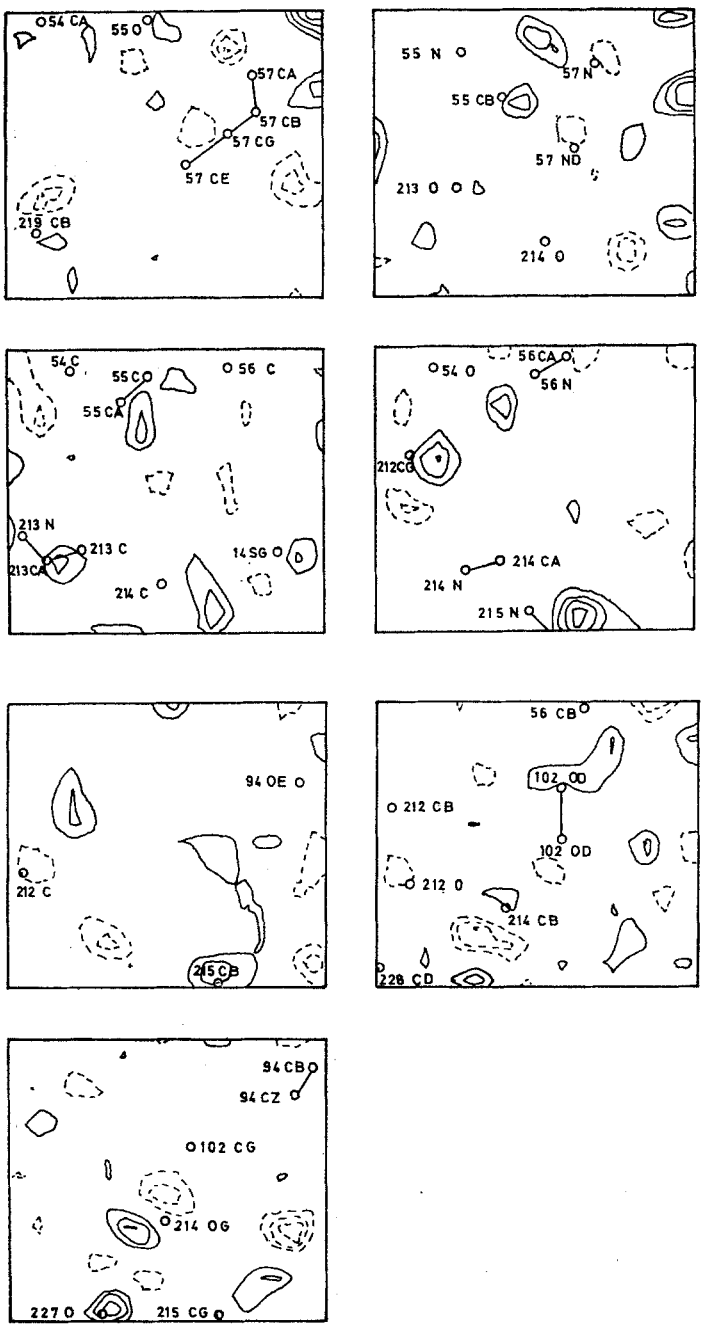


Fig. 2. Final difference Fourier map anhydro-complex versus calculated anhydro-complex Identical cut-out as shown in Fig. 1. Contours from 0.1 e/Å^3 in 0.05 e/Å^3 steps


54 CA
55 O
57 CA
57 CB
57 CG
57 CE
219 CB
55 N O
57 N
55 CB
57 ND
213 O O
214 O
54 C
55 C
56 C
55 CA
213 N
213 C
213CA
14 SG O
214 C
54 O
56 CA
56 N
212CG
214 CA
214 N
215 N
94 OE O
212 C
245 CB
56 CB
102 OD
212 CB
102 OD
212 O
214 CB
228 CD
94 CB
94 CZ
102 CG
214 OG
227 O
215 CG

