CONFORMATIONAL CHANGES RESULTING FROM THE CARBAZOYLATION OF BOVINE α-CHYMOTRYPSIN BY *p*-NITROPHENYL N^2-ACETYL-N^1-ARYLMETHYLCARBAZATES

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SUMMARY

Several (N^2-acetyl-N^1-arylmethylcarbazoyl)-α-chymotrypsins with *p*-substituents in the N^1-arylmethyl group have been prepared. Measurements of (a) accessibility of tryptophyl residues to modification by 2-hydroxy-5-nitrobenzyl bromide, (b) intrinsic fluorescence spectra in the absence and presence of sodium dodecyl sulphate, (c) thermal perturbation spectra indicate that, in general, tryptophyl residues are less accessible to solvent than in the free enzyme and the modified enzymes are more stable than α-chymotrypsin to denaturation by heat or sodium dodecyl sulphate. N^2-Acetyl-N^1-*p*-dimethylaminobenzylcarbazoyl-α-chymotrypsin, however, contains more accessible tryptophyl residues than the other derivatives and is thermally less stable although it is more stable than the free enzyme.

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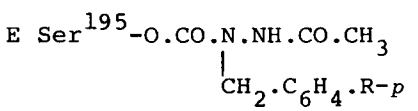
p-Nitrophenyl N^2-acetyl-N^1-benzylcarbazate, the aza-analogue of *N*-acetylphenylalanine *p*-nitrophenyl ester, acylates α-chymotrypsin rapidly and stoicheiometrically at pH 5-7 and the resulting carbazoylated enzyme (I; R=H) does not undergo deacylation readily (1). We now present evidence which indicates that (N^2-acetyl-N^1-arylmethylcarbazoyl)-α-chymotrypsins with small substituents (e.g. I; R=F) differ in conformation from analogues with bulky substituents (e.g. I; R=Me_2N) and from the parent enzyme.



(I)

MATERIALS AND METHODS

α-Chymotrypsin (salt-free, thrice crystallized)(Sigma Chemical Co., Kingston-on-Thames, England) was purified by chromatography on CM-Sephadex (2). The operational molarity of solutions of α-chymotrypsin was determined spectrofluorimetrically with 4-methylumbelliferyl *p*-(*N,N,N*-trimethylammonium) cinnamate as titrant (3). *p*-Nitrophenyl and ethyl esters of N^2-acetyl-N^1-arylmethylcarbazic acids were synthesized by the methods of Elmore and Smith (1) and will be detailed elsewhere. *N^2-Acetyl-N^1-hexahydrobenzylidenehydrazine* (86%) prepared by the interaction of acethydrazide and hexahydrobenzaldehyde in ethanol, had m.p. 88-89°C (Found: C,64.1; H,9.7; N,16.6. $C_9H_{16}N_2O$ requires C,64.3; H,9.6; N,16.7%). Hydrogenation at atmospheric pressure in ethanol over Adam's Pd catalyst gave *N^2-acetyl-N^1-hexahydrobenzylhydrazine* (65%) which had m.p. 40-41°C after recrystallization from ethyl acetate - light petroleum (b.p. 40-60°C) (Found: C,63.3; H,10.6; N,16.6. $C_9H_{18}N_2O$ requires C,63.9; H,10.1; N,16.6%). To a mixture of the foregoing compound (10mmol) and triethylamine (10mmol) in methylene chloride (15ml), a solution of *p*-nitrophenyl chloroformate (10mmol) in methylene chloride (15ml) was added dropwise at 0°C and the mixture was left overnight at 4°C. The solution was washed successively with cold 1M-HCl and water, dried ($MgSO_4$), and evaporated under reduced pressure. *p-Nitrophenyl N^2-acetyl-N^1-hexahydrobenzylcarbazate* (40%) was recrystallized from ethyl acetate-light petroleum (b.p. 40-60°C) when it had m.p. 116-118°C (Found: C,57.1; H,6.2; N,12.3. $C_{16}H_{21}N_3O_5$ requires C,57.3; H,6.3; N,12.5%). In order to prepare the carbazoylated enzymes, a solution of α-chymotrypsin (70mg) in 0.05M-potassium phosphate buffer (4.5ml), pH 6.0, was treated with a 25mM-solution (0.5ml) of the appropriate *p*-nitrophenyl carbazate derivative in tetrahydrofuran at room temperature. When titration of an aliquot (3) showed that the enzyme had completely reacted, the solution was desalted on a column (2.2x15cm) of Sephadex G-25 equilibrated with 1mM-HCl and the appropriate fractions were freeze-dried. No appreciable deacylation of (N^2-acetyl-N^1-benzylcarbazoyl)-α-chymotrypsin was observed after storage at -20°C for a year.

