Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, N.Y., Wiley, p 493.
Goldbarg, J. A., and Rutenburg, A. M. (1958), *J. Biol. Chem. 221,* 491.
Hunter, A., and Downs, C. E. (1945), *J. Biol. Chem. 157,* 427.
Lee, H. J., LaRue, J. N., and Wilson, I. B. (1971), *Anal. Biochem. 41,* 397.
Little, G. H. (1970), Ph.D. Thesis, The Medical College of Georgia.
Little, G. H., and Behal, F. J., (1971), *Biochim. Biophys. Acta 243,* 312.
Makinen, K. K., and Hopsu-Havu, V. K. (1967a), *Enzymology 32,* 333.
Makinen, K. K., and Hopsu-Havu, V. K. (1967b), *Enzymology 32,* 347.
Pfleiderer, G. (1970), *Methods Enzymol. 19,* 514.
Pfleiderer, G., and Femfert, U. (1969), *FEBS Lett. 4,* 265.
Starnes, W. L., and Behal, F. J. (1974), *Biochemistry 13,* 3221.
Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. 1. New York, N.Y., Academic Press, p 667.

Catalysis by Serine Proteases and Their Zymogens. A Study of Acyl Intermediates by Circular Dichroism†

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ABSTRACT: *p*-Nitrophenyl *p'*-guanidinobenzoate and methylumbelliferyl *p'*-guanidinobenzoate, which are active site titrants for trypsin, and *p*-nitrophenyl *p'*-dimethylsulfonioacetamidobenzoate and methylumbelliferyl *p'*-trimethylammoniocinnamate, which are active site titrants for chymotrypsin, are also hydrolyzed by the respective zymogens. Hydrolysis in each case proceeds via the formation of acyl-zymogens. The acylation rates for the zymogens are 10^3–10^7 times slower than for the enzymes whereas the deacylation rates of acyl-enzymes and acyl-zymogens are comparable. These findings are consistent with the idea that the diminished catalytic activity of these zymogens is due primarily to their distorted substrate binding sites. The circular dichroic spectra of the acyl-enzymes show induced negative ellipticities in the region of absorption of the acyl group, due to binding of the group in an asymmetric environment. The circular dichroic spectra of the acyl-zymogens do not, but conversion of the acyl-zymogens to acyl-enzymes changes the circular dichroic spectra to those characteristic of the acyl-enzymes. α-Carbamyl-ε-guanidinated trypsin is a derivative which resembles trypsinogen in lacking activity toward specific ester substrates but possessing low activity toward *p*-nitrophenyl *p'*-guanidinobenzoate. The circular dichroic spectrum of the acyl-enzyme formed during hydrolysis of *p*-nitrophenyl *p'*-guanidinobenzoate by this derivative resembles that of guanidinobenzoyltrypsinogen, and not that of guanidinobenzoyltrypsin. These circular dichroism studies confirm that the same serine residue is involved in catalysis by both enzymes and zymogens. They demonstrate directly that the acylating group is in a different environment in each and indicate that this specific environment is a determinant in the catalytic activity of each. Thus the circular dichroic spectra of these acyl intermediates provide a sensitive probe of the subtle conformational changes which occur on zymogen activation. The results support the previous conclusion that the major feature of the activation of trypsinogen and chymotrypsinogen is the rearrangement of the substrate binding site and that the appearance of a new amino terminus causes this rearrangement.

Several zymogens, including trypsinogen and chymotrypsinogen, display toward certain substrates or pseudosubstrates a weak intrinsic activity prior to activation (Neurath et al., 1973). An extensive study of the hydrolysis of the ester *p*-nitrophenyl *p'*-guanidinobenzoate by chymotrypsinogen and trypsinogen demonstrated that the same functional groups are involved in the catalysis by the enzymes and their parent zymogens, and that the inferior activities of the zymogens are due primarily to an underdeveloped binding site and only secondarily to a less efficient catalytic apparatus (Gertler, 1973; Gertler et al., 1974a,b). This conclusion was supported by the observation that methanesulfonyl fluoride, an active site titrant which lacks the potential for interacting with the binding pocket of the enzyme, reacted with trypsin and trypsinogen at comparable rates (Morgan et al., 1974). Since hydrolysis of NPGB[1] by chymotrypsin and chymotrypsinogen proceeds via the formation of a *p*-guanidinobenzoyl intermediate which decomposes only slowly at acid pH, this acyl intermediate could be isolated and studied. The circular dichroic (CD) spectrum of *p*-guanidinobenzoylchymotrypsinogen differed significantly from that of *p*-guanidinobenzoylchymotrypsin

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[1] Abbreviations used are: NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; pGB, *p*-guanidinobenzoyl; Dip, diisopropylphosphoryl; NPSA, *p*-nitrophenyl *p'*-(dimethylsulfonioacetamido)benzoate; pSA, *p'*-(dimethylsulfonioacetamido)benzoyl; MUGB, methylumbelliferyl guanidinobenzoate; MUTMAC, methylumbelliferyl *p'*-trimethylammoniocinnamate; TMAC, *p'*-trimethylammoniocinnamoyl; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

indicating a perturbation of the *p*-guanidinobenzoyl (pGB) group in the acyl-zymogen as compared to the acyl-enzyme (Gertler et al., 1974a). We have extended these CD measurements to other acyl intermediates, stable at neutral pH, in order to probe more deeply the mode of binding of substrates to enzymes and zymogens and to assess the conformational changes which accompany zymogen activation. In particular we have made extensive use of fluorescent active site titrants which react not only with trypsin but also with thrombin, factor X_a, and plasmin (Jameson et al., 1973). The sensitivity of this method is some hundred times greater than even spectrophotometric methods which rely on the release of the strongly absorbing *p*-nitrophenolate ion.

