98. Hormone-Receptor Interactions. Carboranylalanine (Car) as a Phenylalanine Analogue: Reactions with Chymotrypsin

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Summary

In order to test the effects of the replacement of phenylalanine by carboranylalanine (Car) in biological ligand-acceptor interactions, Z · Ala-Ala-Car · OH (**1**) and Ac · Car · OEt (**2**) were synthesized and their reactions with chymotrypsin studied. The two compounds proved to be good inhibitors with K(i) values of $3 \cdot 10^{-4}$ M (**1**) and $8.6 \cdot 10^{4}$ M (**2**); the K(i) of Z · Ala-Ala-Phe · OH (**1a**) is $1 \cdot 10^{-3}$ M. The inhibition constants were determined by a new photolytic technique, inhibition of photoaffinity labelling by Z · Ala-Ala-Phe(pN$_3$) · OH. Ac · Car · OEt is not hydrolysed by chymotrypsin. The findings indicate that the carboranyl group can interact with the 'phenyl recognition site' of the enzyme to produce the binding that is characteristic of aromatic amino acid residues. However, some kind of distortion in the region of the 'mechanistic site' must be postulated in order to account for the failure of hydrolysis. Some possible effects of the replacement of aromatic amino acids by Car in peptide hormones on hormone-receptor interactions are discussed.

Introduction. – The synthesis of a new phenylalanine analogue, L-carboranyl alanine (Car), has been described recently [1]. The size of the icosahedral side chain is approximately that of a phenyl ring rotating about its 1,4 axis (such rotations can actually be observed in proteins [2]). The idea was to replace phenylalanine in biologically active peptides by Car and, if such peptides still exhibited affinity for their natural receptors, to use them for the study of ligand-acceptor interactions by autoradiographic techniques, or to use them as specifically localized sources of α-particles (upon neutron irradiation) in therapy or biological experimentation [1].

The purpose of the work described here was to study the actual replaceability of Phe by Car in a simple, well-known, biologically significant ligand-acceptor situation. To this end, the interaction of Car derivatives with chymotrypsin was studied and compared with that of the corresponding Phe derivatives.

We chose two Car derivatives: benzyloxycarbonyl-alanyl-alanyl-carboranylalanine, Z · Ala-Ala-Car · OH (L-L-L) (**1**), and ethyl acetyl-carboranylalaninate,

Ac · Car · OEt (DL) (**2**). The peptide is an analogue of the chymotrypsin inhibitor Z · Ala-Ala-Phe · OH (**1a**) of which more than 20 Phe replacements have been studied [3] [4]. The advantage of this system is the favorable interaction of the aromatic amino acid residue with the corresponding 'recognition site' [5] of chymotrypsin, a process that has been described quantitatively in terms of the standard free energy of association [3]. The second derivative, **2**, should not only allow the study of the aromatic recognition site, but also of the adjacent 'mechanistic site' that is responsible for the hydrolytic mechanism [5]. This site contains the proton relay system Ser(195), His(57), and Asp(102). Incorrect positioning of the $-COOEt$ moiety and/or induced perturbations of the mechanistic site will lead to a reduction of hydrolytic velocity compared to Ac · Phe · OEt or Ac · Tyr · OEt, good substrates for chymotrypsin.

For the determination of the inhibition constants, $K(i)$, we used a new photolytic method, inhibition of photoaffinity labelling, that we intend to publish together with a detailed investigation [6] on the photoaffinity labelling of chymotrypsin by means of benzyloxycarbonyl-alanyl-alanyl-(*p*-azido)phenylalanine, Z · Ala-Ala-Pap · OH (L-L-L) (**3**) [4]. The advantage of the method over the usual inhibition of Ac · Tyr · OEt hydrolysis [3] is the small amount of inhibitor necessary. If the photolysis solution is reduced to 0.1 ml, only about 10 μmol of inhibitor are needed for a complete $K(i)$ determination instead of about 1 mmol with the titration technique. The basic idea is that the photolytic inhibitor **3** with $K(i) = 8.54 \cdot 10^{-4}$ M in the dark [4] is successively replaced from the enzyme active site by increasing amounts of the non-photolytic inhibitor to be tested. Thus, upon irradiation, ever smaller amounts of the enzyme become inactivated by covalent attachment of **3** to the active site [6]. The amount of functional enzyme remaining is determined by standard methods [3].