35

Fig. 2

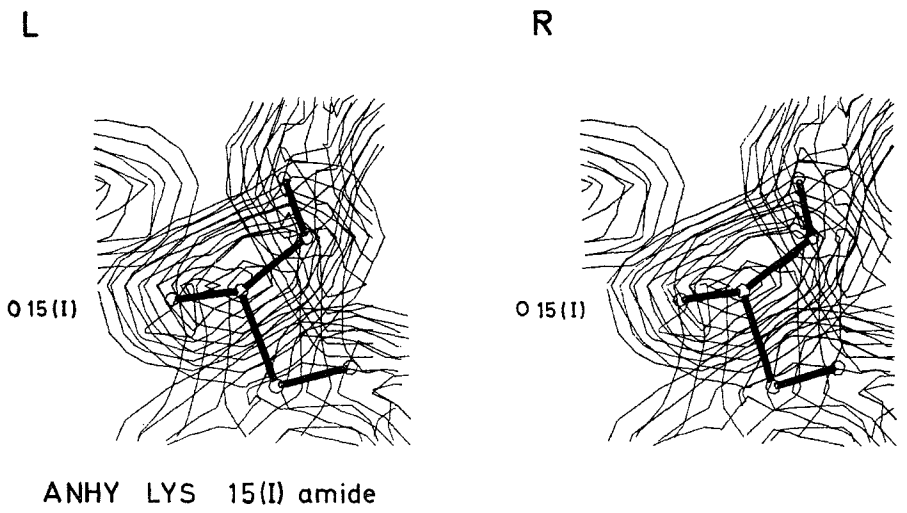


Fig. 3. Electron density map and model fit of Lys 15 (I) amide. Contours from 0.21 e/Å^3 in steps of 0.21 e/Å^3. The Lys 15 (I) O is −41° out of the plane formed by C$^\alpha$, C, N. Ala 16 (I) C$^\alpha$ is in the upper right-hand corner

the anhydro-trypsin preparations. Further residual density peaks are very similar to that observed in the difference map of the native complex (Huber *et al.*, 1974). The resulting coordinate set of the anhydro-complex could then directly be compared with the coordinate set of the native complex. The errors to be expected in these analyses have been estimated to be smaller than 0.2 Å and 5° in atomic positions and in dihedral angles respectively (Huber *et al.*, 1974).

Fig. 3 is a stereo-diagram of the electron-density map of the anhydro-complex at the Lys 15 (I)— Ala 16 (I) amide. It is comparable with Fig. 7 in Huber *et al.* (1974). Electrondensity and model show as large a tetrahedral deformation at C of Lys 15 (I) as in the native complex. An additional angular variable has been introduced describing the degree of pyramidalization at Ser 195 C$^\alpha$. θ^5 allows a rotation of C$^\beta$ around an axis through C$^\alpha$ parallel to N and C. θ^5 is —54° for an ideally tetrahedral C$^\alpha$ and O° for an ideally trigonal C$^\alpha$. It is determined to —15° in the anhydro-complex. This angle is analogous to θ^4 describing the pyramidalization at C Lys 15 (I) (Huber *et al.*, 1974). Table 1 compares the conformational angles at the active site residues of the native and anhydro-complex. They show no significant difference except at θ^5 which demonstrates a trigonal Ser 195 C$^\alpha$ atom in the anhydro-species.

A remarkable observation is that θ^4 is identical within the limits of error in both structures. The high degree of pyramidalization at C of Lys 15 (I) is not influenced by the presence of Ser 195 O$^\gamma$ in the native structure to a measurable extent. Nucleophile-electrophilic center interaction (Bürgi-Dunitz interaction) (Bürgi *et al.*, 1973) plays little role in the deformation of C of Lys 15 (I). Instead the numerous and intimate interactions between the Lys 15 (I)— Ala 16 (I) amide and the enzyme appear to deform the C atom. These values are tabulated in Table 2. The contact distances are identical for the two molecules within the

Table 1. Conformational angles of the contact residues of native (first line) and anhydro-complex (second line). The nomenclature recommended by IUPAC-IUB (1970) is used with additional definitions as given by Diamond (1966, 1971, 1974)