Materials and Methods

Once recrystallized bovine trypsinogen, lyophilized bovine trypsin, three times recrystallized bovine chymotrypsinogen, and three times recrystallized bovine α-chymotrypsin were products of Worthington Biochemical Corp. Purified acid protease from *Aspergillus oryzae*, α-carbamyl-ϵ-guanidinated trypsin, and ϵ-guanidinated trypsinogen were prepared according to Robinson et al. (1973a). Bovine enterokinase was a gift of Dr. L. E. Anderson of this laboratory (Anderson et al., 1975).

p-Nitrophenyl *p'*-guanidinobenzoate (NPGB) was purchased from Cyclo Chemical Co. *p*-Nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide (NPSA) was a gift of Dr. E. Shaw. Methylumbelliferyl *p'*-guanidinobenzoate (MUGB) and methylumbelliferyl *p'*-trimethylammoniocinnamate (MUTMAC) were obtained from Cambrian Biochemicals. The chemical structures of these titrants are shown in Figure 1.

pGB-chymotrypsin and pGB-chymotrypsinogen were prepared as described previously (Gertler et al., 1974a). Other acyl-enzymes and acyl-zymogens were prepared in a similar manner. In all cases the degree of acylation was determined by active site titration using spectrophotometric or spectrofluorometric assays. The zymogens were first activated at acid pH by an acid protease purified from *Aspergillus oryzae*. Titrations with NPGB and NPSA were carried out as described by Chase and Shaw (1967) and Wang and Shaw (1972), and those with MUGB and MUTMAC by the method of Jameson et al. (1973). Fluorescence measurements were made using a Turner 210 spectrofluorometer, at wavelengths of 365 and 445 nm for excitation and emission, respectively.

Ultraviolet spectra were measured with a Cary 15 spectrophotometer at room temperature. Circular dichroic spectra were recorded with a Cary 60 spectropolarimeter equipped with a circular dichroism attachment using cells of 1-cm path length. Prior to spectral studies the native proteins used as standards were further purified by gel filtration on Sephadex G-25 in 1 m*M* HCl to remove any autolysis products.

All other procedures including the preparation of Dip-enzymes and Dip-zymogens, determination of acylation and deacylation rates, and determination of protein have been described previously (Gertler et al., 1974a).

Results

Reaction of Trypsin and Trypsinogen with NPGB and MUGB. Trypsin and trypsinogen react stoichiometrically with NPGB or MUGB to produce guanidinobenzoyl-enzyme or -zymogen which deacylate only slowly, even at neutral pH. The second-order rate constants for the acylation

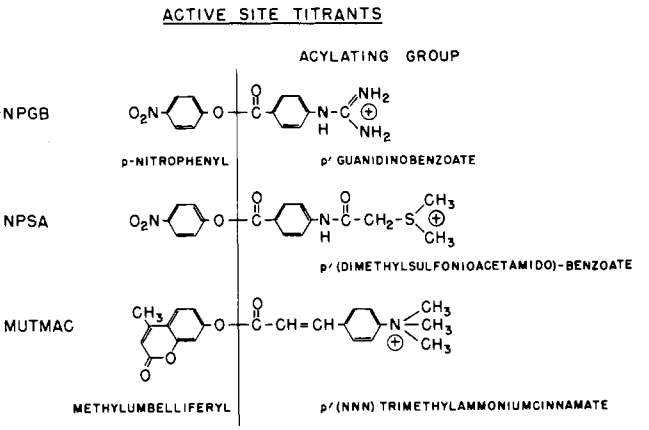

FIGURE 1: Chemical structures of the active site titrants.

of trypsin and trypsinogen by NPGB (k_{II}) have been determined previously to be 2×10^7 and 25 min^{-1} M^{-1}, respectively (Chase and Shaw, 1967; Gertler et al., 1974a). The rate constants for the acylation by MUGB are similar ($>1 \times 10^6$ and 25 min^{-1} M^{-1}, respectively). Since both enzyme and zymogen acylate rapidly and deacylate slowly, the acyl intermediates could be readily obtained by gel filtration after reaction with the appropriate acylating agent. The rate constant for the deacylation (k_3) of pGB-trypsin prepared from either reagent was found to be 0.0014 min^{-1} in agreement with the value of Wang and Shaw (1972). The rate constant for the deacylation of pGB-trypsinogen was determined to be 0.0007 min^{-1}. During the very slow deacylation reaction a small (<10%) amount of autoactivation of the zymogen was seen to occur which may result in a small overestimation in the apparent rate of deacylation.

Spectral Properties of pGB-trypsin and pGB-trypsinogen. The contributions of the pGB-chromophore to the absorption spectra of pGB-trypsin and pGB-trypsinogen were almost identical. In both cases the wavelength of maximum absorption of the native proteins was displaced from 278 to 276 nm in the acyl derivatives. The difference spectra between native and acyl-proteins showed a broad absorption band with a maximum at 270 nm. ($\Delta\epsilon_{M270}$ after correction for incomplete acylation was in each case 2×10^4.) This represents a significant red shift in the absorption maximum of the pGB group and indicates an alteration of the environment of the group upon binding to the proteins. The absorbance changes were reversed completely by deacylation.

