Leaving Group Specificity in the Chymotrypsin-Catalyzed Hydrolysis of Peptides. A Stereochemical Interpretation†

Alan R. Fersht,* D. M. Blow, and Jacques Fastrez‡

ABSTRACT: Rate constants for the attack of amines on various specific and nonspecific acyl-chymotrypsins were determined. It was found that for the attack on specific acyl-enzymes, such as acetylphenylalanine-chymotrypsin, there is a specificity of 100- to 200-fold for alaninamide, tyrosinamide, and other L-amino acids which do not have a free α-carboxyl group. This was not found for nonspecific acyl-enzymes such as furoyl-chymotrypsin. It is inferred from the principle of microscopic reversibility and demonstrated by calculation that the acylation of chymotrypsin by amides and peptides involves a specificity for the leaving group. The stereospecificity is accounted for by the model for the association of the bovine pancreatic trypsin inhibitor with the active site of chymotrypsin. There is a binding site for the carbonyl oxygen of the leaving group in polypeptide substrates involving the α, β, and γ carbons of Met-192, while the side chain of the leaving group makes contact with the side chains of Cys-42 and His-57. This favorable interaction cannot be made in the Michaelis complex without a simultaneously unfavorable interaction of the α carbon of the leaving group with the oxygen of Ser-195 forcing it toward the position it should take up in the transition state of the reaction. There is catalysis and specificity due to strain; the binding energy of the leaving group is not fully realized until the transition state of the reaction. We give a detailed stereochemical mechanism for the acylation of chymotrypsin by a polypeptide substrate.

The specificity of the protease chymotrypsin has been investigated using synthetic ester and amide substrates (Hein and Niemann, 1961). The inherently high reactivity of esters causes chymotrypsin to hydrolyze a wide range of nonspecific as well as specific ester substrates. These reactions have afforded considerable information on the steric requirements for the substrate acyl moiety. Amides are so unreactive that the only substrates accessible to experimental observation are those of the specific amino acids L-tryptophan, L-tyrosine, and L-phenylalanine. A recent report suggests that there is also specificity in the amino moiety of such amide substrates (Baumann *et al.*, 1970). Owing to the experimental difficulty in observing the hydrolysis of amides of other acids there is no information concerning the mutual interaction of specificity in the acyl and the amino portions of amide substrates.

The reverse reaction of the acylation of chymotrypsin by amides is the deacylation of the acyl-enzymes by amines. This is more easily measured (Inward and Jencks, 1965) and the specificity can be studied by means of this reverse reaction. The accompanying paper (Fastrez and Fersht, 1973b) justifies this approach.

We have reexamined the specificity of chymotrypsin in the light of new crystallographic results. Blow *et al.* (1972) have proposed a model for the association of a trypsin inhibitor with chymotrypsin and trypsin. This predicts the stereochemistry of the hydrolyzed peptide bond of the inhibitor.

Experimental Section

Materials and apparatus have been described previously (Fastrez and Fersht, 1973b). Furoyl-chymotrypsin was prepared according to Inward and Jencks (1965) using α-chymotrypsin. Furylacryloyl-chymotrypsin was prepared by a similar procedure except that the enzyme and furylacryloylimidazole were incubated for only 15 min at pH 4.5 and then the pH was lowered to 3.5.

Kinetic Methods. The rate constants for the deacylation of furoyl-chymotrypsin in the presence of nucleophiles at 25°, ionic strength 1.0, and carbonate buffer at pH 9.05, were determined either by the change in absorbance at 265 nm (Inward and Jencks, 1965) or by the binding of proflavine (10^{-4} M) at 465 nm (Bernhard *et al.*, 1966). The deacylation of furylacryloyl-chymotrypsin was monitored under the same conditions at 320 nm (Bernhard *et al.*, 1965).

AcTrp-chymotrypsin was prepared by incubating 5 mM AcTrp with 4×10^{-5} M α-chymotrypsin in 1 M KCl at pH 3.0 (unbuffered) (*cf.* Miller and Bender, 1968). This was placed in one syringe of the stopped-flow mixer at 25°, the other syringe containing 10^{-4} M proflavine in pH 9.05 carbonate buffer at ionic strength 1.0. The deacylation of AcTrp-chymotrypsin was monitored by the proflavine binding at 465 nm after mixing.

The rate constants for the attack of nucleophiles on AcPhe-chymotrypsin were determined from product ratios as described by Fastrez and Fersht (1973b). [^{3}H]AcPhe-OMe (5 mM) was hydrolyzed by δ-chymotrypsin (10^{-5} M, pH 9.30, 5% Me_2SO, 25°, ionic strength 0.95) in the presence of the relevant nucleophile and the products were separated by high-voltage electrophoresis. The ratio of [^{3}H]AcPhe-nucleophile to [^{3}H]AcPhe gives the rate constant relative to that for water (which is 144 sec^{-1}). In some cases rate constants were determined by stopped-flow spectrophotometry using AcPhe-*p*-nitrophenyl ester to generate the acyl-enzyme *in situ* (Fastrez and Fersht, 1973b).

The experimental conditions are summarized in Table I.

