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Cold Adaption of Enzymes: Structural Comparison Between Salmon and Bovine Trypsins

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ABSTRACT **The crystal structure of an anionic form of salmon trypsin has been determined at 1.82 Å resolution. We report the first structure of a trypsin from a phoikilothermic organism in a detailed comparison to mammalian trypsins in order to look for structural rationalizations for the cold-adaption features of salmon trypsin. This form of salmon trypsin (ST II) comprises 222 residues, and is homologous to bovine trypsin (BT) in about 65% of the primary structure. The tertiary structures are similar, with an overall displacement in main chain atomic positions between salmon trypsin and various crystal structures of bovine trypsin of about 0.8 Å. Intramolecular hydrogen bonds and hydrophobic interactions are compared and discussed in order to estimate possible differences in molecular flexibility which might explain the higher catalytic efficiency and lower thermostability of salmon trypsin compared to bovine trypsin. No overall differences in intramolecular interactions are detected between the two structures, but there are differences in certain regions of the structures which may explain some of the observed differences in physical properties. The distribution of charged residues is different in the two trypsins, and the impact this might have on substrate affinity has been discussed.**

Key words: crystal structure, cold adaption, catalytic efficiency, protein stability, anionic, ectotherm

INTRODUCTION

Members of the large family of enzymes known as serine proteases play an important role in many biological systems, including digestion, blood clotting, and fertilization. Extensive studies have been carried out in order to reveal the molecular mechanism of these enzymes. Trypsin, a highly substrate-specific member of the serine protease family, has in particular been thoroughly studied. A series of crystal structure determinations of trypsin in its native form,[1–4] as well as cocrystallized with,[2,5] or covalently bonded to[6,7] a variety of small molecules, have been reported. These and other crystal structures[8–10] have all in various ways lead to a better understanding of the substrate binding and the catalytic mechanism of trypsin, and also to a better understanding of the general molecular mechanism. Time-resolved X-ray crystallography, by the Laue technique,[11,12] has recently been used to study an acyl-trypsin intermediate in the trypsin catalysis.[13,14] This has lead to interesting new information about the hydrolysis. Furthermore, crystal structures of gene modified trypsins have given additional knowledge about the catalytic mechanism,[5] and of the substrate binding mechanism.[15–18]

The crystal structure studies mentioned above have, with one exception, been carried out on trypsins from mammalian sources. The structure of trypsin from the bacteria *Streptomyces griseus*[4] is, in spite of the evolutionary gap, found to be remarkably similar to the mammalian enzymes. Kinetic studies of trypsins and other serine proteases from cold-blooded species have shown that they in many aspects act differently to their warm-blooded homologs. Enzymes from cold-adapted species [e.g., trypsin,[19,20] elastase,[21] and chymotrypsin[22,23] from cod, trypsins from salmon,[24,25] anchovy,[26] and capelin,[27] chymotrypsin from rainbow trout[28]] are found to have higher catalytic efficiency (expressed by k_{cat}/K_m) than their mammalian equivalents. This effect is required by the need for poikhilothermic organisms to compensate for the lower working temperature.[19] For some of the described enzymes the increased catalytic efficiency is due to a higher turnover number (increased k_{cat}), but others also show an increase in substrate affinity (decreased K_m). The anionic salmon trypsin studied here is 30–40 times more efficient than bovine trypsin in

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Coordinates for the structure have been deposited with the Protein Data Bank at Brookhaven, entry 2TBS.

the hydrolysis of a particular substrate.[24] This is predominantly accounted for by a decrease in K_m, but there is also an approximately 2-fold increase in k_{cat}.

The fish enzymes also tend to be less stable at extreme pH and high temperature than their warm-blood counterparts.[19,20] Thus bovine trypsin is found to be stable at pH 3 but unstable at high pH, while most of the fish trypsins studied are unstable at pHs below 5. Anionic salmon trypsin, for example, is very unstable at pH below 5,[24] but is reasonably stable at pH down to about 5, at which pH the present crystals have been grown and the structure solved.[29] Cod trypsin is reported to be unstable at pH below 5. The pH optimum for the hydrolysis of some specific substrates by bovine trypsin is 7.8–8.0,[30] while the corresponding optimum values reported for some salmon trypsins lie in the pH range 9–11.[31] Thus, there seems to be a shift toward the alkaline side both in terms of stability and activity for fish trypsins compared to their mammalian equivalents.

Trypsins from Atlantic salmon,[24] Atlantic cod,[19] Greenland cod,[20,32] capelin,[27] and krill[33] have been reported to be less stable at high temperatures than the bovine enzyme. The anionic salmon trypsin shows considerably lower temperature stability at acidic pH, compared to bovine trypsin.[24] Low and Somero[34] have shown that a series of enzymatic reactions of ectotherms have lower free energy, enthalpy, and entropy of activation than the corresponding reactions in warm-blooded organisms, and this in turn leads to considerable differences in catalytic efficiencies. Some authors[19,21] suggest that the pronounced differences are due to greater flexibility of the fish enzymes.

The special features shown for enzymes from cold-adapted species compared to their counterparts from warm-blooded species are probably not attributable to a single or even a few structural differences, but rather to a complex interplay of such differences which do not lend themselves to simple explanations. Crystal structure studies of enzymes from ectothermic species were initiated in order to search for possible structural evidences for the special features. In this paper we compare trypsins from North Atlantic salmon (*Salmo salar*) with that from cattle.

METHODS

Salmon trypsin was isolated from the fish pyloric caeca by affinity chromatography methods, and crystallized from an ammonium sulfate solution by the method of hanging drop.[35] The data collection, structure determination, and refinement have been reported.[29] Some additional refinement results following recently available gene sequences will be reported here.

The structure is analyzed and compared to other structures from the Brookhaven Protein Data Bank,[36] using the programs FRODO,[37] CHAIN,[38] X-PLOR,[39] Quanta (Molecular Simulations, Inc.), and the CCP4-package (SERC Daresbury Laboratory, 1986).

RESULTS AND DISCUSSION

Results of the Additional Refinement

The structure of salmon trypsin was primarily solved and refined with minor knowledge of the primary structure[29]—only the 23 first amino acids of the N-terminal were sequenced.[35] During the last stage of refinement, a full gene sequence of salmon trypsin became available, but it did not match the crystallographic results completely. Later, other gene loci, coding for salmon trypsin were identified,[40] and one of these, ST II (Fig. 1) showed good agreement with the present crystal structure.

The previous model of salmon trypsin had to be remodeled in a few regions, and this improved the overall quality of the electron density map. The new amino acid sequence showed a deletion of one residue compared to the first sequence and model. This deletion (at position 146, Fig. 1) occurs in a region of the structure which had been omitted from the first refinements due to uninterpretable electron density. In the maps derived from the corrected model, this section has become much clearer and the region is now included in the refinement and map calculations. However, the three last residues in the C-terminal part (residue 243–245) are still not visible and therefore not included in the calculations. For five residues (Lys-23, Arg-62, Lys-74, Tyr-97, and Asn-178), there are no side chain density beyond the β-carbon atom, although the sequence information indicates longer side chains. The occupancies for side chain atoms beyond Cβ of these residues were therefore set to zero during refinement.

The quality of the resulting model and electron density maps are good. Rms deviations from ideality are 0.020 Å for bonds, and 0.040 Å and 0.046 Å for 1–3 and 1–4 angle distances, respectively. The rms deviation from ideality for some other parameters are given in Table I. The crystallographic R-factor for the final model is 0.165 using the 14483 reflection $> 3\sigma$ in the resolution range 6.0–1.8 Å. The mean coordinate error is estimated to be less than 0.15 Å, using the method suggested by Luzatti.[41] Average B-factors are 18.7 Å^2 for all atoms, 16.9 Å^2 for protein atoms, 14.8 Å^2 for main chain atoms, and 19.2 Å^2 for side chain atoms.

The present model includes 164 solvent molecules treated as water oxygen atoms with unit occupancies.

The Overall Molecular Structure

The overall fold of the polypeptide chain of salmon trypsin is similar to that of other trypsins with known 3-D structure, and also to the folding of other members of the serine protease family. A detailed

TABLE I. Final Refinement Parameters and Standard Deviations

No. of protein atoms*		1622
No. of solvent atoms		164
	Rms deviation from ideal values	
	Standard deviation	$\sigma^{\dagger}$
Distance restraints (Å)		
Bond distance (1–2)	0.020	0.01
Angle distance (1–3)	0.040	0.02
Planar distance (1–4)	0.046	0.03
Plane restraint (Å)	0.015	0.02
Chiral volum (Å^3)	0.042	0.12
Nonbonded contact restraints (Å)		
Single torsion contact	0.172	0.50
Multiple torsion contact	0.321	0.50
Possible hydrogen bond	0.221	0.50
Conformational torsion angle restraint (°)		
Planar (ω)	4.1	3
Staggered	15.2	15
Orthonormal	27.6	20
Final *R*-factor in the resolution range 6.0–1.82 Å		
For 16,047 reflections > 1σ(F)	0.178	
For 15,282 reflections > 2σ(F)	0.173	
For 14,474 reflections > 3σ(F)	0.165	

*Including Ca^{2+} and benzamidine.
†The weight on each restraint corresponds to 1/σ.

description of the trypsin molecule was given by Stroud et al.,[42,43] of chymotrypsin by Birktoft and Blow,[44] and of elastase by Sawyer et al.[45]

Sequence work on the gene from Atlantic salmon has revealed that the gene codes for at least 4 different trypsins.[40] The present crystallographic work indicates, as mentioned above, that the structure corresponds to the primary structure ST II (Fig. 1), and there is no indication of a mixture of different trypsins in the crystals. The present form of salmon trypsin (ST II) comprises 222 residues, one less than for bovine trypsin (BT). The deletion (residue 146) occurs in the region described by Marquart et al.[2] as the "autolysis loop."

The sequence homology among known trypsins are in general high: 65% between ST II and BT, 66% between ST II and rat trypsin (RT), and 71% between ST I and ST II (Fig. 1). The six disulfide bridges present in bovine trypsin are found at the same positions in salmon trypsin. A metal ion, presumably a calcium ion in ST II, has similar environments in ST II, BT, and RT.