Chemical modification (4) of tryptophan residues was carried out by allowing an aqueous solution (5ml) of α-chymotrypsin (0.2μmol) to react during 30 min with 2-hydroxy-5-nitrobenzyl bromide (5mg in 0.75ml of acetone) at pH 3.0 and the extent of reaction was determined spectrophotometrically using the value ε_{410}=18450 $M^{-1}cm^{-1}$ in 2M-NaOH.

Intrinsic emission fluorescence spectra were measured at 25.0°C as described previously (3) by exciting at 292nm and recording the emission between 310nm and 420nm. Each spectrum was measured in triplicate and 1.81μM-L-tryptophan in 1mM-HCl was used as a fluorescence intensity standard. Protein solutions (0.1mg/ml) in thrice-distilled water were adjusted to pH 3.0 with 1M-HCl and centrifuged before use. Protein concentrations were compared by the methods of Lowry, Rosenbrough, Farr, and Randall (5). The behaviour of α-chymotrypsin and its carbazoylated derivatives in sodium dodecyl sulphate at 25.0°C was followed by observing the changes in emission at 356nm after excitation at 290nm. Protein samples (0.1mg/ml) were dissolved in 25mM-potassium phosphate buffer, pH 7.413, which was 0.67mM with respect to sodium dodecyl sulphate.

Thermal perturbation difference spectra were obtained by the general method of Biltonen and Lumry (6) on a Unicam SP800B double-beam recording spectrophotometer using 10mm quartz cells. The temperature of the sample was monitored by an immersed calibrated thermocouple. Protein samples (0.5mg/ml) were dissolved in thrice-distilled water and the solutions were adjusted to pH 3.0 with 1M-HCl and centrifuged before use. The transition temperature for each protein was obtained from a graph of the decrease in absorbance at 293nm versus temperature.

RESULTS AND DISCUSSION

Evidence presented earlier (1) indicated that tryptophyl residues move into a less polar environment when α-chymotrypsin is acylated with *p*-nitrophenyl N^2-acetyl-N^1-benzylcarbazate. The accessibility of tryptophyl residues in α-chymotrypsin and various carbazoylated derivatives to 2-hydroxy-5-nitrobenzyl bromide at pH 3.0 is recorded in Table 1. The results for α-chymotrypsin and the derivative (I; R=H) agree with those reported earlier (1.7). It is known that 2-hydroxy-5-nitrobenzyl bromide reacts with α-chymotrypsin and chymotrypsinogen at Trp^{215} (8,9) which forms part of the binding site. The results in Table 1 show that the accessibility of tryptophyl residues to 2-hydroxy-5-nitrobenzyl bromide is higher for α-chymotrypsin than for all carbazoylated derivatives with the exception of the *p*-dimethylamino derivative (I; R=NMe_2). It is not known at present if the lower accessibility of tryptophyl residues in carbazoylated derivatives of α-chymotrypsin is due to (a) a conformational change following carbazoylation, (b) steric occlusion of tryptophyl residues by the substituent, or (c) a combination of both effects. Presumably, these factors do not apply to N^2-acetyl-N^1-*p*-dimethylaminobenzylcarbazoyl-α-chymotrypsin and it is possible that the *p*-dimethylaminobenzyl substituent does not reside in the 'tosyl hole'.
It has been suggested (10) that the presence in formyl-L-phenylalanine of a *p*-iodo substituent, which is sterically similar to the dimethylamino group, precludes binding of the side chain in

TABLE I

Properties of carbazoylated derivatives of α-chymotrypsin

Enzyme derivative	Number of Trp residues modified	Fluorescence properties		
		Relative peak height	Wavelength of maximum emission (nm)	Transition temperature (^{o}C)
α-Chymotrypsin	2.02	1.00	336	48.0
I; R=H	0.40	1.25	335	58.0
I; R=Me	0.30	1.24	335	58.1
I; R=F	0.51	1.22	336	58.0
I; R=Br	0.59	1.26	336	58.0
I; R=NO_2	0.57	1.00	335	59.0
I; R=OMe	0.58	1.10	336	57.0
I; R=CN	0.75	0.99	335	58.0
I; R=CF_3	1.57	1.12	336	55.0
I; R=NMe_2	2.26	0.65	337.5	52.5
N^2-acetyl-N^1-hexahydrobenzyl-carbazoyl-chymotrypsin	1.51	1.14	335.5	----

the 'tosyl hole'. On the other hand, reaction of *p*-nitrophenyl N^2-acetyl-N^1-*p*-dimethylaminobenzylcarbazate with α-chymotrypsin involves the active site, since the modified enzyme is not titratable with 4-methylumbelliferyl *N,N,N*-trimethylammonium cinnamate and does not bind proflavin.