Calculation of Rate Constants. We have just established the validity of the following scheme for the chymotrypsin-catalyzed hydrolysis of amides (Fastrez and Fersht, 1973b). It has been suggested previously (Fersht, 1971) that values of

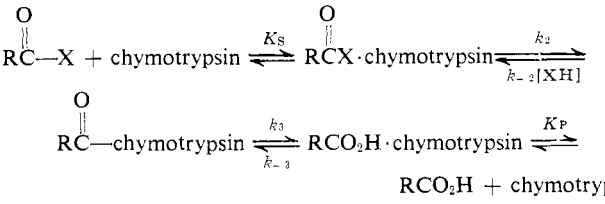
† From the MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England. *Received December 13, 1972.*

‡ Chargé de Recherches du Fonds National Belge de la Recherche Scientifique.

TABLE 1: Experimental Conditions for the Determination of Deacylation Rate Constants.

Acyl-Enzyme[a]	Nucleophile[a]	pK_a	Concn Range (M)	pH	Method
Furoyl-chymotrypsin[b]	Alaninamide	8.24	0–0.17	9.05	Kinetic
	Glycinamide	8.22	0–0.58	9.05	Kinetic
Furylacryloyl-chymotrypsin	Alaninamide	8.24	0–0.68	9.05	Kinetic
	Glycinamide	8.22	0–0.68	9.05	Kinetic
	Hydrazine	8.20	0–0.64	9.05	Kinetic
	Ammonia	9.45	0–0.9	9.30	Kinetic
	Ethylamine	10.97	0–0.9	10.9	Kinetic
AcTrp-chymotrypsin[b]	Alaninamide	8.24	0–0.085	9.05	Kinetic
	Hydrazine	8.22	0–0.32	9.05	Kinetic
AcPhe-chymotrypsin[c]	Alaninamide[d]	8.24	0–0.02	9.30	Kinetic
	Glycinamide[d]	8.22	0–0.8	9.30	Kinetic
	Hydrazine	8.20	0–0.2	9.30	Kinetic
	Alanine	9.69	0–0.35	9.30	Kinetic
	Glycinamide	8.22	0.1–0.5	9.30	Product ratio
	Alaninamide	8.24	0.01–0.1	9.30	Product ratio
	Hydrazine	8.20	0.5–0.9	9.30	Product ratio
	Formylhydrazide	2.81	1.0	9.30	Product ratio
	Glycylglycine	8.25	0.1–0.5	9.30	Product ratio
	Tyrosinamide	7.7	0.1	9.30	Product ratio
	Tryptophanamide	7.7	0.05–0.0125	9.30	Product ratio
	Lysine methyl ester[e]		0.1	9.30	Product ratio

[a] L-Amino acids unless otherwise stated. [b] α-Chymotrypsin, 25°, μ = 1.0. [c] δ-Chymotrypsin, 25°, μ = 0.95, 5% Me_2SO. [d] Fastrez and Fersht (1973b). [e] pK_a estimated to be ~7.5.



RCO_2H + chymotrypsin

k_{-2} and k_2/K_S may be calculated from the overall equilibrium constant for the reaction. For example, if

$$K = \frac{[RCX]}{[RCO_2H][XH]}$$

and subscripts "0" and "1" denote the reference and second compounds respectively, then

$$\frac{K_0}{K_1} = \frac{(k_2/K_S)_1\,(k_{-2})_0}{(k_2/K_S)_0\,(k_{-2})_1} \qquad (1)$$

If K_0, K_1, $(k_2/K_S)_0$, and $(k_{-2})_0$ are known then $(k_{-2})_1$ may be calculated from $(k_2/K_S)_1$ or *vice versa*.

Values of K may be taken from the general data and equations of Fersht and Requena (1971) for the hydrolysis of amides, from Jencks *et al.* (1971) for anilides, and from the data of Jencks and Gilchrist (1964) and Gerstein and Jencks (1964) for ester hydrolysis as K is somewhat insensitive to the nature of the acyl portion. For example, the equation of Fersht and Requena (1971) predicts that the K for both AcPhe-$GlyNH_2$ and AcPhe-$AlaNH_2$ should be 3×10^4 M^{-1} and values of 2×10^4 and 9×10^3 M^{-1} were found respectively (Fastrez and Fersht, 1973b). Calculations based on the general data should be correct at the very worst to within a factor of 5. Comparative data, such as the calculation of relative values of k_{-2} for anilides, should be of high accuracy.

The calculations for the attack on AcTyr-chymotrypsin are based on the value of k_{-2} for hydroxylamine. This was taken to 90 sec^{-1} based on a k_3 for AcTyr-chymotrypsin of 200 sec^{-1} and the partition experiments of Caplow and Jencks (1964) under conditions of high enzyme and low hydroxylamine concentrations.

Results

Reactivity of Acyl-Enzymes with Nucleophiles. Rate constants were measured for the attack of nucleophiles on AcPhe-chymotrypsin, furoyl-chymotrypsin, AcTrp-chymotrypsin, and furylacryloyl-chymotrypsin, and calculated for the attack on AcTyr-chymotrypsin. The latter values are approximate but are probably correct to at least within a factor of 5. The ratios of the rate constants for the attack of alaninamide, glycinamide, and hydrazine to that of 55 M water on AcPhe-chymotrypsin are 44, 11.5, and 2:1, respectively, while the corresponding ratios for AcTyr-chymotrypsin are 100, 14, and 2.2:1. The similarity between observed and calculated values for these similar acyl-enzymes is encouraging. The wide range of data for AcTyr-chymotrypsin is useful for comparative purposes.