Coordinate differences

Table II shows average differences in atomic positions of main chain atoms for different trypsin structures after least-squares fitting of the models. The models are taken from the Brookhaven Protein Data Bank. Coordinate sets for bovine trypsin, as found with different inhibitor complexes, in different crystal forms, and at different pH, are compared. The differences, in the range 0.15–0.30 Å for the bovine trypsin structures probably indicate a level of model uncertainty where also packing and pH effects are taken into account. The average differences in main chain position between the present structure of salmon trypsin and the bovine trypsin structures are about 0.8 Å. The corresponding difference between salmon trypsin and RT is slightly larger (0.9 Å). Trypsin from the *Streptomyces griseus* is homologous in primary structure with bovine trypsin in only 30% of the chain, but is still relatively similar in tertiary structure (1.78 Å).

In Figure 2 the Cα skeletons of ST II and BT are superimposed after least squares fitting of the main chain atoms of the two structures, and in Figure 3, the mean rms difference between ST II and BT is shown for each residue along the poly-peptide chain. The most extreme difference is in the autolysis loop in BT (residues 144–150), where the largest displacement between ST II and BT is 11 Å. Other differences greater than one rms are found in the regions 22–26, 60–63, 96–97, 114–117, 125, 173–174, 223, and the residues 242–245 of the C-terminal helix (Fig. 3), and most of these occur where different side chains in ST II and BT allow or require different main chain conformations. For example, some of the differences are seen for residues where glycines are replaced with other residues (23, 62, 223), thus longer side chains force the peptides to take different conformations. Furthermore, some of the differences are seen for flexible regions where residues are free to take many different conformations, but where also the measured positions are more uncertain.

Temperature factors

The average temperature factor for the main chain atoms in ST II is 14.8 Å^2, compared to the corresponding value of 13.0 Å^2 for BT. Figure 4 shows a comparison of average temperature factors for main chain atoms in ST II and BT, plotted as the average value for each residue along the respective polypeptide chains. The variations in *B*-factors along the chain of the two trypsins are rather similar, but there are some marked regional differences with the most predominant one running from residue 86 to residue 99. The average main chain atomic temperature factor for these 14 residues of ST II is 8.5 Å^2 higher than for the corresponding residues in BT. The loop from residue 144 to 150 is rather flexible in both crystal structures, but with higher *B*-values for ST II. The residues of the C-terminal of the structures, residues 231–245 of the C-terminal helix, are refined to gradually increasing temperature factors along the chain. This effect is most pronounced for the ST II structure where the two last residues of the helix are not visible in the electron

	I	E	E	E	E	E	I	E	E	E	E	E	E	E	I	I	I	I	E	E	E	E	E	I	I	I
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	37	38	39	40	41	42	43	44
ST I	Ile	Val	Gly	Gly	Tyr	Glu	Cys	Arg	Lys	Asn	Ser	Ala	Ser	Tyr	Gln	Ala	Ser	Leu	Gln	Ser	Gly	Tyr	His	Phe	Cys	Gly	Gly
ST II	Ile	Val	Gly	Gly	Tyr	Glu	Cys	Lys	Ala	Tyr	Ser	Gln	Ala	His	Gln	Val	Ser	Leu	Asn	Ser	Gly	Tyr	His	Phe	Cys	Gly	Gly
ST III	Ile	Val	Gly	Gly	Tyr	Glu	Cys	Lys	Ala	Tyr	Ser	Gln	Thr	His	Gln	Val	Ser	Leu	Asn	Ser	Gly	Tyr	His	Phe	Cys	Gly	Gly
ST IV	Ile	Val	Gly	Gly	Tyr	Glu	Cys	Lys	Ala	Tyr	Ser	Gln	Pro	His	Gln	Val	Ser	Leu	Asn	Ser	Gly	Tyr	His	Phe	Cys	Gly	Gly
RT	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Gln	Glu	Asn	Ser	Val	Pro	Tyr	Gln	Val	Ser	Leu	Asn	Ser	Gly	Tyr	His	Phe	Cys	Gly	Gly
BT	Ile	Val	Gly	Gly	Tyr	Thr	Cys	Gly	Ala	Asn	Thr	Val	Pro	Tyr	Gln	Val	Ser	Leu	Asn	Ser	Gly	Tyr	His	Phe	Cys	Gly	Gly

	I	I	E	E	E	E	E	I	I	I	I	I	E	E	E	E	E	E	I	E	I	E	I	I	E	E	E
	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	69	70	71	72
ST I	Ser	Leu	Ile	Ser	Ser	Thr	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Tyr	Lys	Ser	Arg	Ile	Gln	Val	Arg	Leu	Gly	Glu	His	Asn
ST II	Ser	Leu	Val	Asn	Glu	Asn	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Tyr	Lys	Ser	Arg	Val	Glu	Val	Arg	Leu	Gly	Glu	His	Asn
ST III	Ser	Leu	Val	Asn	Glu	Asn	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Tyr	Lys	Ser	Arg	Val	Glu	Val	Arg	Leu	Gly	Glu	His	Asn
ST IV	Ser	Leu	Val	Asn	Glu	Asn	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Tyr	Gln	Ser	Arg	Val	Glu	Val	Arg	Leu	Gly	Glu	His	Asn
RT	Ser	Leu	Ile	Asn	Asp	Gln	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Tyr	Lys	Ser	Arg	Ile	Gln	Val	Arg	Leu	Gly	Glu	His	Asn
BT	Ser	Leu	Ile	Asn	Ser	Gln	Trp	Val	Val	Ser	Ala	Ala	His	Cys	Tyr	Lys	Ser	Gly	Ile	Gln	Val	Arg	Leu	Gly	Glu	Asp	Asn

	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E
	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99
ST I	Ile	Ala	Val	Asn	Glu	Gly	Thr	Glu	Gln	Phe	Ile	Asp	Ser	Val	Lys	Val	Ile	Met	His	Pro	Ser	Tyr	Asn	Ser	Arg	Asn	Leu
ST II	Ile	Lys	Val	Thr	Glu	Gly	Ser	Glu	Gln	Phe	Ile	Ser	Ser	Ser	Arg	Val	Ile	Arg	His	Pro	Asn	Tyr	Ser	Ser	Tyr	Asn	Ile
ST III	Ile	Lys	Val	Thr	Glu	Gly	Ser	Glu	Gln	Phe	Ile	Ser	Ser	Ser	Arg	Val	Ile	Arg	His	Pro	Asn	Tyr	Ser	Ser	Tyr	Asn	Ile
ST IV	Ile	Gln	Val	Thr	Glu	Gly	Ser	Glu	Gln	Phe	Ile	Ser	Ser	Ser	Arg	Val	Ile	Arg	His	Pro	Asn	Tyr	Ser	Ser	Tyr	Asn	Ile
RT	Ile	Asn	Val	Leu	Glu	Gly	Asn	Glu	Gln	Phe	Val	Asn	Ala	Ala	Lys	Ile	Ile	Lys	His	Pro	Asn	Phe	Asp	Arg	Lys	Thr	Leu
BT	Ile	Asn	Val	Val	Glu	Gly	Asn	Glu	Gln	Phe	Ile	Ser	Ala	Ser	Lys	Ser	Ile	Val	His	Pro	Ser	Tyr	Asn	Ser	Asn	Thr	Leu

	E	E	I	I	I	I	I	E	I	E	E	E	E	E	E	E	E	E	E	E	E	I	E	E	E	E	E
	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	127
ST I	Asp	Asn	Asp	Ile	Met	Leu	Ile	Lys	Leu	Ser	Lys	Pro	Ala	Ser	Leu	Asn	Ser	Tyr	Val	Ser	Thr	Val	Ala	Leu	Pro	Ser	Ser
ST II	Asp	Asn	Asp	Ile	Met	Leu	Ile	Lys	Leu	Ser	Lys	Pro	Ala	Thr	Leu	Asn	Thr	Tyr	Val	Gln	Pro	Val	Ala	Leu	Pro	Thr	Ser
ST III	Asp	Asn	Asp	Ile	Met	Leu	Ile	Lys	Leu	Ser	Lys	Pro	Ala	Thr	Leu	Asn	Thr	Tyr	Val	Gln	Pro	Val	Ala	Leu	Pro	Thr	Ser
ST IV	Asp	Asn	Asp	Ile	Met	Leu	Ile	Lys	Leu	Ser	Lys	Pro	Ala	Thr	Leu	Asn	Thr	Tyr	Val	Gln	Pro	Val	Ala	Leu	Pro	Thr	Ser
RT	Asn	Asn	Asp	Ile	Met	Leu	Ile	Lys	Leu	Ser	Ser	Pro	Val	Lys	Leu	Asn	Ala	Arg	Val	Ala	Thr	Val	Ala	Leu	Pro	Ser	Ser
BT	Asn	Asn	Asp	Ile	Met	Leu	Ile	Lys	Leu	Lys	Ser	Ala	Ala	Ser	Leu	Asn	Ser	Arg	Val	Ala	Ser	Ile	Ser	Leu	Pro	Thr	Ser

	E	E	E	E	E	E	E	I	I	I	I	I	I	I	E	I	E	E	E	E	E	E	E	E	E	E	I
	128	129	130	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155
ST I	Cys	Ala	Ser	Ser	Gly	Thr	Arg	Cys	Leu	Val	Ser	Gly	Trp	Gly	Asn	Leu	Ser	Gly	Ser	Ser	Ser	Asn	Tyr	Pro	Asp	Thr	Leu
ST II	Cys	Ala	Pro	Ala	Gly	Thr	Met	Cys	Thr	Val	Ser	Gly	Trp	Gly	Asn	Thr	Met	-	Ser	Ser	Thr	Ala	Asp	Ser	Asn	Lys	Leu
ST III	Cys	Ala	Pro	Ala	Gly	Thr	Met	Cys	Thr	Val	Ser	Gly	Trp	Gly	Asn	Thr	Met	-	Ser	Ser	Thr	Ala	Asp	Ser	Asn	Lys	Leu
ST IV	Cys	Ala	Pro	Ala	Gly	Thr	Met	Cys	Thr	Val	Ser	Gly	Trp	Gly	Asn	Thr	Met	-	Ser	Ser	Thr	Ala	Asp	Lys	Asn	Lys	Leu
RT	Cys	Ala	Pro	Ala	Gly	Thr	Gln	Cys	Leu	Ile	Ser	Gly	Trp	Gly	Asn	Thr	Leu	Ser	Ser	Gly	Val	Asn	Glu	Pro	Asp	Leu	Leu
BT	Cys	Ala	Ser	Ala	Gly	Thr	Gln	Cys	Leu	Ile	Ser	Gly	Trp	Gly	Asn	Thr	Lys	Ser	Ser	Gly	Thr	Ser	Tyr	Pro	Asp	Val	Leu