The effect of the pGB group on the CD spectra of the acyl-enzyme and acyl-zymogen were, however, very different. Figure 2 compares the CD spectra of trypsin and trypsinogen with those of their pGB derivatives. Acylation of trypsin results in a large decrease in the ellipticity in the region 250–300 nm. No such decrease is seen upon acylation of the zymogen. The difference CD spectrum between trypsin and pGB-tryspin shows a broad bell-shaped curve with a maximum negative ellipticity at 270 nm, identical with the difference absorption spectrum. This coincidence suggests that the ellipticity changes are due largely to the chromophore itself and not to the changes in the environment of residues in the protein such as tyrosyl, tryptophanyl, or phenylalanyl groups, although environmental changes cannot be ruled out. The CD spectra of Dip-trypsin and Dip-trypsinogen are identical with those of the respective native proteins.

The characteristic negative ellipticity of pGB-trypsin dis-

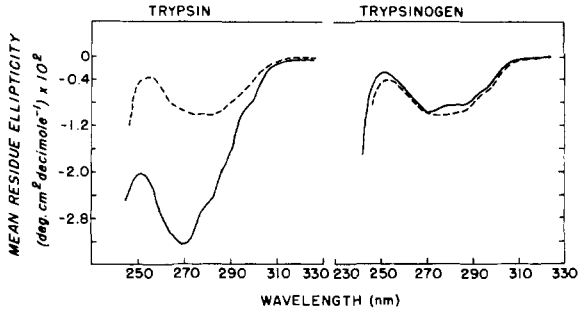

FIGURE 2: Circular dichroic spectra of native trypsin or trypsinogen (- - -) and pGB-trypsin or pGB-trypsinogen (—) in 0.1 *M* NaCl-0.02 *M* Hepes (pH 8.2). The degree of acylation of pGB-trypsin was 98% and of pGB-trypsinogen, 58%.

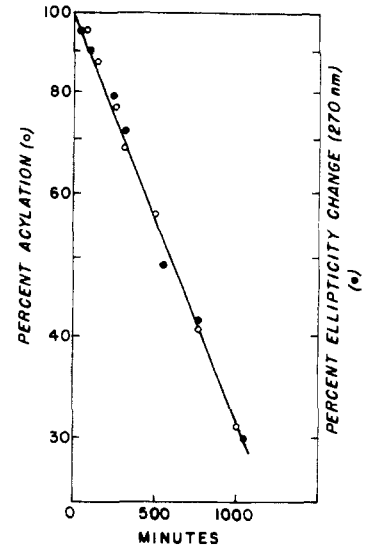

FIGURE 3: The deacylation of pGB-trypsin at pH 8.2, 25°C (●). Percent ellipticity change at 270 nm; (O) active sites assay using MUGB.

appeared on deacylation at pH 8.2 (Figure 3). There was a direct correlation between the loss of the negative ellipticity measured at 270 nm and the formation of active trypsin which could be assayed by active site titration. The rate constant for the deacylation reaction was calculated from both curves to be 0.0014 min^{-1}, the value previously reported by Chase and Shaw (1969).

Activation of a sample of pGB-trypsinogen at acid pH by the acid protease from *Aspergillus oryzae* resulted in a rapid change in the CD spectrum from that of pGB-trypsinogen to that characteristic of pGB-trypsin. Similarly activation by enterokinase at neutral pH resulted in the same change. The rate of change of the CD spectrum was directly correlated to the appearance of enzyme activity in parallel experiments employing native zymogen. Indeed, the method represents an extremely accurate way of determining enterokinase activity at neutral pH. Since the product of the reaction, pGB-trypsin, is inactive no errors due to autocatalytic activation of trypsinogen can occur.

The CD spectrum of the final product of activation, both at acid and neutral pH, was identical with that of pGB-trypsin prepared by direct acylation (after corrections were made for incomplete acylation of the zymogen). The CD spectra of the acylated proteins appear therefore to provide a direct probe of the environment of the group which acylates the serine residue of the active site.

The conversion of trypsinogen to trypsin involves the cleavage of the peptide bond between Lys_6 and Ile_7 and the appearance of a new α-amino group on Ile_7. Robinson et al. (1973b) investigated the role of this group in enzyme function by preparation of a derivative of trypsin in which all the lysyl residues had been converted to homoarginyl residues. The derivative was uniquely suited for such studies since only the α-amino group of Ile_7 but none of the ϵ-amino groups was free. Modification of the ϵ-amino groups had no apparent effect on the catalytic activity of the enzyme. However, carbamylation of the α-amino group of ϵ-guanidinated trypsin produced a derivative which was inactive toward specific ester substrates but reacted slowly with pseudosubstrates such as Dip-F and NPGM. For example, the second-order rate of acylation of this derivative by NPGB is 160 min^{-1} M^{-1} which is slightly higher than that for trypsinogen but some 10^6 times slower than that for native or ϵ-guanidinated trypsin.

The CD spectra of pGB-guanidinated trypsin and pGB-guanidinated trypsinogen were identical with those of pGB-trypsin and pGB-trypsinogen, respectively. Thus, guanidination of the lysine residues had no apparent effect on the mode of binding of the pseudosubstrate. However, the CD spectrum of pGB α-carbamyl-ϵ-guanidinated trypsin was the same as that of the acylated zymogen, not the acylated enzyme (cf. Figure 2). Blocking of the amino-terminal isoleucine by a carbamyl group or by the $Val\text{-}Asp_4\text{-}Lys$, as in trypsinogen, therefore results in similar decreases of catalytic activity and apparently a similar mode of binding of the substrate.