The data are presented in Table II and the calculations for AcTyr-chymotrypsin illustrated in Figure 1.

SPECIFIC ACYL-ENZYMES AND AMINES. $AlaNH_2$, $GlyNH_2$, and hydrazine have similar pK_a values (8.2). $GlyNH_2$ is five to six times more reactive toward AcPhe-chymotrypsin and AcTyr-chymotrypsin than is the normally highly reactive hydrazine.

TABLE II: Rate Constants for the Attack of Amines on Acyl-Chymotrypsins (Acyl-CT).

Nucleophile	k_{-2} (sec^{-1} M^{-1})				
	AcPhe-CT[a]	AcTyr-CT[b]	AcTrp-CT[c]	Furoyl-CT[c]	Furylacryloyl-CT[c]
Water[d]	2.62	3.64	1.4	3.36×10^{-5}[e]	5.42×10^{-5}
Hydrazine	2.94×10^{2}	450	40	6.3×10^{-1}[e]	1.28×10^{-2}
Hydroxylamine		90			
Ammonia	(8)[f]	24	(9)[f]	1.4×10^{-2}[e]	Low
Ethylamine				3.6×10^{-2}[e]	1.8×10^{-2}
Thiosemicarbazide		6.2			
Formylhydrazide	1.23×10^{2}	470			
Semicarbazide	1.0×10^{2}	120			
p-Nitroaniline		0.3			
p-Acetylaniline		4.2			
p-Trimethylammoniumaniline		2.7			
m-Chloroaniline		1.1			
p-Chloroaniline		5			
m-Methoxyaniline		2.7			
p-Methylaniline		7.8			
p-Methoxyaniline		36			
p-Dimethylaminoaniline		170			
Glycinamide	1.66×10^{3}	2.8×10^{3}		6.94×10^{-2}	5.3×10^{-3}
Glycylglycine	6.78×10^{2}				
Alaninamide	6.34×10^{3}	2.0×10^{4}	716	1.5×10^{-1}	2.8×10^{-2}
Tyrosinamide	1.7×10^{4}				
Tryptophanamide	6.63×10^{3}				
Lysine methyl ester	1.85×10^{3}				
Alanine	<70				

[a] From product ratios with δ-chymotrypsin at pH 9.30, 25°, ionic strength 0.95, 5% Me_2SO. [b] Calculated from the data summarized in Fastrez and Fersht (1973a) pH 7.8–8, 25°, for α-chymotrypsin. [c] α-Chymotrypsin at 25°, pH 9.05, $\mu = 1.0$. [d] Observed deacylation rate constant divided by 55 M. [e] Inward and Jencks (1965). [f] Estimated from free energies of hydrolysis and k_2/K_S. These values are approximate only.

$AlaNH_2$ and the L-amino acid amides are more reactive still. L-Lysine methyl ester is of similar reactivity to $GlyNH_2$ while glycylglycine is slightly lower. Unsubstituted L-Ala is of low reactivity. Formylhydrazine is relatively reactive. Hydrazine is somewhat more reactive than the other nonspecific amines. $AlaNH_2$ is 800 times more reactive than ammonia.

NONSPECIFIC ACYL-ENZYMES AND AMINES. Hydrazine is four times more reactive than $AlaNH_2$ toward furoyl-chymotrypsin while the relative reactivities are reversed toward furylacryloyl-chymotrypsin. $AlaNH_2$ is only five times more reactive than ammonia with furoyl-chymotrypsin. In the case of the specific acyl-enzymes $AlaNH_2$ is *800* times more reactive than ammonia. There is a specificity for $AlaNH_2$ (and the other L-amino acid amides) relative to simple amines such as ammonia and hydrazine for the reaction with the specific acyl portions.

In none of the cases was there any evidence for the saturation of a nucleophile binding site.

Water is relatively unreactive compared with amines toward furoyl-chymotrypsin (Caplow and Jencks, 1964; O'Leary and Kluetz, 1971). This does not seem a characteristic of nonspecific substrates as furylacryloyl-chymotrypsin is similar to AcPhe-chymotrypsin and AcTyr-chymotrypsin in its relative reactivity toward amines and water.

k_2/K_S FOR THE HYDROLYSIS OF SUBSTRATES BY CHYMOTRYSPIN. In Table III the values for AcPhe derivatives are presented. The value of k_2/K_S (k_{cat}/K_M), k_{-2}, and K were directly measured for $AcPhe\text{-}AlaNH_2$ (Fastrez and Fersht, 1973b). k_{-2} was measured for the other nucleophiles. Values of K were extrapolated from that for $AcPhe\text{-}AlaNH_2$ using the finding of Fersht and Requena (1971) that log K varies as $0.51pK_a$ (amine). The calculated values of k_2/K_S parallel the measured activity of α-chymotrypsin toward analogous Cbz-Tyr derivatives (Morihara *et al.*, 1969) apart from the Gly-Gly com-

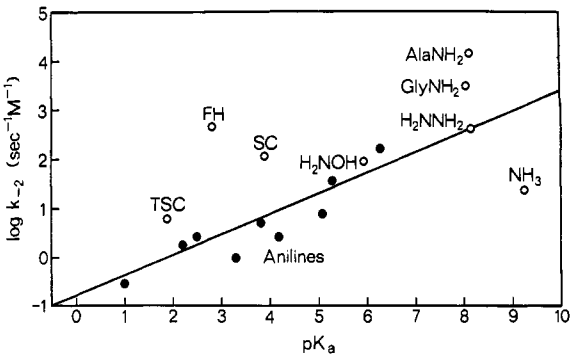


FIGURE 1: Brønsted plot of log k_{-2} for the attack of nucleophiles on AcTyr-cymotrypsin against the pK_a of the nucleophile. The filled circles are for the anilines, FH = formylhydrazine, SC = semicarbazide, TSC = thiosemicarbazide. The solid line through the points for the anilines is of slope 0.4. The data are approximate only.