	E	I	I	E	I	I	I	I	E	E	E	E	I	E	E	E	E	E	E	E	I	E	E	E	I	I	I
	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182
ST I	Arg	Cys	Leu	Asp	Leu	Pro	Ile	Leu	Ser	Ser	Ser	Ser	Cys	Asn	Ser	Ala	Tyr	Pro	Gly	Gln	Ile	Thr	Ser	Asn	Met	Phe	Cys
ST II	Gln	Cys	Leu	Asn	Ile	Pro	Ile	Leu	Ser	Tyr	Ser	Asp	Cys	Asn	Asn	Ser	Tyr	Pro	Gly	Met	Ile	Thr	Asn	Ala	Met	Phe	Cys
ST III	Gln	Cys	Leu	Asn	Ile	Pro	Ile	Leu	Ser	Tyr	Ser	Asp	Cys	Asn	Asn	Ser	Tyr	Pro	Gly	Met	Ile	Thr	Asn	Ala	Met	Phe	Cys
ST IV	Gln	Cys	Leu	Asn	Ile	Pro	Ile	Leu	Ser	Tyr	Ser	Asp	Cys	Asn	Asn	Ser	Tyr	Pro	Gly	Met	Ile	Thr	Asn	Ala	Met	Phe	Cys
RT	Gln	Cys	Leu	Asp	Ala	Pro	Leu	Leu	Pro	Gln	Ala	Asp	Cys	Lys	Ser	Ser	Tyr	Pro	Gly	Lys	Ile	Thr	Ser	Asn	Met	Phe	Cys
BT	Lys	Cys	Leu	Lys	Ala	Pro	Ile	Leu	Ser	Asp	Ser	Ser	Cys	Lys	Ser	Ala	Tyr	Pro	Gly	Gln	Ile	Thr	Ser	Asn	Met	Phe	Cys

	I	I	E	E	E	E	E	E	I	I	E	E	E	I	E	I	I	I	I	I	I	E	E	E	I	I	I
	183	184B	184	185	186	187	188B	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	209	210	211
ST I	Ala	Gly	Phe	Met	Glu	Gly	Gly	Lys	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Glu	Leu	Gln	Gly
ST II	Ala	Gly	Tyr	Leu	Glu	Gly	Gly	Lys	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Glu	Leu	Gln	Gly
ST III	Ala	Gly	Tyr	Leu	Glu	Gly	Gly	Lys	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Glu	Leu	Gln	Gly
ST IV	Ala	Gly	Tyr	Leu	Glu	Gly	Gly	Lys	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Glu	Leu	Gln	Gly
RT	Leu	Gly	Phe	Leu	Glu	Gly	Gly	Lys	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Asn	Gly	Gln	Leu	Gln	Gly
BT	Ala	Gly	Tyr	Leu	Glu	Gly	Gly	Lys	Asp	Ser	Cys	Gln	Gly	Asp	Ser	Gly	Gly	Pro	Val	Val	Cys	Ser	Gly	Lys	Leu	Gln	Gly

	I	I	I	I	I	E	E	E	E	E	E	E	E	I	I	I	I	I	E	I	E	E	E	E	E	I	I
	212	213	214	215	216	217	219	220	221B	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238
ST I	Val	Val	Ser	Trp	Gly	Tyr	Gly	Cys	Ala	Gln	Arg	Asn	Lys	Pro	Gly	Val	Tyr	Thr	Lys	Val	Cys	Asn	Tyr	Arg	Ser	Trp	Ile
ST II	Val	Val	Ser	Trp	Gly	Tyr	Gly	Cys	Ala	Glu	Pro	Gly	Asn	Pro	Gly	Val	Tyr	Ala	Lys	Val	Cys	Ile	Phe	Asn	Asp	Trp	Leu
ST III	Val	Val	Ser	Trp	Gly	Tyr	Gly	Cys	Ala	Glu	Pro	Gly	Asn	Pro	Gly	Val	Tyr	Ala	Lys	Val	Cys	Ile	Phe	Asn	Asp	Trp	Leu
ST IV	Val	Val	Ser	Trp	Gly	Tyr	Gly	Cys	Ala	Glu	Pro	Gly	Asn	Pro	Gly	Val	Tyr	Ala	Lys	Val	Cys	Ile	Phe	Asn	Asp	Trp	Leu
RT	Val	Val	Ser	Trp	Gly	Tyr	Gly	Cys	Ala	Gln	Lys	Gly	Lys	Pro	Gly	Val	Tyr	Thr	Lys	Val	Cys	Asn	Tyr	Val	Asn	Trp	Ile
BT	Ile	Val	Ser	Trp	Gly	Ser	Gly	Cys	Ala	Gln	Lys	Asn	Lys	Pro	Gly	Val	Tyr	Thr	Lys	Val	Cys	Asn	Tyr	Val	Ser	Trp	Ile

	E	E	E	E	E	E	E
	239	240	241	242	243	244	245
ST I	Ser	Ser	Thr	Met	Ser	Ser	Asn
ST II	Thr	Ser	Thr	Met	Ala	Ser	Tyr
ST III	Thr	Ser	Thr	Met	Ala	Ser	Tyr
ST IV	Thr	Ser	Thr	Met	Ala	Thr	Tyr
RT	Gln	Gln	Thr	Val	Ala	Ala	Asn
BT	Lys	Gln	Thr	Ile	Ala	Ser	Asn

Fig. 1. Comparison of primary structures of trypsins from salmon (ST I, ST II, ST III, ST IV), cattle (BT), and rat (RT). Internal and external residues are indicated with I and E, respectively. The numbering system is the one adopted from chymotrypsinogen.[60]

density map, most likely due to dynamic disorder. A comparison of the average temperature factors as a function of distance from the molecular center of mass for some trypsin structures (Fig. 5) shows good agreement between the curves for ST II and BT2. The corresponding curve for BT, however, shows lower values for atoms outside a radius of about 10 Å from the center than BT2 and ST II. This discrepancy is probably due to packing and pH effects.

Internal residues and hydrophobicity

There are 74 sequence differences between ST II and BT, but most of these (68) are changes of surface residues which are not expected to affect the tertiary structure to any great extent. Seventy-two residues in ST II have a water accessible surface area of less than 10 Å^2, and are considered to be internal. Only six of the internal residues are different in ST II and

TABLE II. Mean Displacement (Å) in Main Chain Atomic Position Among Different Trypsin Structures After Least-Squares Fitting of the Models*

	ST II	RT	SGT	BT	BT2	BT3	BT4	BT5	BT6	BT7
ST II	—	0.86	1.78	0.73	0.78	0.77	0.78	0.81	0.79	0.77
RT		—	1.39	0.53	0.47	0.53	0.53	0.53	0.51	0.53
SGT			—	1.38	1.36	1.38	1.38	1.43	1.42	1.38
BT				—	0.26	0.15	0.25	0.26	0.20	0.15
BT2					—	0.28	0.29	0.35	0.30	0.27
BT3						—	0.26	0.26	0.20	0.15
BT4							—	0.32	0.30	0.26
BT5								—	0.21	0.26
BT6									—	0.22
BT7										—

*ST II = benzamidine-inhibited salmon trypsin, pH 5.0, from the present study; RT = 2TRM, benzamidine-inhibited rat trypsin, mutant Asn-102, pH 8.0[5]; SGT = 1SGT, benzamidine-inhibited *Streptomyces griseus* trypsin[4]; BT = 3PTB, benzamidine-inhibited bovine trypsin, pH 7.0, orthorhombic[2]; BT2 = 1TLD, bovine trypsin, pH 5.3[1]; BT3 = 1TPO, bovine trypsin, pH 5.0, orthorhombic[2]; BT4 = 3PTN, bovine trypsin, trigonal[3]; BT5 = 1NTP, monoisopropylphosphoryl-inhibited bovine trypsin, neutron data[61]; BT6 = 4 PTP, diisopropylphosphoryl-inhibited bovine trypsin[7]; BT7 = 1TPP, *p*-aminophenyl-pyruvate-inhibited bovine trypsin.[2]

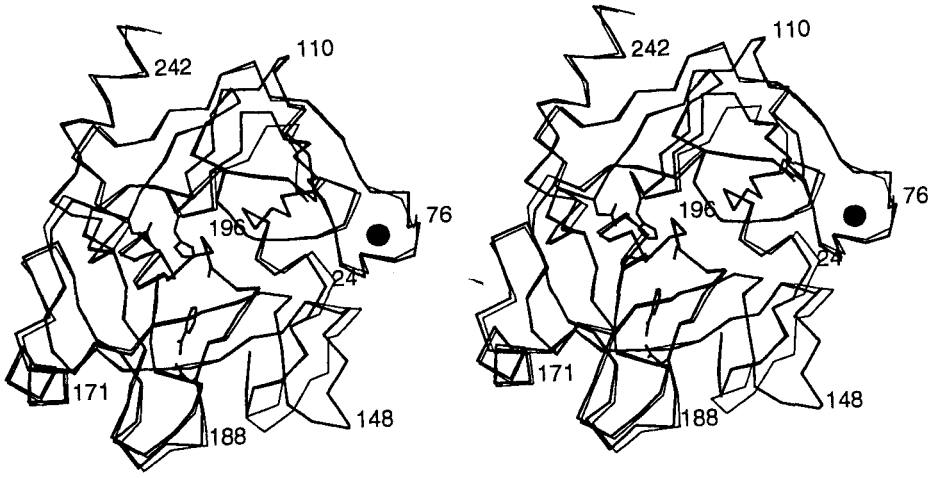

Fig. 2. The Cα-traces of ST II (heavy lines) and BT (thin lines) after superposition of the two structures. The side chains of the catalytic residues Asp-102, His-57, and Ser-195, the side chain of Asp-189 in the bottom of the specificity pocket, and the benzamidine molecule are indicated for ST II, along with a van der Waals sphere of the calcium ion.

BT. Three of these (63, 138, and 212) are changes from isoleucines in BT to valines in ST II, one (160) is a change from alanine to isoleucine, one (238) from leucine to isoleucine, and one (229) from threonine to alanine (Fig. 1). The Leu and Ile of residue 238 in BT and ST II, respectively, occupy about the same space in the two structures. The side chains of residue 138 and 160 are directed toward each other in both BT and ST II, and the reduced size of residue 138 in ST II compared to BT is compensated for by the increased size of residue 160, so that the sum of the space occupied by the two residues is about the same. The reduced size of residues 63, 212, and 229 in ST II compared to BT leaves more free space in the interior of ST II.