The CD spectra of pGB-tryspin and pGB-trypsinogen at pH 4.0 were essentially the same as those at pH 8.2. The results can therefore be contrasted to those obtained in the previous study of pGB-chymotrypsin and pGB-chymotrypsinogen at pH 4.0 (Gertler et al., 1974a). Whereas the changes in absorption spectra upon acylation of chymotrypsin and chymotrypsinogen by NPGB were similar to those seen here for trypsin and trypsinogen, the changes in CD spectra upon acylation differed considerably. pGB-chymotrypsin did not show an altered ellipticity in the region 250-300 nm which could be attributed to the pGB chromophore but pGB-chymotrypsinogen showed a marked increase in ellipticity due to this group, much greater than that seen upon acylation of trypsinogen.

Reaction of Chymotrypsin and Chymotrypsinogen with NPSA. NPSA was introduced by Wang and Shaw (1972) as an active site titrant for chymotrypsin. The ester is also hydrolyzed by chymotrypsinogen (Gertler et al., 1974b). Catalysis proceeds via an acyl intermediate and again the acylation rate of the zymogen is far slower than that of the enzyme. The second-order rate constants are 12.9×10^5 min^{-1} M^{-1} for chymotrypsin and 8.2 min^{-1} M^{-1} for chymotrypsinogen at pH 8.2. The deacylation rate constants at the same pH were determined to be 0.012 min^{-1} for the acyl-enzyme and 0.009 min^{-1} for the acyl-zymogen.

Spectral Properties of pSA-chymotrypsin and pSA-chymotrypsinogen. The contributions of the pSA chromophore to the absorption spectra of pSA-chymotrypsin and pSA-chymotrypsinogen were determined as the difference spectra between native and acyl-proteins. A broad absorption band was found in both cases with a maximum at 277 nm ($\Delta\epsilon_{M277} = 1.5 \times 10^4$). This again marks a significant red shift in the absorption maximum from that of the group in free solution (260 nm). The CD spectra of chymotrypsin, chymotrypsinogen, and their pSA derivatives are shown in Figure 4.

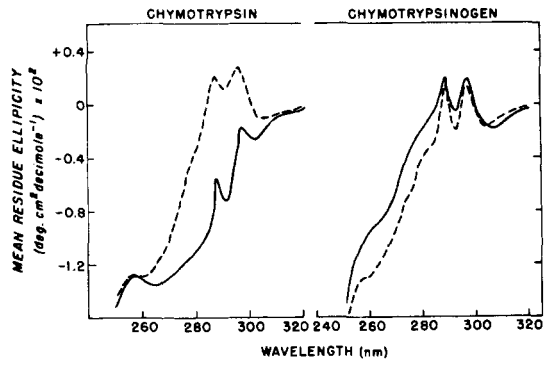

FIGURE 4: Circular dichroic spectra of native chymotrypsin or chymotrypsinogen (- -) and pSA-chymotrypsin or pSA-chymotrypsinogen (—) in 0.1 *M* NaCl–0.02 *M* Hepes (pH 8.2). The degree of acylation of pSA-chymotrypsin was 99% and of pSA chymotrypsinogen 60%.

As in the case of the reaction of NPGB with trypsin, a considerable decrease in ellipticity was induced upon acylation of the enzyme. Acylation of the zymogen caused a small increase over the same region.

Activation of pSA-chymotrypsinogen at acid pH by the protease from *Aspergillus oryzae* resulted in change of the CD spectrum to one similar to that obtained for pSA-chymotrypsin. The rate of change of the spectrum (at 280 nm) could be correlated with the appearance of enzyme activity in parallel experiments employing native zymogen (Figure 5).

The negative ellipticity due to the pSA chromophore in pSA-chymotrypsin and the positive ellipticity due to the group in the acyl-zymogens disappeared upon deacylation at pH 8.2. The CD spectra of the acyl derivatives at pH 4.0 were essentially the same as those at pH 8.2.

Reaction of Chymotrypsin and Chymotrypsinogen with MUTMAC. MUTMAC was introduced by Jameson et al. (1973) as a fluorometric active site titrant for chymotrypsin. We have used it to assay accurately as little as 0.01 nmol of the enzyme. The substrate is also hydrolyzed by chymotrypsinogen at a rate almost 100 times faster than those of the benzoic acid esters studied (k_{II} acylation = 1660 min^{-1} M^{-1}) but still considerably slower than that of the enzyme ($k_{II} > 1 \times 10^6$ min^{-1} M^{-1}). The deacylation rates of both acyl-enzyme and acyl-zymogen are comparable and slow (k_3 enzyme = 0.011 min^{-1}, k_3 zymogen = 0.0066 min^{-1}).

Spectral Studies of TMAC-chymotrypsin and TMAC-chymotrypsinogen. The CD spectra of TMAC-chymotrypsin and TMAC-chymotrypsinogen are shown in Figure 6. At pH 8.2, a large decrease in ellipticity was induced upon acylation of the enzyme, but a small increase upon acylation of the zymogen. The changes are similar to those seen for pSA derivatives (Figure 4). At pH 4.0 a large negative ellipticity is again seen in the region of absorption of the chromophore in the acyl-enzyme. However, in contrast, a large positive ellipticity is seen in that region for the acyl-zymogen. This positive ellipticity of the acyl-zymogen is dependent upon the pH of the sample, being greatest at pH 3.0 and disappearing at pH 8.5. The change is not dependent on the ionization of any single group.