TABLE III: k_{cat}/K_M for the Hydrolysis of AcPhe Derivatives by δ-Chymotrypsin.[a]

Substrate	k_{cat}/K_M (sec^{-1} M^{-1})	k_{cat}/K_M Rel to AcPhe-$GlyNH_2$	Rel Reactivities of Cbz-Tyr Derivatives[b]
AcPhe-$GlyNH_2$	15[c]	1	1
AcPhe-$AlaNH_2$	126[d]	8.4	7
AcPhe-$TrpNH_2$	270[c]	18	
AcPhe-$TyrNH_2$	680[c]	45	21
AcPhe-GlyGly	25[c]	1.7	0.1
AcPhe-$NHNH_2$	1.3[c]	0.1	

[a] 25°, pH 9.30, 5% Me_2SO, μ = 0.95. [b] 30°, pH 8.8, α-chymotrypsin (Morihara *et al.*, 1969). [c] Calculated. [d] Measured.

pound. Under their conditions the activity should be a measure of k_2/K_S.

Values of k_2/K_S for furoylamides were calculated from the data of Inward and Jencks (1965) based on the values of k_2/K_S and k_{-2} for trifluoroethyl furoate and the values of K from the general measurements of Fersht and Requena (1971). These must be among the slowest enzymatic rate constants ever determined!

The relative values of k_2/K_S for AcTyr and furoyl derivatives are also given. For the nonspecific amino groups the AcTyr derivatives hydrolyze about 1–2 $\times$ 10^6 times faster. For the specific residue $AlaNH_2$ the increase is 2.4 $\times$ 10^8

TABLE IV: k_{cat}/K_M for the Hydrolysis of Furoyl Derivatives.[a]

Substrate	k_{cat}/K_M (sec^{-1} M^{-1})	$\frac{k_{cat}/K_M[AcTyr]}{k_{cat}/K_M[Furoyl]}$[b]
Furoylmethoxyamide	5.5×10^{-5}	
Furoyltrifluoromethylamide	5.9×10^{-7}	
Furoylhydroxamate[c]	5×10^{-5}	8.5×10^5
Furoylhydrazide	2.8×10^{-6}	7.8×10^5
Furoylglycinamide	3.7×10^{-7}	7.5×10^7
Furoylalaninamide	1.8×10^{-6}	2.4×10^8
Furoylmorpholide	2.6×10^{-9}	
Furoylamide	1.5×10^{-6}	3.5×10^6
Furoylethylamide	5.8×10^{-9}	

[a] Calculated for 25°, μ = 1.0. [b] Ratio of k_{cat}/K_M of AcTyr derivative to furoyl. [c] Assuming 50% OH attack on furoyl-chymotrypsin (Caplow and Jencks, 1964).

Discussion

Hydrazine is a very reactive nucleophile in nonenzymatic reactions. For example, it reacts 300 times faster than glycinamide or glycylglycine with methyl formate despite the three nucleophiles having similar pK_a values (Blackburn and Jencks, 1968). Hydrazine maintains this high reactivity toward a nonspecific acyl-enzyme, furoyl-chymotrypsin (Inward and Jencks, 1965). Yet, toward a specific acyl-enzyme, AcPhe-chymotrypsin, hydrazine is 20 times less reactive than alaninamide and 100 times less than tyrosinamide. There is a specificity toward carboxyl-blocked L-amino acids in the reaction of specific acyl-enzymes. It is inferred from the principle of microscopic reversibility that chymotrypsin has a specificity toward derivatives of acyltryosines, tryptophans, and phenylalanines which have a carboxyl-blocked L-amino acid for the leaving group. It is seen in Table III that the values of k_{cat}/K_M for the hydrolysis of AcPhe-$AlaNH_2$ and AcPhe-$TyrNH_2$ are 100 and 500 times greater, respectively, than that for AcPhe-$NHNH_2$. This is not due to inherent chemical differences in reactivity since in nonenzymatic reactions hydrazides may be several hundred times more reactive than amides formed from amines of similar pK_a (Fersht, 1971).

The specificity for the leaving group of substrates of specific acyl portions is seen clearly on examination of the ratios of k_{cat}/K_M for AcTyr and furoyl derivatives (Table IV). Whereas AcTyr-$NHNH_2$, etc., are hydrolyzed about 2 $\times$ 10^6 times faster than the analogous furoyl derivatives, AcTyr-$AlaNH_2$ is hydrolyzed 2 $\times$ 10^8 times faster.

These observations suggest that for efficient catalysis the substrate has to be firmly anchored at both ends to the enzyme.