Studies of T4 lysozyme mutants[46] have shown that even a single amino acid mutation in the core of the enzyme can have relatively dramatic effects on the stability. Especially mutations imposing larger cavities in the enzyme give rise to greater energy of destabilization. Comparison of the primary structures of ST II and BT in terms of hydrophobic properties shows that the amino acids in ST II are on the average more hydrophobic than those in BT. Using the hydrophobicity scale of Engelman et al.,[47] the average value for the residues of ST II and BT are −0.28 and −0.50, respectively. The corresponding values for internal residues only are 1.28 and 1.31 for ST II and BT, respectively. The differences in size and hydrophobicity of internal residues between the two trypsins may be a reason for the observed differences in stability.

Charged residues

The p*I* values of anionic salmon trypsin are found to be 4.8 and 4.9.[24] The sum of negatively and pos-

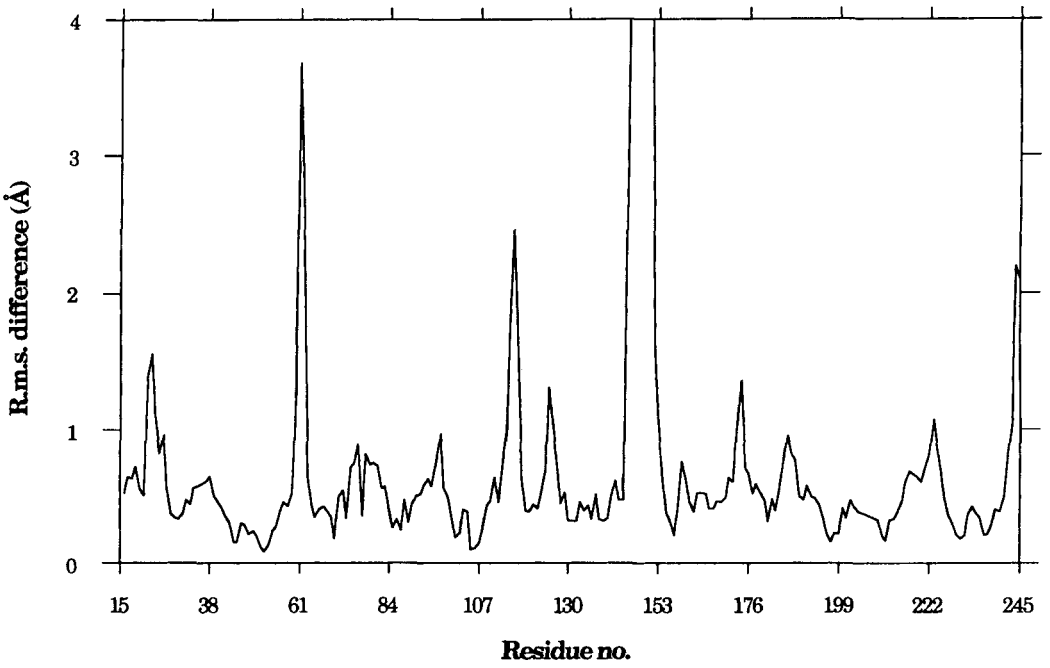

Fig. 3. Rms differences (Å) in main chain atomic positions between ST II and BT, plotted for each residue along the chain.

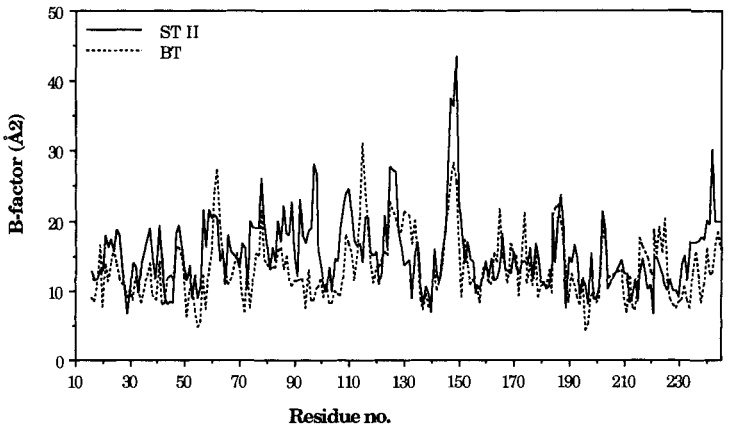

Fig. 4. A chart of the average temperature factors (Å^2) of the main chain atoms of ST II (solid lines) as compared to the BT (dashed lines).

itively charged side chains from the salmon trypsin amino acid composition given in Table III shows a net charge of −4 for ST II and ST III, −5 for ST IV, and +3 for ST I. These sequences probably correspond to one cationic form (ST I) and three anionic forms as described earlier.[40] The corresponding sum of charged amino acid side chains in BT is +6, which accounts for a measured p*I* of 10.8.[48]

The correspondence between trypsin sequences and p*I* values is not obvious. However, it seems likely that ST II is one of the anionic forms with p*I* of about 5. The measured isoelectric point depends not only on the net charge, but also on the distribution of the charged groups. There are four basic side chains more and six fewer acidic side chains in BT than in ST II. Five of the basic side chains are conserved between ST II and BT (Lys-60, Arg-66, Lys-107, Lys-188, and Lys-230), and one, residue 87, is a lysine in BT and an arginine in ST II. The distributions of the remaining basic side chains, 6 in ST II and 10 in BT, are quite different in the two trypsins. Five of them in ST II, not found in BT, are in the N-terminal domain, while the 10 which are unique for BT are found in the C-terminal domain (8) and in the interdomain loop (2). Seven acidic side chains are conserved between the two trypsins (Glu-70,

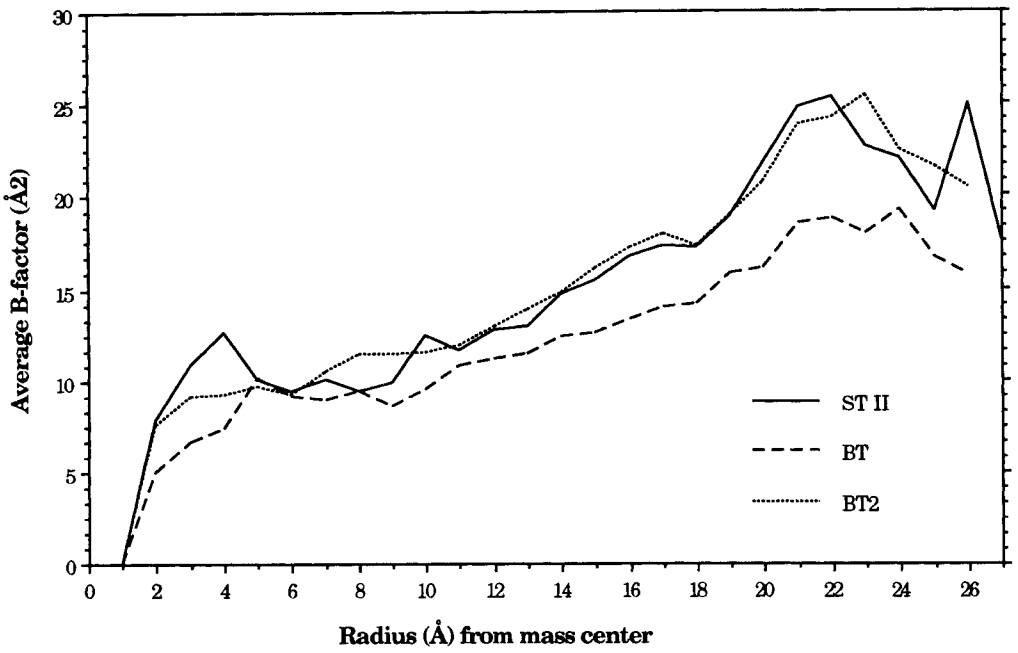

Fig. 5. The average temperature factors (Å^2) for all protein atoms plotted as a function of distances from the mass-center for ST II, BT, and BT2. The plotted values are averages of shells of 1 Å.

TABLE III. Comparison of the Amino Acid Composition in Trypsins From the North Atlantic Salmon and Cattle*

	ST I	ST II	ST III	ST IV	Bovine trypsin[2,30]
Gly	23	23	23	23	25
Asp	7	7	7	7	6
Glu	6	9	9	9	4
Asn	15	17	17	17	16
Gln	8	7	7	9	10
Lys	8	8	8	7	14
Arg	8	4	4	4	2
His	4	5	5	5	3
Ile	11	11	11	11	15
Val	16	18	18	18	17
Leu	15	13	13	13	14
Ala	11	14	13	13	14
Pro	8	10	10	11	8
Trp	4	4	4	4	4
Phe	4	4	4	4	3
Tyr	11	13	13	13	10
Cys	12	12	12	12	12
Met	5	6	6	6	2
Ser	39	26	26	24	34
Thr	8	11	12	12	10
Total	223	222	222	222	223

*There is one deletions of a residue, 146, in ST II, ST III, and ST IV compared to bovine trypsin. Nomenclature of the salmon trypsins (ST I–ST IV) is from Figure 1.

Glu-77, Glu-80, Asp-102, Glu-186, Asp-189, and Asp-194), and 9 acidic side chains in ST II, not present in BT, are distributed with 5 and 4 in the C- and N-terminal parts, respectively. The three acidic side chains unique for BT are distributed with 1 in the N-terminal and 2 in the C-terminal part of the structure. In both trypsins the calcium ion neutralizes some of the negative charge in the appropriate region of the N-terminal domain.

This means that not only is ST II anionic and BT cationic, but that there is also a difference in polarity between the two structures with the N-terminal part of ST II considerably more positively charged than BT, while the C-terminal part of BT is more positively charged than in ST II (Fig. 6). Thus, the region of the molecule that is on the same side as the entrance to the active site is also more positively charged in BT than in ST II.

Glycine residues

Glycine residues are known to permit higher chain flexibility, and differences in glycine content in proteins may be one reason for differences in overall dynamic behavior. Three residues in ST II, namely, Lys-23, Arg-62, and Ser-148 are glycines in BT, and one, Asn-223, is glycine in ST II. Thus there are more glycines in BT than in ST II. These four regions are also shown to be among those with the highest rms differences between the two structures (Fig. 3). However, by comparing the temperature factors of the main chain atoms for these regions (Fig. 4), no tendency for the glycines mentioned above to impose differences in chain flexibility between the two trypsins can be seen.