Amino acid	ϕ	ψ	w	τ	χ^1	χ^2	χ^3	χ^4	χ^5	θ^1	θ^2	θ^3	θ^4	θ^5
Ala 55	−103	153	166	100										
	− 94	152	171	108										
Ala 56	− 69	− 19	176	128										
	− 67	− 29	179	121										
His 57	− 85	− 3	168	106	62	−103								
	− 80	− 11	156	111	69	−107								
Cys 58	− 90	2	170	120	− 50	− 86	− 86	−146		10	55			
	− 80	6	173	127	− 38	− 87	− 80	−142		10	55			
Tyr 59	− 56	112	164	116	177	−113								
	− 58	119	171	116	172	−100								
Asn 100	− 58	123	167	114	−150	−161								
	− 54	120	164	114	−146	−149								
Asn 101	82	52	−170	104	− 80	140								
	84	59	−156	108	− 84	140								
Asp 102	− 92	76	177	101	−169	0								
	−108	77	175	90	−186	9								
Ile 103	−148	139	−174	112	−167	172								
	−145	143	−174	110	−167	169								
Met 104	−146	149	177	107	132	164	122							
	−150	145	176	107	138	164	103							
Asp 189	−162	169	155	107	−154	−172								
	−165	172	163	106	−159	−182								

Ser 190	− 95	156	−168	116	48								
	−104	160	−166	110	59								
Cys 191	−142	−176	−178	106	−154	38	86	171	−9	62			
	−148	−171	−172	105	−155	39	86	174	−9	62			
Gln 192	− 56	133	−169	113	− 1	− 90	161						
	− 69	133	−169	110	1	− 98	176						
Gly 193	113	− 28	−170	109									
	116	− 31	−170	107									
Asp 194	− 92	− 27	169	116	− 67	142							
	− 89	− 26	174	114	− 67	145							
Ser 195	− 33	143	−166	121	− 83								−54
	− 35	134	−177	121									−15
Gly 196	77	− 21	−171	122									
	92	− 22	−171	115									
Gly 197	− 84	173	−163	115									
	− 88	172	−163	117									
Val 213	− 59	125	−173	113	−176								
	− 58	129	−169	114	−179								
Ser 214	−125	− 68	171	121	−156								
	−130	− 70	171	118	−153								
Pro 13 (I)	− 90	− 40	165	107					171	178	167		
	− 91	− 33	168	108					183	198	166		
Cys 14 (I)	− 44	153	−162	119	127	90	97	−118	175	142			
	− 54	155	−165	119	125	87	98	−115	175	142			
Lys 15 (I)	−129	57	159	108	− 61	177	176	176				−35	
	−130	58	150	108	− 56	182	185	176				−41	
Ala 16 (I)	− 99	172	175	108									
	− 96	174	174	111									

Table 2. Contacts between enzyme and inhibitor around the Lys 15 (I) – Ala 16 (I) amide. anhydro-complex (second number). This listing comprises

	13 O	14 N	38 S^{γ}	14 S^{γ}	14 C^{β}	14 C^{α}	14 C	14 O	15 N	15 N^{ζ}
216 N	H 33/32									
215 C^{β}	37/35	38/40								
99 $C^{\delta 1}$			39/38	40/39						
99 $C^{\delta 2}$			39/39	38/39						
99 C^{γ}				38/39						
57 N^{δ}				39/39						
57 C^{δ}					39/38					
57 N^{ϵ}					38/38				40/38	
214 O					37/					
192 $NO^{\epsilon 2}$							40/40	H 29/29		
214 O					36/37	35/35	39/39		H 33/33	
195 O^{γ}									H 32	
57 N^{δ}				39/						
215 C^{α}	37/35									
215 C	39/38									
216 O	33/32									
414 WAT										H 28/28
416 WAT										H 28/29
190 O										H 25/26
215 O										36/36
226 C^{α}										39/40
190 C										34/35
190 O^{γ}										35/35
215 C										
219 O										
191 N										
191 C^{α}										
191 C										
191 O										
192 N										
192 C^{α}										
192 C										
193 N										
193 C^{α}										
193 C										
194 N										
195 N										
195 C^{β}										
195 C^{α}										
41 O										
42 S^{γ}										
192 C^{β}										