Leaving Group Specificity and the Model of Blow et al. (1972). The specificity of chymotrypsin is nicely accounted for by the model for the association of bovine pancreatic trypsin inhibitor with chymotrypsin and trypsin proposed by Blow *et al.* (1972). Under certain conditions (Wilson and Laskowski, 1971; Rigbi, 1971) the peptide bond Lys-15-Ala-16 is cleaved by trypsin. It is strongly suggested that this bond is situated at the active site of the enzyme in a reactive conformation. The Ala-16 leaving group of the inhibitor makes at least fifteen contacts with the chymotrypsin (or trypsin). These are listed in Table V and illustrated in Figure 2. The contacts are mainly with the residues including and surrounding the catalytically important Ser-195 and His-57.

Some important contacts and consequences are as follows. (1) The carbonyl oxygen of Ala-16 makes hydrophobic contacts with the α, β, and γ carbons of Met-192. In a dipeptide such as AcPhe-Ala the negatively charged carboxylate oxygens will be repelled by the hydrophobic region. This can explain why chymotrypsin is not an exo(carboxy)peptidase and why Ala is unreactive toward AcPhe-CT. (2) The methyl (β carbon) makes hydrophobic contacts with the γ sulfur of Cys-42 and the imidazole ring of His-57. The Ala-16 methyl group points away from the mass of the enzyme and so larger hydrophobic residues may be accommodated and the β carbon still make the required hydrophobic bonds. Further contacts may be made by the side chain; AcPhe-$TyrNH_2$ is five times more reactive than AcPhe-$AlaNH_2$. Gly derivatives lacking the β carbon are of lower reactivity. D-Amino acids cannot be accommodated (Kozlov *et al.*, 1972); the side chain would have to point into the enzyme.

Strain Energy in Michaelis Complex Causes Specificity. There is no net binding energy from the hydrophobic contacts. AcPhe-$GlyNH_2$, AcPhe-$AlaNH_2$, and AcPhe-NH_2 have similar K_M values (Baumann *et al.*, 1970). Similarly, there is no evidence for the saturation of a nucleophile binding site for the attack of nucleophiles on the nonspecific furoyl-chymotrypsin (Inward and Jencks, 1965) or on AcPhe-chymotrypsin or AcTrp-chymotrypsin. The hydrophobic binding energy is used to lower the activation energy of the chemical steps. Specificity is expressed in k_{cat} and not K_M.

Blow *et al.* (1972) mentioned that the model for the association of the inhibitor and the enzyme leaves a few contacts

TABLE V: Contacts Made by the Reactive Peptide Bond (Lys-15–Ala-16) and by Ala-16 of Pancreatic Trypsin Inhibitor, with Chymotrypsin, According to the Model of Blow *et al.* (1972).[a]

	Lys-15			Ala-16				
Inhibitor Atom	C^{α}	C	O	N	C^{β}	C^{α}	C	O
Chymotrypsin residue								
Cys-42					S^{γ}4.0C			
His-57				$N^{\epsilon 2}$3.9C	$C^{\delta 2}$4.0C $N^{\epsilon 2}$3.5C	$N^{\epsilon 2}$4.1C		
Met-192			C^{α}3.7C					C^{γ}3.7C C^{β}3.4C C^{α}3.6C
Gly-193			N3.4CH[b]				N3.7C	N3.4CH[b]
Ser-195	O^{γ}4.0C C^{β}3.7S	O^{γ}2.9C C^{β}2.9*	N2.8CH O^{γ}2.9C C^{β}2.6*	O^{γ}2.9CH C^{β}3.4C	O^{γ}3.2C	O^{γ}2.7* C^{β}3.7C		

[a] Each entry in the table names an atom belonging to the residue named on the left, which is in contact with an inhibitor atom. It gives the distance in ångströms between the two atoms, according to the model of Blow *et al.* (1972), followed by code symbols as follows: C, the indicated distance may be within ±0.2 Å of a good contact distance; S, the indicated distance is 0.2–0.5 Å too short for a favorable contact; *, the indicated distance is too short for a favorable contact, by more than 0.5 Å; H, the indicated atoms may be chemically able to form a hydrogen bond. (This does not necessarily mean that such a bond is stereochemically feasible.) [b] Henderson (1970) proposed that hydrogen bonds from NH-193 and NH-195 will stabilize the carbonyl oxygen position for an acyl-enzyme (in this case O-Lys-15). In the model here, which represents a Michaelis complex, the NH-193···O-15 distance makes a long hydrogen bond. Blow *et al.* assumed the NH-193···O-15 contact to be a hydrogen bond; and not the NH-193···O-16 contact. In a tetrahedral intermediate the NH-193···O-15 contact would shorten slightly.

which are 0.5 Å shorter than accepted van der Waals contact distances. Ignoring contacts involving the flexible parts of side chains of Lys-15, Arg-17, and Arg-39 of the inhibitor which can easily be relieved, six such contacts remain. Some of these bad contacts are likely to be due to the inherent errors of the coordinates and of the assumption that both the inhibitor and the enzyme are rigid bodies. However, the three shortest contacts, all involving Ser-195 of the enzyme, seem significant. These three contacts are indicated diagrammatically in Figure 3.