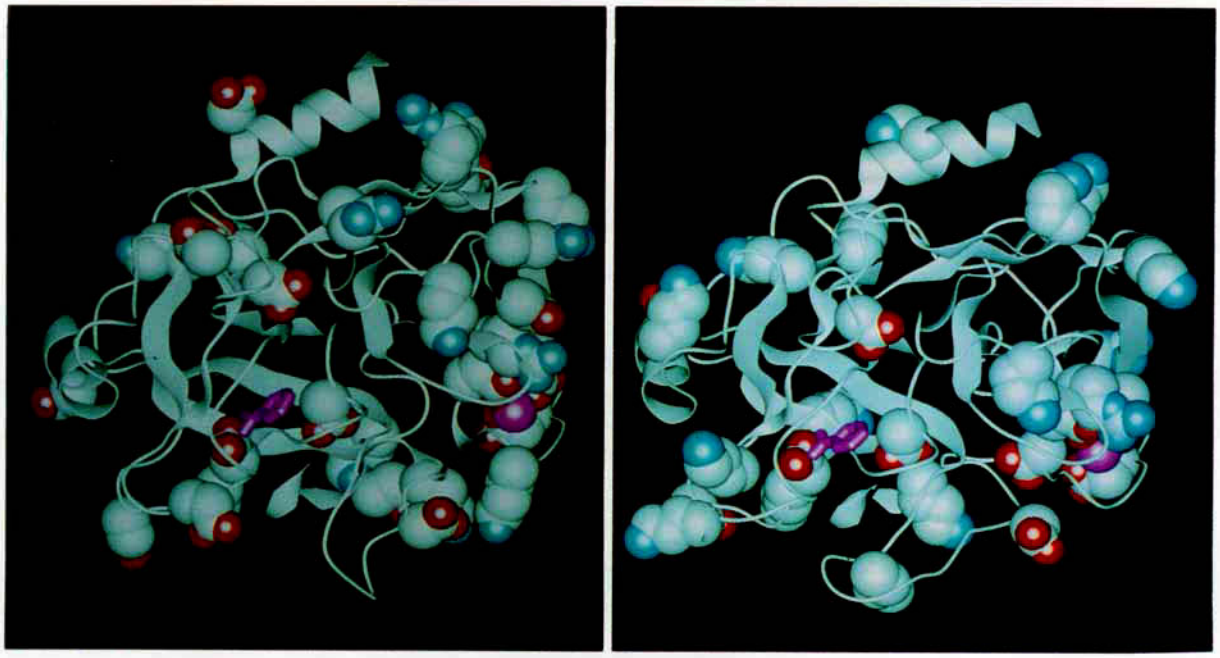

Fig. 6. Backbone structures of ST II (to the left) and BT (to the right). Side chains atoms of the charged residues are depicted as van der Waals spheres. oxygen atoms of acidic side chains and nitrogen atoms of basic side chains are colored in red and blue, respectively. The benzamidine molecule in the active site pocket and the calcium ion are indicated in indigo.

The Secondary Structure and H-Bonding Pattern

A schematic representation of the polypeptide chain of salmon trypsin is given in Figure 7. The β-sheet and α-helical structure elements are marked along with the six disulfide bridges. Differences in the amino acid sequences for ST II and BT are underlined.

Trypsin structures can be divided into N-terminal and C-terminal domains. Either domain consists mainly of a six stranded β-barrel structure,[49] with two short segments of α-helical structure in the C-terminal domain. The first helix runs from residues 164 to residue 173, and the second helix from residue 231 to the C-terminus. The N-terminal β-barrel (residues 30–108) and the C-terminal β-barrel (residues 134–229) are connected by three polypeptide stretches: (1) residues 16–29, (2) residues 109–133, and (3) the C-terminal helix, residues 230–245 (Fig. 2).

A comparison of main chain–main chain hydrogen bonding patterns between ST II and BT shows that there is high conformity between the two structures. The main differences in structural elements are found in the surface loops and in some of the reverse turns, while the core of the proteins are highly conserved in secondary as well as in primary structure. In Figure 8 the numbers of hydrogen bonds in ST II and BT are plotted as a function of spheres with increasing radius from the mass center. In this context the distance criterion "hydrogen bonding donor to acceptor distances shorter than 3.4 Å" is used for selecting hydrogen bonds. Hence, this comparison does not take into account differences in strength between the individual hydrogen bond interactions in the two structures. Keeping this limitation in mind, the plots do not indicate any overall difference in intramolecular electrostatic interactions between ST II and BT.

β-sheet and helices

One-third of the residues in ST II take part in β-sheet structure according to their φ and ψ angles. The residues possessing β-sheet conformation in ST II are giving rise to a total of 66 hydrogen bonds shorter than 3.4 Å. The classification of hydrogen bonds is based on the O···N distance, and the C–O···N or Cα–N···O angles. The average O···N distance of the main chain–main chain hydrogen bonds between β-sheet secondary structure elements is 2.90 Å in the present structure, and the corresponding 66 hydrogen bonds are present in BT too with an average length of 2.92 Å. This indicates that the double β-barrel is very well conserved, and not affected by the 15 differences in primary structure found for the residues taking part in β-sheet structure.

The two stretches of α-helix, residues 164–173, and residues 231–245, are very similar in ST II and BT. The φ and ψ torsion angles of the α-helical residues are all close to the predicted values,[49] and the

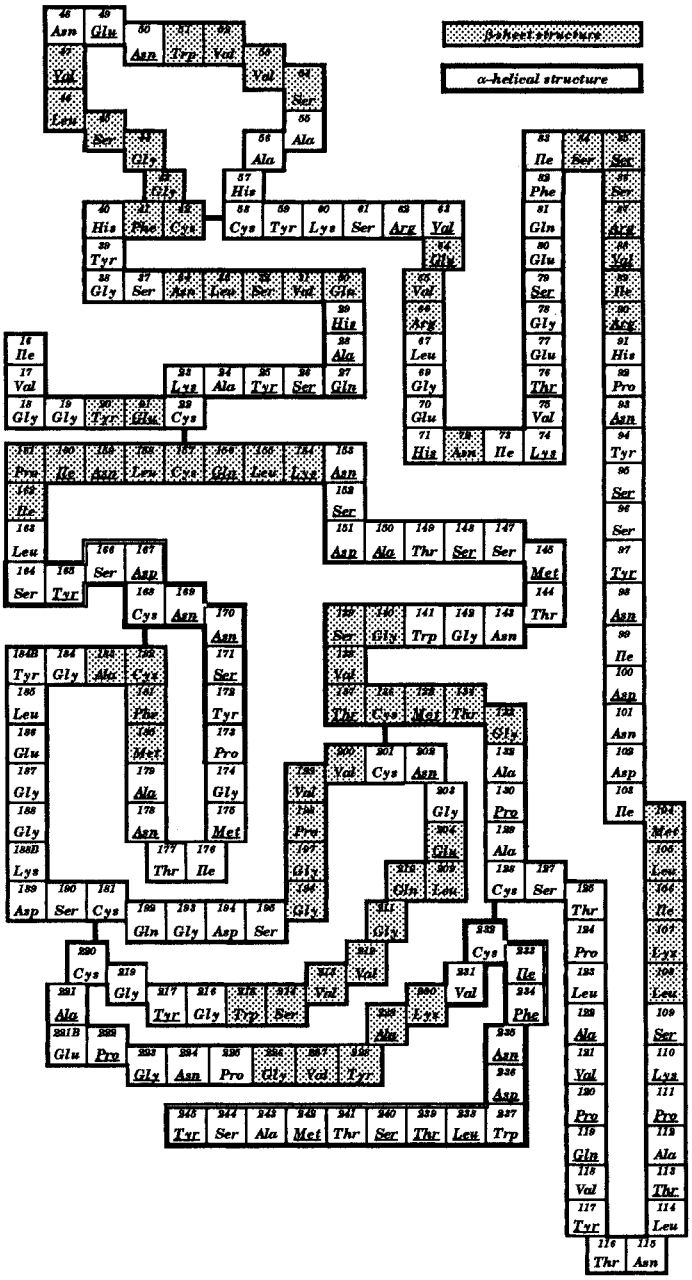

Fig. 7. Schematic representation of the primary and secondary structure of salmon trypsin. β-sheet and α-helical structural elements are indicated, along with the six disulfide bridges. Amino acids which are different in ST II and BT are underlined.

same set of intrahelical main chain–main chain H-bonds is found in the two structures.

The C-terminal helix can be seen as an "arm" along the "body" of the spherical protein (Fig. 2) and makes interactions with the N-terminal β-barrel. The side chains of these helical residues form contacts with the side chains of the antiparallel β-sheet strands formed by the residues 87–92, and 101–107, and to the side chains of the residues 51, 123–128, and 178–179. Since the primary structure is conserved between ST II and BT for only 6 of the 15 residues of the helix, these intramolecular contacts are different in the two trypsins. In BT there are five H-bonding or ion-pair interactions between side chain atoms of the helix and the rest of the molecule. The side chain of Asn-233 forms hydrogen bonds to residues 178 and 179, and the side chain of Tyr-234 forms a hydrogen bond to Asn-101. These interactions may have stabilizing effects on the contact areas and also on the helix by preventing the arm from

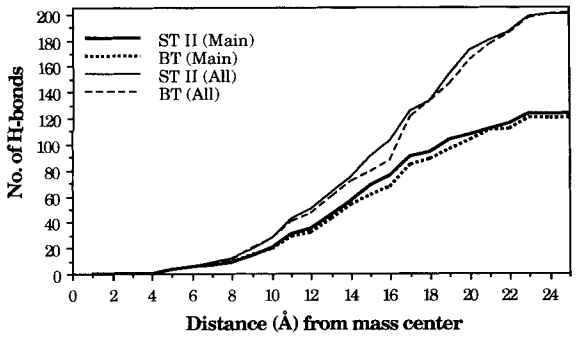

Fig. 8. Number of hydrogen bonds within a certain distance from the mass center of ST II and BT plotted as a function of this distance. Only intramolecular hydrogen-binding interactions between protein atoms are considered.

moving. Residues Ile-233 and Phe-234 of ST II cannot make such interactions. The interactions between the helix and the rest of the molecule in ST II are therefore predominantly hydrophobic. At the C-terminal end of the helix, the carboxyl group of BT forms ion-pair interactions with Lys-87 and Lys-107, as well as interactions between the side chain of Asn-245 and Lys-87. In BT the C-terminal is further stabilized by a hydrogen bond between the side chain of Asn-245 and the main chain of the helix. There is no interpretable electron density for residues 243–245 in the maps of ST II, indicating disorder of the C-terminal residues. Residue 245 of ST II is a tyrosine, which has a side chain which is unable to fold back to form hydrogen bonds with the main chain of the helix. Residues 87 and 107 in ST II are arginine and lysine, respectively, and could be candidates for ion-pair formation with the carboxyl group of the C-terminal in ST II too. However, a tyrosine in this position would probably force a slightly different conformation on the main chain, and it is clear that in ST II, no hydrogen bond interactions can be formed between the tyrosine side chain and Arg-87 or Lys-107.