Discussion

Recent work from our laboratory (Gertler et al., 1974a; Robinson et al., 1973b) has shown that chymotrypsinogen and trypsinogen are able to catalyze the hydrolysis of the ester *p*-nitrophenyl *p'*-guanidinobenzoate. The reaction proceeds via the formation of an acyl-zymogen analogous to the acyl-enzyme intermediate in the chymotrypsin-catalyzed hydrolysis of ester substrates (Bender and Killheffer, 1973), e.g.

$$CTg + NPGB \xrightarrow{k_{II}} pGB\text{-}CTg \xrightarrow{k_3} CTg + pGBOH$$

The second-order rate constant for the acylation (k_{II}) was several orders of magnitude lower for chymotrypsinogen than for chymotrypsin. The rate constants for deacylation (k_3) differed by less than two orders of magnitude and were in both cases dependent on the ionization of a single group. The present study extends these investigations to the determination of the kinetic parameters for the hydrolysis of other ester substrates by chymotrypsinogen and trypsinogen. The use of fluorescent substrates has greatly increased the sensitivity of the measurements and thus has enabled the investigations to be carried out with far less material. The results, summarized in Table I, show that for each substrate the second-order acylation rate constant is considera-

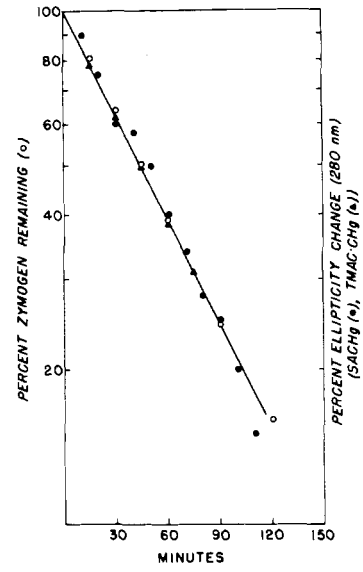

FIGURE 5: The activation of chymotrypsinogen, pSA-chymotrypsinogen, and TMAC-chymotrypsinogen by an acid protease from *Aspergillus oryzae* in 0.1 *M* NaCl–0.02 *M* sodium acetate (pH 4.0). (○) Percent zymogen remaining; (●, ▲,) percent ellipticity change.

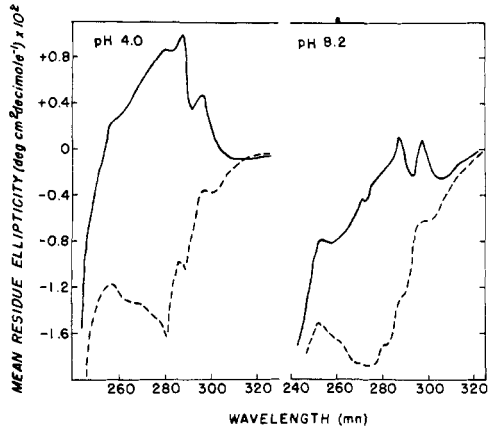

FIGURE 6: Circular dichroic spectra of TMAC-chymotrypsin (- - -) and TMAC-chymotrypsinogen (—) in 0.1 *M* NaCl–0.02 *M* sodium acetate (pH 4.0) and in 0.1 *N* NaCl–0.02 *M* Hepes (pH 8.2). The degree of acylation of TMAC-chymotrypsin was 83% and of TMAC-chymotrypsinogen, 75%.

Table I: Comparative Rates of Reaction of Enzymes and Zymogens with Various Pseudosubstrates.

Reaction		k_{II}	k_3 (min^{-1})		k_3
Substrate	Zymogen	(Enzyme/Zymogen)	Enzyme	Zymogen	(Enzyme/Zymogen)
NPGB	+ Trypsinogen	7.7×10^6	0.0014	0.0007	2.0
MUGB	+ Trypsinogen	$>1 \times 10^5$	0.0014	0.0007	2.0
NPGB[a]	+ Chymotrypsinogen	1.6×10^7	0.62	0.0089	74.0
MUGB	+ Chymotrypsinogen	$>1 \times 10^5$			
NPSA	+ Chymotrypsinogen	1.6×10^5	0.012	0.0091	1.3
MUTMAC	+ Chymotrypsinogen	$>1 \times 10^3$	0.011	0.0066	1.7
DipF[b]	+ Trypsinogen	6.5×10^4			
MSF[c]	+ Trypsinogen	49.0			

[a] Gertler et al., 1974b. [b] Morgan et al., 1972. [c] Morgan et al., 1974.

bly lower for the zymogen than for the enzyme. This rate constant is the product of terms for both acylation and binding. Since Gertler et al. (1974a) have demonstrated that the affinity of trypsinogen for the competitive inhibitor, *p*-aminobenzamidine, was three to four orders of magnitude lower than that of trypsin, it was suggested that the principal reason for the difference between the acylation rates of zymogen and enzyme is weaker binding of the substrate to the zymogen.