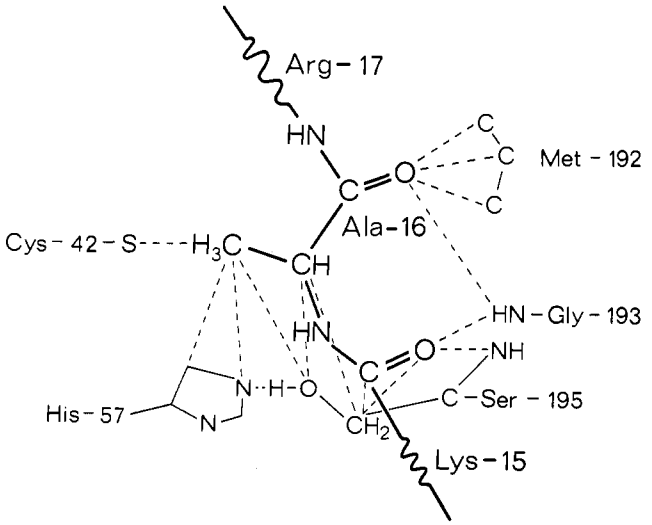


FIGURE 2: Interactions of the reactive peptide bond (Lys-15–Ala-16) of the pancreatic trypsin inhibitor and chymotrypsin. The inhibitor residues are in heavier type.

One of these contacts, that involving the nucleophilic γ oxygen of Ser-195, can be relieved by rotation about a side-chain bond. This is precisely the rotation believed to occur in going to the acyl-enzyme (Henderson, 1970). The other two short contacts in the model bring the carbonyl group of Lys-15 into about the right position for formation of a tetrahedral intermediate, and a satisfactory model for the acyl-enzyme complex with the inhibitor can be built once this rotation is allowed to occur (Blow *et al.*, 1972). The rotation of serine oxygen would lengthen and weaken the hydrogen bond with the imidazole of His-57.

Acylation of Chymotrypsin by a Peptide Substrate. Some of these structural features can be used to interpret the acylation of chymotrypsin by a peptide substrate. The rate constant for this reaction depends on the energy difference between the Michaelis complex and the transition state.

If the alanine moiety of AcPhe-AlaNH_2 was oriented ex-

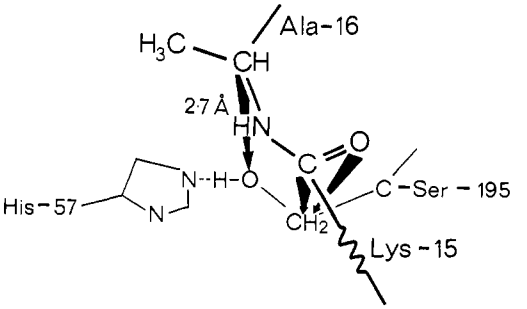


FIGURE 3: Schematic illustration showing the three contacts of less than 2.9 Å introduced in the model for the interaction of chymotrypsin with the pancreatic trypsin inhibitor.

actly as described for the inhibitor complex, between Cys-42 and Met-192, this could only be achieved by making unfavorable short contacts with Ser-195. This strain can be relieved by driving the serine oxygen toward its position in the transition state for the formation of an acyl-enzyme.

An alternative model is that the favorable contacts for the alanine moiety cannot be realized in the Michaelis complex due to the interference of Ser-195. Only when the serine oxygen is driven toward its position in the transition state can the alanine settle comfortably into its hydrophobic environment.

In both of these cases the energy difference between the transition state and the Michaelis complex is lowered as the alanine moiety cannot make the favorable contacts with Met-192 and Cys-42 until the serine oxygen is rotated toward its position in the transition state. The first model involves a strained Michaelis complex, the strain being relieved in the transition state. The second model involves a Michaelis complex which is not strained but is not utilizing the binding potential of the substrate alanine moiety. The actual case probably lies between these two extremes, there being some distortion of the geometry around Ser-195 in the Michaelis complex and better alanine binding in the transition state.

The requirement for the presence of a specific acyl portion in order for there to be specificity in the leaving group is also consistent with the strain hypothesis. If the acyl end of the substrate is floppy or held in the wrong orientation strain cannot be fully realized. The acyl portion is, of course, the major specificity site.

The first model for the acylation of chymotrypsin by a (peptide) substrate based on the known structure of the enzyme was proposed by Steitz *et al.* (1969) and Henderson (1970). This accounted for the specificity on the acyl portion and described the events necessary for the formation of the covalent acyl-enzyme. We may now expand this model to describe the events on the leaving group side and the energetic changes that increase the rate constant for the reaction. We propose that the acylation of chymotrypsin by a peptide proceeds as follows. (1) The acyl side chain of the substrate binds in the hydrophobic binding pocket of the enzyme as described by Steitz *et al.* (1969) for *N*-formyl-L-tryptophan. There is a long hydrogen bond between the (acyl) carbonyl oxygen of the substrate and the backbone NH of Gly-193 and a better hydrogen bond between the oxygen and the NH of Ser-195. There is a long hydrogen bond between the hydrogen of the *N*-acylamino portion of the substrate and the carbonyl of Ser-214 (Steitz *et al.*, 1969). There is strain in the enzyme–substrate complex due to the leaving group having to be accommodated between Met-192 and Ser-195. The nucleophilic γ oxygen of Ser-195 begins to rotate toward the substrate (acyl) carbonyl carbon; this oxygen has to rotate 120° to form the acyl-enzyme (Henderson, 1970). (2) A tetrahedral intermediate is formed by the γ oxygen of 195 rotating sufficiently to form the covalent bond with the (acyl) carbonyl of the substrate. The proton simultaneously moves towards the imidazole of His-57. The carbonyl oxygen bond length increases, strengthening the hydrogen bonds with the NH groups. The *N*-acylamino hydrogen bond with Ser-214 possibly shortens (Robertus *et al.*, 1972). The strain between the α carbon of the substrate amino portion and the γ oxygen of Ser-195 is relieved in the process, and a hydrogen bond might be formed between the NH of Gly-193 and the CO of the peptide beyond the one being cleaved (Table V). (3) The tetrahedral adduct of Ser-195 and the substrate decomposes to give the acyl-enzyme by transfer of the proton from His-57 imidazolium group to the nitrogen of the substrate. (4) There is no (observable) binding of the leaving group to the enzyme.