There seem to be no effective electrostatic intramolecular interactions between the C-terminal helix and the rest of the molecule in ST II, while several such interactions stabilize both ends of the corresponding helix in BT. This could explain the more pronounced increase in average *B*-factors along the C-terminal helix of ST II than in BT, and the observed disorder of residues 243–245 (Fig. 4). This indicates that the C-terminal helix of ST II acts more as a "waving arm," than is the case in BT.

Particularly for the BT structure, the C-terminal helix also may act as a lock for the double domain structure. The internal rigidity of such a helix, and the hydrogen bonding interactions between the helix and both domains of the structure are probably factors restricting relative mobility of the two β-barrels. Examination of the crystal packing shows that the C-terminal helices in ST II and BT are not involved in close intermolecular contacts which could influence the *B*-factors.

TABLE IV. Hydrogen-Binding Contacts Between the N-Terminal and C-Terminal β-Barrels in ST II and BT*

Main chain–main chain	ST II distance (Å)	BT distance (Å)
N 43–O 195	2.78	2.87
O 71–N 155	3.12	—
N 73–O 153	2.84	2.87
Main chain–side chain		
O 139–OE1 Gln-30	3.19	—
O 193–NE2 His-40	2.92	3.05
O 71–NE1 Trp-141	3.01	2.79
O 245–NZ Lys-87	—	2.87
O 245–NZ Lys-107	—	2.59
OXT 245–NZ Lys-107	—	3.37
O 102–OG1 Thr-229	—	2.73
Side chain–side chain		
OE1 Gln-30–OG Ser-139	2.74	2.74
ND2 Asn-74–OD1 Asp-153	—	2.98
OD2 Asp-102–OG Ser-214	2.51	2.83
OD1 Asp-100–OG1 Thr-177	2.63	2.88
OD1 Asp-100–ND2 Asn-179	—	3.16
ND2 Asn-101–OH Tyr-234	—	2.68

*Nomeclature of the salmon trypsin structure (ST II) and the bovine trypsin structure (BT) is from Table II.

Interdomain contacts

From above, the double domain structure is well conserved between ST II and BT. The two β-barrels are probably also relatively rigid, allowing flexibility only in the loops which connect the β-sheet strands. The three polypeptide stretches connecting the two β-barrels are, along with a series of interdomain hydrogen bonds and hydrophobic interactions, important for the relative orientation of the two β-barrels.

The catalytic residues of Asp-102, His-57, and Ser-195 are sited at the junction between the two domains, thus making this contact region of great importance. There are several other contact regions and some parts of the two domains seem to be more strongly connected than others. The regions of the two β-barrels, close to the catalytic residues, are more loosely connected than the regions at the opposite side of the molecule. This is in particular true for ST II. The hydrogen bonds between Asp-102 OD1 and Ser-214 OG, and between Asp-100 OD1 and Thr-177 OG1, both side chain–side chain interactions, are the only interdomain hydrogen bond in this region of ST II (Table IV).

In BT, the interdomain hydrogen bonding pattern involving residues near the specificity pocket is very similar to that found for ST II. In the contact regions

TABLE V. Comparison of Hydrogen Bonds (Å) and Other Nonbonded Distances (Å) in the Active Site Region for Some Trypsin Structures*

	ST II ben. pH 5.0	BT ben. pH 7.0	BT2 native pH 5.3	BT3 ben. pH 5.0	BT8 ben. pH 5.3	SGT native pH 6.2
His-57 Nd1–Asp-102 Od2	2.74	2.67	2.68	2.57	2.67	2.83
His-57 Nd1–Asp-102 Od1	3.53	3.47	3.44	3.34	3.45	3.39
His-57 N–Asp-102 Od1	2.75	2.76	2.91	2.75	2.84	2.96
His-57 Ne2–Ser-195 Og	3.17	2.96	3.10	2.83	3.23	3.05
Asp-102 Od2–Ser-214 Og	2.51	2.83	2.72	2.73	2.76	2.55
Asp-189 Od1–Gly-221 N	2.87	2.84	2.94	2.73	2.96	2.97
Asp-189 Od2–Ser-190 N	2.94	2.86	2.79	2.96	2.81	2.88
Asp-189 Od1–BEN N2	2.82	2.87	—	—	2.89	—
Asp-189 Od2–BEN N1	2.77	2.92	—	—	2.75	—
Ser-214 O–His-57 Nd1	3.49	3.76	3.77	3.58		3.76
Ser-214 O–OW 324 (710)	2.78	2.84				—
Ser-195 Og–OW 415 (702)	2.70	3.16	(SO4)	3.43		—
BEN N1–OW 341 (416)	3.17	3.13	—	—		—
BEN N1–Ser-190 Og	3.05	3.04	—	—		—
BEN N2–Gly-219 O	2.91	2.82	—	—		—
BEN C4–Ser-195 Og	3.47	3.68	—	—		—
BEN C3–Ser-195 Og	3.58	3.65	—	—		—
Asp-189 Od2–OW 302 (562)	3.19	2.84	2.69	2.91	2.56	
Asp-189 Od1–OW 303 (704)	2.83	3.42	3.11	3.62	3.87	

*pH of crystallization and presence of benzamidine inhibitor (ben.) is indicated. The BT water numbering system is given in parentheses. Nomenclature of the salmon trypsin structure (ST II), the bovine trypsin structures (BT, BT2, BT3), and *Streptomyces griseus* trypsin (SGT) is from Table II. Data from BT8 are from Bartunik et al.[1] for the structure HTB1 (their notation).

near the catalytic residues, however, fewer hydrogen bonds are found in ST II than in BT. In BT a hydrogen bond is present between OD1 of Asp-100 and of ND2 of Asn-179. ST II has an alanine in position 179 and cannot form such an interaction. The hydrogen bond between the main chain carbonyl oxygen atom of Asp-102 and OG1 of Thr-229 found in BT is not present in ST II, because Thr-229 in BT has been changed to an alanine in ST II. In addition to these differences, the same region of the N-terminal domain in BT seems to be more extensively linked to the C-terminal domain via salt bridges and hydrogen bonds to the C-terminal helix and C-terminal carboxyl group than is the case for ST II. Ion-pairs in this region might be important for maintaining the relative orientation of the two domains of the molecules.

Although several hydrogen bonds connect the two domains, hydrophobic interactions may be at least as important. The structure is relatively open in the region between the two domains except for contacts between the N-terminal residues and residues close to the specificity pocket. Due to differences in amino acid sequences between BT and ST II in the contact regions there seems to be more free space in this region for ST II. The reduced volume of occupied space in this contact area for ST II compared to BT corresponds to no more than that of approximately 3–4 methyl groups, but this may still give rise to reduced hydrophobic interactions between the two domains in ST II.

The Active Site

Comparative kinetic data[24,25] have shown that salmon trypsin hydrolyzes the substrate *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BApNA) much more efficiently than bovine trypsin. This is predominantly attributed to a decrease in K_m with only a small increase in k_{cat}. These results may indicate possible differences in the substrate binding sites of the two trypsins. It is suggested that there may be a supplementary hydrophobic locus in the substrate binding pocket in salmon trypsin which gives increased affinity for BApNA.[25]

A comparison of nonbonded interatomic distances in the active site cleft and catalytic site region among some trypsin structures is given in Table V.

Catalytic site

The residues known as the "catalytic triad," Asp-102, His-57, and Ser-195, have similar conformation in all the trypsins listed in Table V. The imidazole ring of the central His-57 of the triad makes hydrogen bonds to Asp-102 and Ser-195 (Fig. 9). The Nε2 atom of the imidazole ring is undoubtedly protonated at pH 5. There is, however, no clear relation between pH and Nε2 His-57 to Oγ Ser-195 distances for the structures listed in Table V. It is suggested that the role of Asp-102 is to stabilize the charge of His-57 during catalysis. Both the side chain and main chain of Asp-102 is extensively hydrogen bonded to other parts of the molecule. The carboxyl

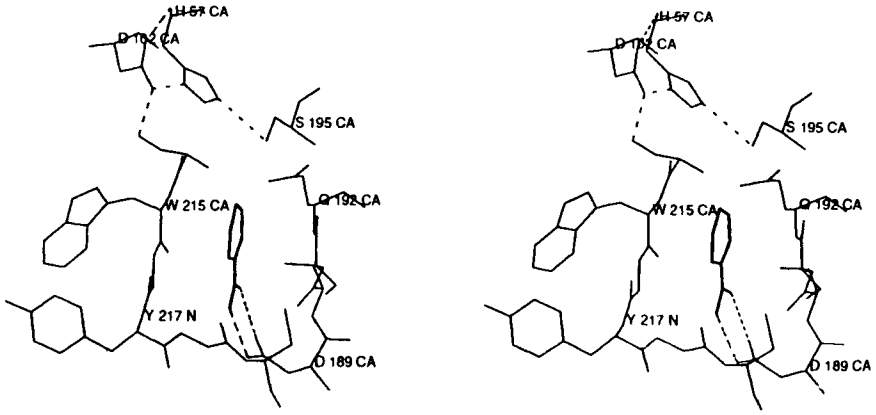

Fig. 9. The active site cleft and catalytic site of ST II with the benzamidine inhibitor (heavy lines) bound to the side chain of Asp-189 in the bottom of the binding pocket. The residues 214–220 and 190–192 form the hydrophobic walls of the primary binding pocket. The residues of the catalytic triad of Asp-102, His-57, and Ser-195 are indicated. Hydrogen bonds are drawn in dashed lines.

oxygen atoms of Asp-102 is hydrogen bonded to the imidazole ring, as well as to the main chain peptide nitrogen of His-57. In BT, Asp-102 is further stabilized via a hydrogen bond between its carbonyl oxygen atom and the side chain hydroxyl group of Thr-229. ST II has an analine in position 229, thus lacking this interaction. This difference in amino acid side chains also gives more free space in this region of ST II than in BT. Even though this is a highly internal residue, no other groups in ST II fill the space that is occupied by Oγ1 and Cγ2 of residue Thr-229 in BT.