The fluorescence intensities of the carbazoylated derivatives of α-chymotrypsin relative to the native enzyme and the wavelengths of emission maxima are given in Table 1. With the exception of the *p*-dimethylamino derivative (I; R=NMe_2), the carba-

zoylated enzymes have fluorescence intensities approximately equal to or rather greater than that of α-chymotrypsin. The increase in fluorescence intensity might be explained if (a) carbazoylation is accompanied by a conformational change in which one or more tryptophyl residues move into a less polar environment, (b) a residue which quenches the fluorescence of a tryptophyl residue is less effective after reaction with the carbazates, (c) both changes operate together. It has been suggested that His^{40} quenches the fluorescence of tryptophyl residues in α-chymotrypsin (11). The possibility that the enhanced fluorescence intensity of carbazoylated derivatives of α-chymotrypsin results from energy transfer from the aromatic ring of the substituent to a nearby tryptophyl residue can be discounted since the derivative obtained from *p*-nitrophenyl N^2-acetyl-N^1-hexahydrobenzylcarbazate also shows an enhancement of fluorescence. The alicyclic ring of this carbazoylated enzyme could not be involved in energy transfer. The *p*-nitro- and *p*-cyano-derivatives (I; R=NO_2, R=CN) have approximately the same fluorescence intensity as the native enzyme. It has been found, however, that both ethyl N^2-acetyl-N^1-*p*-nitro- and N^2-acetyl-N^1-*p*-cyano-benzylcarbazates, which did not react significantly with the enzyme during the time-course of the experiment, quench the fluorescence of α-chymotrypsin; quenching was complete in the presence of 100-fold excess of ethyl esters. It is probable that the quenching involved Trp^{215} and this masked the normal enhancement which might have been expected. Ethyl N^2-acetyl-N^1-benzylcarbazate did not affect the intrinsic fluorescence of α-chymotrypsin. The *p*-dimethylamino derivative (I; R=NMe_2) is again atypical since its fluorescence intensity is 35% lower than that of α-chymotrypsin. Presumably the converse of explanations (a) or (b) operate in this case; since tryptophyl

residues are more accessible to 2-hydroxy-5-nitrobenzyl bromide, and since maximum emission of the carbazoylated enzyme occurs at a slightly longer wavelength than with α-chymotrypsin, a conformational change is more likely. This view is strengthened by the observation that ethyl N^2-acetyl-N^1-*p*-dimethylaminobenzyl-carbazate does not quench the intrinsic fluorescence of α-chymotrypsin. Further evidence for a conformational change accompanying carbazoylation was obtained from a study of the behaviour of the proteins in 67mM sodium dodecyl sulphate at pH 7.413 and 25.0°C; under these conditions, α-chymotrypsin was unfolded in a few seconds whereas the carbazoylated derivatives (I; R=H, R=OMe) were stable.

The transition temperatures for the thermal unfolding of α-chymotrypsin and its carbazoylated derivatives (I) at pH 3.0 are also recorded in Table 1. Similar results for α-chymotrypsin were obtained by Biltonen and Lumry (6). All the spectral changes were reversed after a few hours at room temperature. Our results indicate that all of the carbazoylated derivatives are more stable than free enzyme to thermal denaturation. Nevertheless, the *p*-dimethylamino derivative (I; R=NMe_2) and, to a lesser extent, the *p*-trifluoromethyl derivative (I; R=CF_3) have lower transition temperatures than the other substituted chymotrypsins. It has previously been observed that diethylphosphoryl-(12) and di-isopropylphosphoryl-derivatives (13,14) of α-chymotrypsin are more stable than the parent enzyme.

In summary, the evidence presented here indicates that the introduction of an N^2-acetyl-N^1-arylmethylcarbazoyl substituent at the active site of α-chymotrypsin causes tryptophyl residues to become less accessible to solvent and stabilizes the molecule to denaturation by heat and by sodium dodecyl sulphate. In

contrast, introduction of the N^2-acetyl-N^1-*p*-dimethylaminobenzyl-carbazoyl substituent gives a modified enzyme with a different conformational state in which tryptophyl residues are more accessible to solvent but which is still somewhat more stable than the free enzyme to thermal denaturation. A kinetic study of the reactions between *p*-nitrophenyl N^2-acetyl-N^1-arylmethyl-carbazates and α-chymotrypsin has been carried out and will be reported later.

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