Deacylation of Acyl-Chymotrypsins. The above description accounts for the lowering of the activation energy of the acylation reaction, starting from the Michaelis complex. Our observations show that in the reverse reaction alaninamide promotes rapid deacylation, but does not bind significantly to the acyl-enzyme to form an acyl–product complex.

In an acyl–product complex, the distance between the nitrogen of the amino leaving group and the carbonyl carbon of the acyl group has to be at least 2.8 Å, compared to a covalent bond distance of 1.3 Å, before the peptide bond is broken. Part of this will be taken up by a slight movement ($\sim$0.5 Å) of the carbonyl carbon as it passes through a tetrahedral conformation to a trigonal planar conformation in the acyl-enzyme; but most of it must be absorbed by the leaving group, which is not covalently anchored to the enzyme. This seems likely to displace the whole alanine moiety from its binding site. Such a displacement would mean that the binding energy of alaninamide to the acyl-enzyme is low, the binding energy being fully realized only when the transition state is approached. As in the case of the acylation step, the result is to reduce the activation energy of the reaction.

Electronic Effects. The attack of amines on furoyl-chymotrypsin is insensitive to their pK_a values, *i.e.*, the β is low (Inward and Jencks, 1965). There are insufficient data for the corresponding reactions with AcTyr-chymotrypsin except for the anilines. The attack of anilines on AcTyr-chymotrypsin involves a β value of 0.4 (see Figure 1). This is close to that expected for rate determining attack (Fersht, 1971). The acylation of chymotrypsin by AcTyr-anilides probably involves rate determining breakdown of a tetrahedral intermediate.

The use of *perturbations* from linear free-energy relationships has been suggested as a criterion for the attribution of rate-determining step in amide solvolysis (Fersht, 1971). The acylation of chymotrypsin by furoylamides (Table IV) is characteristic of that for the breakdown of a tetrahedral intermediate. The furoyl hydroxamate and methoxyamide are characteristically highly reactive, as are to a lesser extent the hydrazide and furoylamide (Fersht, 1971). It should be noted that in this paper the values for the attack of phenol and *p*-nitrophenol on acetylmethoxyamide are too large by a factor of 55 and the reactivity of acetohydroxamic acid is also incorrect. The specific effect of the leaving group in AcTyr derivatives obscures analysis, so that one cannot extrapolate from the furoyl case. For AcTyr derivatives the transition state for the acylation–deacylation reaction is strongly affected by the stereochemical interactions. Similar stereochemical effects could easily influence the rate constants for the breakdown of the tetrahedral intermediate.

References

Baumann, W. K., Bizzozero, S. A., and Dutler, H. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8*, 257.

Bernhard, S. A., Lau, S. J., and Noller, H. (1965), *Biochemistry 4*, 1108.

Bernhard, S. A., Lee, B. F., and Tashjian, Z. H. (1966), *J. Mol. Biol. 18*, 405.

Blackburn, G. M., and Jencks, W. P. (1968), *J. Amer. Chem. Soc. 90*, 2638.

Blow, D. M., Wright, C. S., Kukla, D., Rühlmann, A., Steigemann, W., and Huber, R. (1972), *J. Mol. Biol. 69*, 137.

Caplow, M., and Jencks, W. P. (1964), *J. Biol. Chem. 239*, 1640.

Fastrez, J., and Fersht, A. R. (1973a), *Biochemistry 12*, 1067.
Fastrez, J., and Fersht, A. R. (1973b), *Biochemistry 12*, 2025.
Fersht, A. R. (1971), *J. Amer. Chem. Soc. 93*, 3504.
Fersht, A. R., and Requena, Y. (1971), *J. Amer. Chem. Soc. 93*, 3499.
Gerstein, J., and Jencks, W. P. (1964), *J. Amer. Chem. Soc. 86*, 4655.
Hein, G., and Niemann, C. (1961), *Proc. Nat. Acad. Sci. U. S. 47*, 1341.
Henderson, R. (1970), *J. Mol. Biol. 54*, 341.
Inward, P. W., and Jencks, W. P. (1965), *J. Biol. Chem. 240*, 1986.
Jencks, W. P., and Gilchrist, M. (1964), *J. Amer. Chem. Soc. 86*, 4561.
Jencks, W. P., Schaffhausen, B., Tornheim, K., and White, H. (1971), *J. Amer. Chem. Soc. 93*, 3917.
Kozlov, L. V., Djachenko, E. D., Volkova, L. I., and Antonov, V. K. (1972), *Fed. Eur. Biochem. Soc. 18*, 231.
Miller, C. G., and Bender, M. L. (1968), *J. Amer. Chem. Soc. 90*, 6850.
Morihara, K., Oka, T., and Tsuzuki, H. (1969), *Biochem. Biophys. Res. Commun. 35*, 210.
O'Leary, M., and Kluetz, M. D. (1971), *J. Amer. Chem. Soc. 93*, 7431.
Rigbi, M. (1971), *in* Proceedings of the International Conference on Proteinase Inhibitors, Fritz, H., and Tschesche, H., Ed., Berlin, Walter de Gruyter, p 117.
Robertus, J. D., Kraut, J., Alden, R. A., and Birktoft, J. J. (1972), *Biochemistry 11*, 4293.
Steitz, T. A., Henderson, R., and Blow, D. M. (1969), *J. Mol. Biol. 46*, 337.
Wilson, K. A., and Laskowski, M., Sr. (1971), *J. Biol. Chem. 246*, 3555.