Primary binding site

In trypsin the residues 190–192 and 214–220 form the hydrophobic walls of the active site cleft, while the charged side chain of Asp-189 in the bottom of the "pocket" forms effective bonds to substrates or inhibitors carrying a cationic charge (Fig. 9). Hydrophobic interactions between the walls of the pocket and the substrate, and the negative charge in the bottom of the pocket, are source of specificity for arginine and lysine.

Average displacement in main chain atomic positions between ST and BT for the nine residues forming the specificity pocket is 0.13 Å, and the corresponding value is 0.27 Å if the side chains are also included. There is one amino acid difference between ST and BT among the residues forming the substrate binding cleft, namely residue 217, which is a serine in BT and a tyrosine in ST. However, the change to a bulky tyrosine does not seem to have dramatic effects on the structure of ST II compared to BT in this region. The side chain of residue 217 points away from the active site pocket, and although it mostly makes interactions with the solvent, it is possible that the size of this side chain restricts the mobility of residue 217 and of nearby residues which interact with it.

The benzamidine molecule is sandwiched between the peptide planes of residue 215 and 216 on one side, and the planes of 191 and 192 on the other side, both planes being about 4 Å from the plane of the benzamidine molecule. The benzamidine molecule is further bonded via salt bridges to Asp-189 in the bottom of the pocket (Fig. 9). Both carboxyl oxygen atoms of Asp-189 are in turn hydrogen bonded to water molecules (O 302 and O 303) and to the peptide nitrogen atoms of the residues 190 and 221. The salt bridges between the amidinium nitrogen atoms of benzamidine and the carboxyl oxygen atoms of Asp-189 are relatively symmetric, with N2–Od1 and N1–Od2 distances of 2.82 and 2.77 Å, respectively. N1 of benzamidine also forms hydrogen bond interactions to a water molecule (O 341) and to the side chain of Ser 190. In addition, N2 forms a hydrogen bond to the carbonyl oxygen of Gly-219. The binding of benzamidine appears to be similar in the crystal structures of the benzamidine–trypsin complexes listed in Table V. The salt bridges to the Asp-189 are somewhat shorter in ST II than in bovine and rat trypsins, but do not give any indications of significant differences in binding properties for benzamidine among trypsins from different species. Comparison of the static models reveals no contraction of the active site pocket across the trypsins. Distances between residues making up the hydrophobic walls of the pocket and the benzamidine molecule are not significantly different among any of the trypsins.

Subsites

The BPTI (bovine pancreatic trypsin inhibitor) is found to be an inhibitor also for salmon trypsin,[24] and the BPTI–trypsin complex[50,51] is probably the

best model available for studying interactions between trypsin and a natural substrate. In order to compare the binding sites of BT and ST II, the ST II model was superimposed on the model of the complex between BPTI and bovine trypsin. For the superposition, only main chain atoms from residues of the catalytic triad, primary binding site, oxyanion hole, and S1 and S1′–S4′ subsites were used (residues 57, 102, 195, 189–193, 214–220, 39–41). These regions of ST II fit well to the corresponding residues of the BPTI–trypsin complex with an average displacement of 0.34 Å for the 64 atoms included. The corresponding value using the same atoms to fit BT to the complex is 0.30. Including side chain atoms in the superpositions, the corresponding values are 0.57 and 0.34 Å for ST II and BT, respectively.

This comparison indicates that the interactions with a substrate bonded to ST II are probably very similar to those with a substrate bonded to BT. All the interactions between the binding sites of bovine trypsin and BPTI can also be formed with ST II with only minor adjustments of the involved groups.

The Michaelis complex is stabilized by interactions between residues of the substrate and residues of the N-terminal and C-terminal domains in trypsin. The primary binding site, the S1 subsite, and the oxyanion hole is made up of residues from the C-terminal domain, i.e., those residues in trypsin which bind the N-terminal part of the substrate and thus also stabilize the acyl-enzyme intermediate after cleavage of the substrate. The S1′–S4′ subsite, the residues binding the C-terminal half of the substrate (i.e., the part that is cleaved off first), are formed by residues from the N-terminal domain of the enzyme. Possible relative movement of the two domains, discussed in a previous section, may result in an increased degree of deformation of the substrate, and thus both lower the activation energy for formation of the unfavorable tetrahedral intermediate, and generate a "pulling force" which assists breakdown of the tetrahedral complex to form the acyl enzyme and first reaction product.

In a similar way, relative interdomain movements may be a factor influencing the nucleophilicity of the reactive Ser-195. Both His-57, which act as a general base in the reaction, and Asp-102, which probably stabilizes the charge on His-57, are sited on the N-terminal domain of trypsin, while the activated serine is sited in the C-terminal domain. Mobility of the two domains with respect to each other as a part of the general dynamic behavior of the enzymes may during the course of the reaction alter the polarizability of His-57, which in turn will influence the rates of the acylation as well as deacylation steps in the catalytic cycle. Comparison of differences in dynamic behavior between different trypsins cannot be made from static models alone, but as shown in previous sections of this paper, interdomain interactions in ST II and BT are different. This may be seen in relation to the observed differences in catalytic ability.

The Calcium-Binding Site

A series of pancreatic serine proteases are found to structurally bind a Ca^{2+} ion.[52] The role of the calcium ion seems to be to stabilize the structure against thermal and proteolytic degradation.[4] The environment of the calcium ion in ST II is similar to those found for other crystallographically studied serine proteases, although some of the ligands are different in the various structures. Calcium to ligand distances for some selected structures of serine proteases are listed in Table VI. Five of the six ligands in the nearly octahedral coordination around Ca^{2+} are the same in ST II and BT, namely the side chains of Glu-70 and Glu-80, the main chain of residues 72 and 75, and a water molecule (Fig. 10). The sixth ligand, however, is changed from a water molecule (OW 711) in BT to a carboxylate oxygen atom (Glu-77) in ST II. The side chain of Glu-77 interacts with Ca^{2+} in BT too, but via the water molecule OW 711, which is the sixth ligand in BT. The arrangement of three carboxylate oxygen atoms, two main chain carbonyl oxygen atoms, and a water oxygen atom serving as ligands for calcium is not unique, either among metal binding proteins in general,[53] or for serine proteases.

Pancreatic porcine elastase[54] (PPE) has a similar calcium ion coordination, except that the Glu-77 is replaced by an asparagine (Table VI), and the structure of rat trypsin[5] indicates a glutamate in position 77, which is bonded to the calcium ion.

The calcium-binding loop, running from residue 69 to 80, connects two antiparallel β-strands in the N-terminal domain. The loop is stabilized via direct hydrogen bonds to other parts of the molecule and via several water molecules which form bridges between the loop and other parts of the molecule. There are 4 amino acid differences between ST II and BT in the calcium binding loop, Asp-71 in BT is changed to His in ST, Asn-74 to a Lys, Val-76 to a Thr, and Asn-79 to a Ser (Fig. 2). None of these changes causes major structural differences, but the His-71, Lys-74, and Ser-79 of ST II are all involved in side chain–side chain hydrogen bonding interactions with other parts of the molecule, interactions which are not present in BT. The hydroxyl group of Ser-79 in ST II forms hydrogen bonds to the hydroxyl group of Tyr-25 and to the carbonyl oxygen atom of Gly-69. These interactions replace a "network" of three conserved water molecules in the bovine trypsin structures, namely waters OW 561, OW 706, and OW 714 in BT. The side chain of His-71 in ST II also replaces two water positions which are present in BT, OW 412 and OW 718, respectively, to form direct H-bonding interactions with regions of the structure which are cross linked via water molecules in BT. The disordered side chain of Lys-74 in

TABLE VI. Distances Between the Calcium Ion and Ligands in ST II, Compared to Some Other Serine Proteases*

Ligand	ST II	BT	BT2	BT3	PPE
Od1 Asn-77	—	—	—	—	2.7
Oe2 Glu-77	2.30	—	—	—	—
O 75	2.30	2.28	2.32	2.20	2.4
Oe2 Glu-80	2.52	2.40	2.35	2.46	2.4
Oe1 Glu-70	2.29	2.37	2.21	2.32	2.5
OW 327	2.40	2.29	2.45	2.60	2.5
O72	2.42	2.37	2.31	2.33	2.4
OW 711	—	2.49	2.29	2.18	—

*PPE, pancreatic porcine elastase.[54] Nomenclature of the salmon trypsin structure (ST II) and the bovine trypsin structures (BT, BT2, BT3) is from Table II.

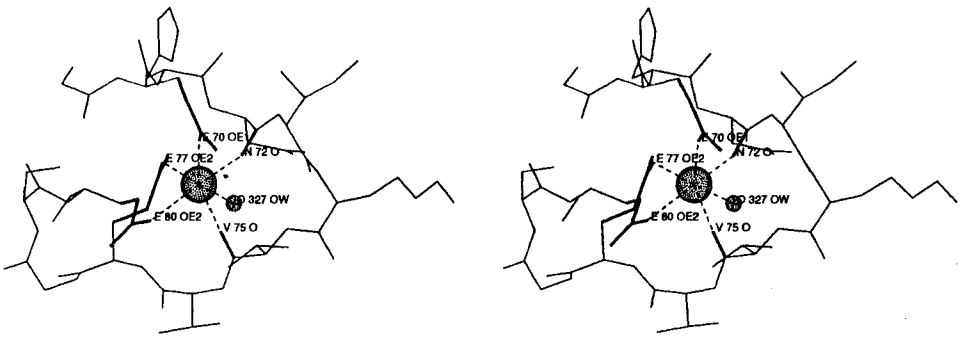

Fig. 10. Residues of the calcium-binding site of ST II, comprising residues 69–80, are drawn in thin lines. The water molecule (OW 327) is indicated with a small filled circle, and other groups interacting with the calcium ion are drawn as heavy lines. Calcium to ligand interactions are indicated with dashed lines.