In contrast to the large difference in acylation rates, the deacylation rates of these acyl-enzymes and acyl-zymogens are more nearly comparable. Though the substrates chosen for this study were those which produce relatively stable acyl intermediates at neutral pH, the deacylation rates are significantly greater than can be explained by alkaline hydrolysis of the ester bond. Bender et al. (1962) have shown that the rate of alkaline hydrolysis of a denatured acylchymotrypsin was comparable with the rate of alkaline hydrolysis of the corresponding ethyl ester. The rate constant for the alkaline hydrolysis of ethyl *p'*-guanidinobenzoate at pH 8.2 is about 9×10^{-6} min^{-1} (Chase and Shaw, 1969) which is 10^{-2}–10^{-3} times slower than the deacylation rates of the *p'*-guanidinobenzoyl proteins studied here. The data therefore suggest the existence of a catalytic apparatus in the zymogen which for the pseudosubstrates used is of similar efficiency to that of the corresponding enzyme. This conclusion is in agreement with crystallographic studies which indicated that the catalytic apparatus, the "charge-relay system", was largely preexistent in the zymogen (Freer et al., 1970). Morgan et al. (1974) have, in addition, shown that methanesulfonyl fluoride, an active-site titrant which lacks the potential for interaction with the substrate binding site of the enzyme, reacts with trypsinogen at a rate only 50 times slower than with trypsin. The kinetic data therefore suggest that the major cause of the diminished catalytic activity of the zymogens toward these pseudosubstrates is defective substrate binding and that there is a lesser impairment related to distortion of the catalytic apparatus.

X ray crystallographic studies have shown that the activation of chymotrypsinogen results in a repositioning of Gly_{193}. The –NH– group of this residue has been postulated to play an important role in the function of chymotrypsin by stabilization of the tetrahedral transition states during acylation and deacylation. One would therefore expect that perturbation of this residue in chymotrypsinogen would decrease the catalytic activity. However, it is also possible that the stabilization of the tetrahedral intermediate is less effective during the deacylation of acyl-enzymes formed from pseudosubstrates because of different orientation of the carbonyl moiety (Henderson, 1970; Robillard et al., 1972). This would account for their slower rate of deacylation[2] and may explain the similarity of the deacylation rates of the corresponding enzymes and zymogens.

Gertler et al. (1974a) in the work which prompted these studies showed that the environment of the acylating group was different in pGB-chymotrypsin and in pGB-chymotrypsinogen. However, the reaction of NPGB with chymotrypsin may not be a typical model. Ethyl *p*-guanidinobenzoate is neither a substrate nor an inhibitor of chymotrypsin indicating that the pGB group binds poorly to this enzyme (Mares-Guia and Shaw, 1967). The acyl moiety does, however, fulfill the specificity requirements for trypsin. Ethyl *p*-guanidinobenzoate is an active site titrant for trypsin and phenylguanidine is a competitive inhibitor. We thought, therefore, that comparison of the circular dichroic spectra of pGB-trypsin and pGB-trypsinogen would give a better indication of the role of substrate binding in the catalysis by enzyme and by zymogen and of the structural changes involved upon zymogen activation. CD spectra indicate that the acylating group is in a different environment in pGB-trypsin than in pGB-trypsinogen. Nevertheless, activation of the pGB-trypsinogen led to the appearance of the CD spectrum characteristic of the acyl-enzyme, offering conclusive proof that the same residue (Ser_{195})[3] is involved in catalysis by both.

Interpretation of the CD spectra in terms of specific interactions between substrate and enzyme is difficult. The positively charged guanidino group fulfills the specificity requirements of trypsin and may be expected to reach deeply into the specificity pocket, forming a salt linkage with the negatively charged Asp_{189} residue[3] at the base of the pocket. This is consistent with the inhibitory properties of phenylguanidine, and the close structural similarity of the group to benzamidine, a strong competitive inhibitor of trypsin which has been shown to bind in this manner (Kreiger et al., 1974). Such a mode of binding would allow interaction of the benzoyl ring with the hydrophobic groups which line the specificity pocket. These interactions might lead to the electronic perturbations necessary to account for the observed CD spectra in GB-trypsin.

The negative ellipticity is not observed for pGB-trypsino-

[2] The deacylation rate of pGB-chymotrypsin is 10^3 times slower than that of *N*-acetyltryptophanylchymotrypsin (Zerner et al., 1964).

[3] Numbers of amino acid residues in the serine proteases refer to those in the sequence of bovine chymotrypsinogen A.

gen suggesting that this mode of binding is not possible in the zymogen. This is in agreement with kinetic data which show that the binding of benzamidine to trypsinogen is some 10^5 times weaker than to trypsin (Gertler et al., 1974a). The negative ellipticity is not observed for pGB α-carbamyl-ϵ-guanidinated trypsin. Blocking of the α-amino group of Ile_7 by the activation of peptide Val-Asp_4-Lys in trypsinogen (or by Val-Asp_4-HAr in guanidinated trypsinogen) or by a carbamyl group in the guanidinated trypsin derivative results therefore in similarly decreased reactivity toward pseudosubstrates and in a characteristic mode of binding of the acylating group.