4′-Thioadenosine 3′,5′-Cyclic Phosphate and Derivatives. Chemical Synthesis and Hydrolysis by Phosphodiesterase†

A. K. M. Anisuzzaman, William C. Lake, and Roy L. Whistler*

ABSTRACT: 9-(2,3-*O*-Isopropylidene-4-thio-β-D-ribofuranosyl)-adenine is phosphorylated with cyanoethyl phosphate and the isopropylidene group removed to yield 9-(4-thio-β-D-ribofuranosyl)adenine 5′-phosphate (**1**), which is cyclized to the desired 4′-thioadenosine 3′,5′-cyclic phosphate (**2**). Reaction of **2** with butyric anhydride gives the N^6,2′-*O*-dibutyryl derivative. 6-Chloro-9-(2,3,5-tri-*O*-acetyl-4-thio-β-D-ribofuranosyl)-purine is allowed to react with *n*-butylamine to produce N^6-(*n*-butyl)-9-(4-thio-β-D-ribofuranosyl)adenine (**4**). The 2′,3′-*O*-isopropylidene derivatives of **4** are phosphorylated and hydrolyzed to the N^6-(*n*-butyl) derivative, which is cyclized to give N^6-(*n*-butyl)-s$^{4'}$cAMP. s$^{4'}$cAMP and its derivatives are examined as alternate substrates for cAMP phosphodiesterase from bovine heart muscle. s$^{4'}$cAMP is hydrolyzed at 95% of the rate observed for cAMP with K_m = 56.8 μM and V_m = 2.08 μmol of P_i per mg of protein per minute. Thus, substitution of sulfur as the heteroatom in the D-ribose ring does not affect the binding or hydrolysis of cAMP by phosphodiesterase. The N^6-(*n*-butyl) and N^6,2′-*O*-dibutyryl derivatives are hydrolyzed at a rate of 12 and 0%, respectively, relative to cAMP. All three of the s$^{4'}$cAMP phosphate analogs are effective inhibitors of the action of phosphodiesterase on cAMP.

The plethora of interest in adenosine 3′,5′-cyclic phosphate has induced us to prepare and examine the analog with sulfur replacing the oxygen atom in the D-ribofuranosyl ring. In addition, we have prepared certain derivatives of this sulfur analog which are analogous to other derivatives showing biochemically interesting properties. We have previously shown that sugar analogs (Shankland *et al.*, 1968; Hoffman and Whistler, 1968), nucleoside analogs (Bobek *et al.*, 1970), and certain nucleotide analogs (Hoffman and Whistler, 1970) having sulfur replacing the ring oxygen atom of the sugar have especially interesting and intriguing biological properties among which is low toxicity (Hoffman and Whistler, 1968). These analogs are proving useful tools in elucidating the biochemistry of the naturally occurring compounds (Hellman *et al.*, 1973; Whistler and Lake, 1972; Barnett *et al.*, 1970).

In the present work 9-(2,3-*O*-isopropylidene-4-thio-β-D-ribofuranosyl)adenine (Hoffman and Whistler, 1970) is phosphorylated and the isopropylidene blocking group removed to give 9-(4-thio-β-D-ribofuranosyl)adenine 5′-phosphate (**1**) in 46% yield. Cyclization of **1** to 9-(4-thio-β-D-ribofuranosyl)-adenine 3′,5′-cyclic phosphate (**2**) (s$^{4'}$cAMP) in 63% yield is accomplished by modification of the method of Smith and coworkers (1961). Reaction of s$^{4'}$cAMP with butyric anhydride produces N^6,2′-*O*-dibutyryl-9-(4-thio-β-D-ribofuranosyl)adenine 3′,5′-cyclic phosphate in 50% yield.

6-Chloro-9-(2,3,5-tri-*O*-acetyl-4-thio-β-D-ribofuranosyl)-purine (Bobek *et al.*, 1970) reacts with *n*-butylamine to give N^6-(*n*-butyl)-9-(4-thio-β-D-ribofuranosyl)adenine (**4**) in 63% yield. Compound **4** exhibits a negative Cotton effect consistent with the β-D configuration (Emerson *et al.*, 1966; Nishimura *et al.*, 1968). The 2′,3′-hydroxyl groups of **4** are protected by isopropylidene blocking to give N^6-(*n*-butyl)-9-(2,3-*O*-isopropylidene-4-thio-β-D-ribofuranosyl)adenine (**5**) in 79%

† From the Department of Biochemistry, Purdue University, Lafayette Indiana 47907. *Received January 2, 1973.* Journal Paper No. 5004 of the Purdue Agricultural Experiment Station, Lafayette, Ind. 47907. This investigation was supported by Grant No. CA12422 from the National Institutes of Health.