ST II seems to have two main conformations, which make interactions via water molecules to O 38 in one conformation and is accessible for ion-pair formation with the side chain of Asp-153 in the second conformation. In sum many more structurally bonded water molecules are involved in the formation of bridges between the calcium-binding loop and other parts of the molecule in BT than in ST II. However, most of these interactions are, due to differences in primary structure, replaced by direct side chain–side chain interactions in ST II. The mean temperature factor of the main chain atoms of the calcium binding loop (Fig. 4) is slightly higher for ST II than for BT, indicating higher mobility in ST II. This, in spite of the fact that movement of this loop in the crystal structure of ST II is heavily restricted due to close intermolecular contacts.[29] The calcium-binding loop in BT is not involved in close intermolecular contacts.

Salmon trypsin does not show the same increase in autolysis in the absence of calcium[24] as reported for bovine trypsin.[30] The autolysis rate has also been found to be less dependent on the presence of calcium for other fish trypsins including capelin[27] and lungfish.[55] This does not necessarily indicate that calcium has a lower stabilizing effect in ST II, since the generally lower autolysis could be due to the presence of fewer exposed autolysis sites.

The detailed mechanism of how the calcium ion stabilizes the structure and reduces autolysis is not known, but the calcium-binding loop in both ST II and BT does make interactions with what is described as the autolysis loop in BT, residues 143–155. ST II forms two main chain–main chain hydrogen bonds between these loops, while BT correspondingly forms one main chain–main chain and one side chain–side chain hydrogen bond.

The Autolysis Sites

Bovine trypsin retains activity after partial digestion at two known sites. Autolysis near Lys-145 leads to the formation of α-trypsin with two polypeptide chains, and autolysis at a second site near Lys-188 leads to the formation of ψ-trypsin with three polypeptide chains.[56]

The electron density maps of ST II give no indication of partly digested enzyme at these sites or any other sites. Residue 188 is a lysine in ST II also, and the BT and ST II structures are very similar in this region. However, the loop from residue 144 to 154 is quite different in the two structures with a one residue deletion, residue 146, in ST II compared to BT.

The polypeptide chain of ST II takes a different direction from BT beyond residue 144, but the enzymes are coincident from residue 154. Residue 145 is a methionine in ST II and is thus not a site for autolysis. The closest possible autolysis site in this region for ST II is Lys-154, but this residue is much more buried and thus less exposed for tryptic digestion. The residues near Lys-145 in bovine trypsin are reported to be disordered and to have high temperature factors in all the available crystal structures. This could be due to partial cleavage, but there are indications suggesting that the disorder is mostly dynamical.[1,57] The corresponding residues in ST II also seem to be flexible. The temperature factors are considerably higher than those of the average structure, and also higher than for the corresponding residues of BT (Fig. 4). Lysine 154 and the adjacent residues in ST II, however, are more rigid. Fontana et al.[58] have shown that stretches of the polypeptide chain with high mobility are more susceptible to proteolytic attack. The lower flexibility of the chain near the potential autolysis site, Lys-154 in ST II, could therefore indicate that this loop is not a major site for autolysis.

CONCLUDING DISCUSSION

The overall tertiary structure of the present form of salmon trypsin is similar to that of bovine trypsin. The average displacement in atomic positions between the salmon trypsin structure and various crystal structures of bovine trypsin is about 0.8 Å. This is significantly more than the corresponding differences among the various coordinate sets of bovine trypsin, which range from 0.15 to 0.35 Å.

The amino acid sequence for this form of salmon trypsin differs from that of BT in about 35% of the residues. There is one deletion in the ST II sequence, namely residue 146, which is present in BT. This deletion occurs in the loop where the atomic displacements are largest (Fig. 3). In bovine trypsin this loop is known as the autolysis loop since tryptic cleavage at Lys-145 is believed to be a part of the mechanism which reduces trypsin activity after the enzyme has played its role. In salmon trypsin, there are no lysines and arginines in this region of the structure and it is therefore not an autolysis site. Most of the observed structural differences between the two trypsins may be explained by differences in primary structure. Different amino acid side chains permit or require different backbone conformations. Differences in hydrogen bonding interactions due to side chain differences give rise to different tertiary structure in some cases. This is particularly evident for loop regions where also the most pronounced differences are seen.

Comparison of temperature factor profiles (Figs. 4 and 5) does not indicate any overall differences in flexibility of the two structures. Some loop regions of the structures have marked different dynamic properties, but direct comparison of the flexibility of external loops between different crystal structures is difficult. Effects due to different crystal packing may, in some cases, be estimated, but long-term interactions from close intermolecular contacts often cannot be ruled out. Intermolecular contacts will particularly reduce mobility of solvent-exposed regions, which in the solvated molecule are freer to take different conformations. The dynamic properties of such regions can thus not be detected from crystallographic temperature factors. The observed decrease in temperature stability of enzymes from cold-adapted species compared to the corresponding mammalian enzymes is most likely due to weaker intramolecular forces in these enzymes. In general, one might consider the number of disulfide linkages, the average hydrophobicity, and the number and strength of hydrogen bonds. From the comparison of the crystal structures of ST II and BT, there are no obvious indications of overall differences in such forces. The six disulfide bridges found in bovine trypsin are conserved in the salmon enzyme. Assuming that the average strength of hydrogen bonds in salmon and bovine trypsin is comparable, there are no significant differences in overall hydrogen-bonding interaction between the two trypsins which could indicate differences in dynamic freedom. The reduced stability may, however, not necessarily arise from a general reduction in strength of intramolecular forces, but from weakened interactions in one or a few important sections of the structure. One such example is the contact region between the C-terminal helix and the rest of the molecule (Fig. 2). This helix may be important for the connection between the two β-barrel domains typical for trypsins structures. Since it is oriented like an arm along the molecule, residues of the helix form several intramolecular contacts to residues of the N-terminal domain, and thus form a link between the C-terminal and N-terminal domains. These intramolecular contacts are, however, quite different in ST II compared to BT. In the latter there are several hydrogen bonds between polar side chains of the C-terminal helix and residues of the N-terminal domain, interactions which are not present in the ST II structure due to differences in amino acid sequence. From the electron density maps of ST II it can also be seen that this region is more flexible than in BT. The lack of well-defined electron density for the last three residues of salmon trypsin is a strong indication of disorder. The salt bridges between the lysines of residues 87 and 107, and the carboxylate groups of the C-terminus are well conserved structural features for bovine trypsin. Residues 87 and 107 in salmon trypsin are arginine and lysine, respectively, and could thus be involved in similar salt bridge formations, but the apparent disorder in this region of the salmon trypsin crystal structure may imply weaker electrostatic interac-

tions. The salmon trypsin crystals were grown at pH 5.0 which may mean that the carboxylate group of the C-terminus is at least partly protonated. Low pH might thus disrupt salt bridge formation and introduce a greater degree of disorder in the C-terminal helix of ST II. Corresponding disorder in the C-terminus is, however, not reported for the low pH structures of bovine trypsin[1] so that these salt bridges may be less important for the stability of the helix in bovine trypsin than in ST II due to the presence of additional interactions between the helix and the rest of the molecule. This may be an important factor for the reduced pH stability of ST II.

The average hydrophobicity of the residues in a protein is, in general, considered to be very important for stability. Although the differences in these values for ST II and BT are relatively small, the internal residues tend to be less hydrophobic, and the external residues more hydrophobic in ST II compared to BT. Both factors may probably reduce the stability of the former.

The difference in glycine content does not seem to be a factor in the greater chain flexibility of salmon trypsin, since the number of glycine residues is lower than in bovine trypsin. Some of the glycine residues do, however, occupy different position in the two trypsins, a factor which will impose differences in flexibility for these regions. A corresponding argument may be used for proline residues, which are known to increase rigidity of polypeptide chains. The number of prolines is higher in ST II than in BT, but some of them are in different positions.

The active site clefts and catalytic sites of the two trypsin structures are similar in both tertiary and primary structure. Only one of the active site pocket amino acids is different between the ST II and BT, namely residue 217, which is a tyrosine in ST II and a serine in BT. This change does not seem to effect the tertiary structure of the active sites to any extent, but may influence the mobility of this region of the structure. The catalytic triad of Ser-195, His-57, and Asp-102 is sited at the junction between the two β-barrel domains, which is typical for the trypsin family of serine proteases. The structures of the two domains are similar in ST II and BT, respectively, but there are distinct differences in the interactions between the N-terminal and C-terminal domains of the two trypsin structures. There are fewer interdomain hydrogen bonds in ST II, compared to BT, and the interdomain hydrophobic interactions are probably also weaker in ST II. Due to smaller hydrophobic amino acids in the contact region between the two domains, more free space and cavities are present in ST II. Amino acid changes yielding cavities in the core of protein molecules have been shown in some cases to give marked differences in the stability.[46] This could be one reason for the lower stability of ST II compared to BT, but it could also be envisaged that weaker interdomain interactions could give rise to more relative interdomain motion during substrate binding and catalysis, thus lowering the activation energy for the formation of enzyme substrate intermediate complexes. Since the catalytic site is at the junction of the two domains, such motion would affect the relative orientation of the catalytic residues, and also the structure of the substrate binding sites.

The double domain structure of trypsin may be seen as a "hinge" connection between the two domains. The part of the structures which may be seen as the "hinge" in trypsins is relatively similar in BT and ST II, while the more loosely connected region of the two domains, near the catalytic residues, is quite different. It might be envisaged that the lower number of interdomain interactions in this region for ST II compared to BT allows greater relative movement of the two domains, thus giving rise to a more "dynamic" behavior in respect to substrate binding, catalysis, and the release of products. Relative rigid body motion of domains is a well known phenomena for many enzymes and other proteins,[59] but little is known about this kind of movement for the trypsin family of enzymes.

The distributions of acidic and basic residues in the two trypsins are different. The increased number of acidic side chains in ST II compared to BT, giving rise to the anionic character of the former, is distributed in such a way that the C-terminal domain of ST II is considerably more negatively charged, while the N-terminal domain is only slightly more negatively charged compared to the corresponding regions of BT. In particular, there are more acidic amino acids on the same side as the active site entrance in ST II, compared to BT. It could thus be envisaged that ST II is more effective in locating the positively charged arginine and lysine side chains of a substrate, and also is more efficient in guiding the substrate into the binding cleft. This could give rise to an increased affinity for the positively charged substrates and substrate analogues of ST II.

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