Interpretation of the data for the acylchymotrypsins is less clear. The acylchymotrypsins which deacylate slowly show a negative ellipticity similar to that seen for pGB-trypsin which also deacylates slowly. Since the two enzyme molecules are similar in shape, especially in the region of the substrate binding site, it is tempting to propose a similar mode of substrate binding in all cases. Henderson (1970) has argued convincingly that the reason for the slow deacylation of indoleacryloylchymotrypsin was the tight binding of the molecule to the hydrophobic residues which line the pocket in a mode which did not allow easy access of the activated water molecule necessary to hydrolyze the acyl intermediate. The induced negative ellipticity of the slowly deacylating acyl-enzymes may be characteristic of this type of binding. The more rapid deacylation of pGB-chymotrypsin may be explained by a more mobile acyl group which allows the approach of the necessary water molecule.

However, although each of the chymotrypsin substrates studied contains the aromatic ring typical of good substrates, each also has a positively charged substituent on the ring. In chymotrypsin the binding pocket is hydrophobic and at its base is Ser_{189} in place of the aspartyl residue in trypsinogen. The energy necessary for burying a positive charge in the pocket may be too high to allow such a mode of binding, though model building studies suggest that it would be possible. These studies do, however, reveal difficulties in rotating large inflexible acyl groups into the hydrophobic binding pocket after attachment to Ser-195. Thus activation of acyl-zymogens probably involves either reshaping of the hydrophobic pocket around a bound acyl chromophore or surface changes at a secondary binding site.

In spite of the difficulties of interpreting the CD spectra in terms of specific interactions, it is clear that in all cases the mode of binding of substrate to the enzyme and zymogen has been different. This agrees with the crystallographic data which showed the main differences between chymotrypsin and chymotrypsinogen to be around the substrate binding site.

The study of CD spectra of acyl-enzyme/acyl-zymogen pairs clearly offers a sensitive approach to the study of conformational changes around the active sites of the zymogen which accompany activation. These changes are in general so small that they can be observed only with great difficulty using other physical techniques (Neurath et al., 1956; Fasman et al., 1966). A similar approach was suggested by Fairclough and Vallee (1971) who studied the changes in CD spectra upon activation of arsanilazochymotrypsinogen. However, the reaction of the zymogen with diazotized *p*-arsanilic acid results in the modification of several residues and the major site of modification, Tyr_{146}, is neither critical to the process of activation nor to the activity of the enzyme. The study of zymogens and enzymes specifically acylated at the active site serine residue has obvious advantages.

Since NPGB and other active site titrants have been shown to react rapidly with several non-pancreatic enzymes (Chase and Shaw, 1969) they might be expected to react also with the zymogens. Study of the kinetics of these reactions and of the circular dichroic spectra of the acyl derivatives should provide considerable information about the mechanism of activation of the zymogens involved, for example, in blood clotting and fibrinolysis. The enzymes thrombin, factor Xa, factor IXa, and plasmin are all homologous to trypsin (Enfield et al., 1974); the activation peptides differ considerably ranging in size from several thousand molecular weight to the hexapeptide of trypsinogen and therefore the mechanism of zymogen activation may also differ.

References

Anderson, L. E., Walsh, K. A., and Neurath, H. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 483.

Bender, M. L., and Killheffer, J. V. (1973), *Crit. Rev. Biochem. 1,* 149.

Bender, M. L., Schonbaum, G. R., and Zerner, B. (1962), *J. Am. Chem. Soc. 84,* 2540.

Chase, T., Jr., and Shaw, E. (1967), *Biochem. Biophys. Res. Commun. 29,* 508.

Chase, T., Jr., and Shaw, E. (1969), *Biochemistry 8,* 2212.

Enfield, D. L., Ericsson, L. H., Fujikawa, K., Titani, K., Walsh, K. A., and Neurath, H. (1974), *FEBS Lett. 47,* 132.

Fairclough, G. F., Jr., and Vallee, B. L. (1971), *Biochemistry 10,* 2470.

Fasman, G. D., Foster, R. J., and Beychock, S. (1966), *J. Mol. Biol. 19,* 240.

Freer, S. T., Kraut, J., Robertus, J. D., Wright, H. T., and Xuong, Ng. H. (1970), *Biochemistry 9,* 1997.

Gertler, A. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 465.

Gertler, A., Walsh, K. A., and Neurath, H. (1974a), *Biochemistry 13,* 1302.

Gertler, A., Walsh, K. A., and Neurath, H. (1974b), *FEBS Lett. 38,* 157.

Henderson, R. (1970), *J. Mol. Biol. 54,* 341.

Jameson, G. W., Roberts, D. V., Adams, R. W., Kyle, W. S. A., and Elmore, D. T. (1973), *Biochem. J. 131,* 107.

Kerr, M. A., Walsh, K. A., and Neurath, H. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 483.

Kreiger, M., Kay, L. M., and Stroud, R. M. (1974), *J. Mol. Biol. 83,* 209.

Mares-Guia, M., and Shaw, E. (1964), *J. Biol. Chem. 240,* 1579.

Mares-Guia, M., and Shaw, E. (1967), *J. Biol. Chem. 242,* 5782.

Morgan, P. H., Robinson, N. C., Walsh, K. A., and Neurath, H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3312.

Morgan, P. H., Walsh, K. A., and Neurath, H. (1974), *FEBS Lett. 41,* 108.

Neurath, H., Rupley, J. A., and Dreyer, W. J. (1956), *Arch. Biochem. Biophys. 65,* 243.

Neurath, H., Walsh, K. A., and Gertler, A. (1973), in Metabolic Conversion of Enzymes, West Berlin, Springer-Verlag, p 301.

Robillard, G. T., Powers, J. C., and Wilcox, P. E. (1972), *Biochemistry 11,* 1773.