Vertical row: Atoms of the trypsin component;
Horizontal row: Atoms of the inhibitor component;
WAT: Bound water molecules.

limits of error. It appears impossible to alter the inhibitor conformation without disrupting many of these interactions or inducing a complementary change in the enzyme. It is evident, however, that the contact residues of the enzyme are part

The contact distances (multiplied by 10) are given in Å for the native (first number) and only part of all contacts between the two components

15 C^{ε}	15 C^{δ}	15 C^{γ}	15 C^{β}	15 C^{α}	15 O	15 C	16 N	16 C^{β}	16 C^{α}	16 C	16 O	17 N
38/36												
							41/37					
				38/38								
			33	32	31	25	33					
36/35												
30/33	34/35											
37/36												
39/40	38/39											
37/35												
39/39												
	39/39											
	38/37											
	39/38	39/40	37/38									
	40/39		36/35		37/38							
			39/40									
			39/39	38/39	33/34	39/40					37/38	
					36/37							
					H 29/30	39/40				36/37	37/38	38/39
					38/40							
					38/39							
					34/35							
					H 31/30	39/38						
			40/39	40/40	32/33	32/32	37/39		39/40			
					37/35	40/39						
								36/35	36/36	38/37		H 29/29
								37/36				
											33/33	

of an intricate intra-molecular hydrogen bonding system, making this part of the molecule particularily rigid. The deformation of C of Lys 15 (I) allows and improves these interactions to account for the unfavourable energy contribution of the pyramidalization (about 1.4 kcal/mole^{-1} (Winkler and Dunitz, 1971)).

The pyramidalization of a carbonyl carbon and the approach of a nucleophilic nitrogen towards the electrophilic center obey a logarithmic relationship (Bürgi

et al., 1973). The values for the C of Lys 15 (I) ($\theta_4 = -34°$ and its distance to the O^γ of Ser 195 = 2.5 Å) observed in the complex do not conform to this particular curve. The contribution of the interactions to the pyramidalization described before might provide an explanation. It is clear, however, that the particular relationship depends on the chemical nature of the reactants.

Fig. 12 in Huber *et al.* (1974) demonstrated that the binding segment of the inhibitor undergoes a conformational change in the main chain to adapt optimally to the enzyme surface. The structure analysis of the anhydro-species showed that also the pyramidalization of C of Lys 15 (I) is mainly due to adaptation of the surfaces of both components. The catalytic residues are not involved.

In the native enzyme the pyramidalization of C facilitates the approach of the nucleophilic Ser 195 O^γ to form a covalent bond followed by further progress of the catalytic reaction. His-57 N^ε forms a hydrogen bond to Ser 195 O^γ in the native complex. The equilibrium contact distance of Ser 195 O^γ to C of Lys 15 (I) of 2.5 Å indicates that the proton resides predominantly on O^γ. The hydrogen bond between O^γ and N^ε cannot be formed in the anhydro-species. N^ε does not accept another hydrogen bond. It is slightly (perhaps not significantly) closer to the N of Ala 16 (I) than in the native complex (3.7 Å and 4.1 Å respectively). The imidazole ring is indeed rigidly linked to Asp 102 and the conformational change required for proton transfer to N of Ala 16 (I) must be energetically very difficult (Huber *et al.*, 1973, 1974; Satterthwait and Jencks, 1974).

The missing hydrogen bond offers a possible explanation for the slightly smaller stability of the anhydro-complex (about 2 kal $mole^{-1}$)(Vincent *et al.*, 1974). We are, however, aware of the fact that the driving force for complex formation is dehydration (Quast *et al.*, 1974). Possible differences in hydration of native and anhydro-trypsin are unknown.

Acknowledgement. PTI (Trasylol®) was a gift from Bayer AG, Wuppertal, which is gratefully acknowledged. We wish to thank Miss B. Theile for helping us with the X-ray photographs. The financial assistance of the Deutsche Forschungsgemeinschaft and SFB 51 is acknowledged.

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