Robinson, N. C., Neurath, H., and Walsh, K. A. (1973a), *Biochemistry 12,* 414.
Robinson, N. C., Neurath, H., and Walsh, K. A. (1973b), *Biochemistry 12,* 420.
Stroud, R. M., Kay, L. M., and Dickerson, R. E. (1974), *J. Mol. Biol. 83,* 185.
Wang, C. C., and Shaw, E. (1972), *Arch. Biochem. Biophys. 150,* 259.
Zerner, B., Bond, R. P. M., and Bender, M. L. (1964), *J. Am. Chem. Soc. 86,* 3674.

Formation of Transient Complexes in the Glutamate Dehydrogenase Catalyzed Reaction†

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ABSTRACT: The reaction of glutamate dehydrogenase and glutamate (gl) with NAD^+ and $NADP^+$ has been studied with stopped-flow techniques. The enzyme was in all experiments present in excess of the coenzyme. The results indicate that the ternary complex (E·NAD(P)H·kg) is present as an intermediate in the formation of the stable complex (E·NAD(P)H·gl). The identification of the complexes is based on their absorption spectra. The binding of the coenzyme to (E·gl) is the rate-limiting step in the formation of (E·NAD(P)H·kg) while the dissociation of α-ketoglutarate (kg) from this complex is the rate-limiting step in the formation of (E·NAD(P)H·gl). The K_m for glutamate was 20–25 m*M* in the first reaction and 3 m*M* in the formation of the stable complex. The K_m values were independent of the coenzyme. The reaction rates with NAD^+ were approximately 50% greater than those with $NADP^+$. Furthermore, high glutamate concentration inhibited the formation of (E·NADH·kg) while no substrate inhibition was found with $NADP^+$ as coenzyme. ADP enhanced while GTP reduced the rate of (E·NAD(P)H·gl) formation. The rate of formation of (E·NAD(P)H·kg) was inhibited by ADP, while it increased at high glutamate concentration when small amounts of GTP were added. The results show that the higher activity found with NAD^+ compared to $NADP^+$ under steady-state assay conditions do not necessarily involve binding of NAD^+ to the ADP activating site of the enzyme. Moreover, the substrate inhibition found at high glutamate concentration under steady-state assay condition is not due to the formation of (E·NAD(P)H·gl) as this complex is formed with K_m of 3 m*M* glutamate, and the substrate inhibition is only significant at 20–30 times this concentration.

Glutamate dehydrogenase (L-glutamate:$NAD(P)^+$ oxidoreductase (deaminating) EC 1.4.1.3) from beef liver is one of the most extensively studied enzymes. It is an allosteric enzyme consisting of six identical protomers (Appella and Tomkins, 1966). The enzyme is activated by ADP and inhibited by GTP when glutamate is used as substrate. Since many of the complexes between glutamate dehydrogenase and its substrates and coenzymes possess characteristic spectra (Fisher, 1973), the enzyme is particularly well suited for fast kinetic studies. Iwatsubo and coworkers (Iwatsubo and Pantaloni, 1967; diFranco and Iwatsubo, 1972; diFranco, 1974) followed the spectral changes during the initial phase of the oxidative deamination of glutamate and suggested the following reaction scheme:

$$E + NAD(P)^+ + gl \rightleftharpoons (E\cdot NAD(P)\cdot gl) \rightleftharpoons (E\cdot NAD(P)H\cdot kg) \rightleftharpoons (E\cdot NAD(P)H) + kg$$

$$(E\cdot NAD(P)H) + gl \rightleftharpoons (E\cdot NAD(P)H\cdot gl) \rightleftharpoons (E\cdot gl) + NAD(P)H$$

E is enzyme and gl and kg are glutamate and α-ketoglutarate, respectively (for simplification the release of NH_3 and H^+ in the reaction sequence will not be discussed in the present paper).

The release of the reduced coenzyme from (E·NAD(P)H·gl) is assumed to be the rate-limiting step under normal assay conditions (Engel and Dalziel, 1969; D'Albis and Pantaloni, 1972; Shafer et al., 1972; diFranco 1974), while at low glutamate concentration the release of α-ketoglutarate from the ternary complex (E·NAD(P)H·kg) is the rate-limiting step (diFranco, 1974). Steady-state kinetics indicate that the enzyme binds its coenzyme and glutamate in a random manner (Engel and Dalziel, 1969, 1970; Silverstein and Sulebele, 1973). There are two binding sites for NAD^+ and $NADP^+$ per protomer, one "active site" and one "nonactive site" (Krause et al., 1974). The "nonactive site" is the same as the ADP activating site (Fisher, 1973). The "active site" binds the two coenzymes in the same manner, while the affinity of the "nonactive site" for NADPH is 10 times lower than for NADH (Krause et al., 1974). The latter finding explains why the previous authors have found only one binding site for $NADP^+$ (Frieden, 1959; Pantaloni and Dessen, 1969; Cross and Fisher, 1970). The higher activity observed with NAD^+ compared to $NADP^+$ has in part been explained by assuming that the second NAD^+ molecule activates the enzyme by binding to the ADP activating site (Pantaloni and Dessen, 1969; Cross and Fisher, 1970, Fisher, 1973; diFranco, 1974).

Recently, Shafer et al. (1972) studied the oxidative deamination of glutamate with enzyme present in excess of the coenzyme. Since the affinity of the "active site" for the

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