Experimental Part

Materials and methods. Photolysis was carried out at 25° with 365 nm radiation from a 200 W mercury lamp in an apparatus described earlier [7]. Chymotryptic activity was measured with a standard assay of the initial velocity of Ac · Tyr · OEt hydrolysis [3]. The determination was carried out at pH = 8.0 and 20° with an automatic titrator (pH-Stat, *Radiometer AB*, Copenhagen). α-Chymotrypsin was thrice recrystallized, salt-free, and lyophilized from *Boehringer GmbH*, Mannheim; Ac · Tyr · OEt from *Fluka AG*, Buchs.

Z · Ala-Ala-Car · OH (L–L–L) (**1**) was obtained by the condensation of Z · Ala-Ala · OSU [3] with L-carboranylalanine [1] in dimethylformamide. Yield 43% colourless crystals from ethyl acetate/2-propanol/diisopropyl ether, m.p. 140°; Rf 0.65 *n*-BuOH/AcOH/H_2O 4:1:1 (*v*/*v*); $[\alpha]_D^{20} = -34.6°$ (*c* = 0.5, EtOH); IR. (cm^{-1}) 2570 (B–H), 1720–1600 (C=O).

Ac · Car · OEt (DL) (**2**) was prepared from ethyl DL-*N*-acetyl-propargylglycinate and bisacetonitrilo-decarborane in benzene with a slight modification of the general method [1]. Yield 46% colourless crystals from ethanol/water, m.p. 85°; Rf 0.59 $HCCl_3$/MeOH 9:1 (*v*/*v*); IR. (cm^{-1}): 2570 (B–H), 1730 (C=O, ester), 1670 (C=O, amide).

Inhibition of photoaffinity labelling. The photolysis solution consisted of: 1) 5 mg α-chymotrypsin in 0.75 ml water; 2) 0.25 ml of a 0.1 M aqueous *p*-aminophenylalanine solution (as an excellent radical scavenger); 3) 0.5 ml of a 4 mM (or 8 mM) solution of Z · Ala-Ala-Phe(pN_3) · OH in 50 mM Tris-hydrochloride buffer, pH 8.0; 4) 0.5 ml of a 0, 2, 4, 8 or 16 mM solution of Z · Ala-Ala-Car · OH (or of another compound to be tested) in an ethanol/water 1:1 (*v*/*v*) Tris-hydrochloride buffer, 50 mM pH 8.0. Because of the extreme hydrophobic properties of Ac · Car · OEt, this compound was dissolved in *t*-butyl alcohol (= solution 4); we found that the enzyme is fully active after storing in aqueous systems containing up to 25% of *t*-BuOH.

The solution for the assay of (remaining) chymotryptic activity consisted of 5) 10 ml of a 10 mM aqueous solution of Ac · Tyr · OEt; 6) 1 ml of 10 mM Tris-hydrochloride buffer, pH 8.0; 7) 1 ml 2M NaCl.

The photolysis solution, 1)+2)+3)+4)=2 ml, was incubated in the dark at 25° for 10 min and photolysed for 10 min. Aliquots of 0.5 ml were taken and diluted with 0.75 ml water; 10 µl of this solution were used for the assay of chymotryptic activity. To this end, the 10 µl were introduced into the mixture of 5)+6)+7)+8 ml water (=20 ml), brought to 20°, and titrated with 0.1 N NaOH. The initial velocity was determined graphically and expressed as per cent of the initial velocity of a non-irradiated probe, prepared and titrated in the dark.

Results and discussion. – The results of the inhibition experiments are shown in *Figure 1*. The K(i) values at pH = 8 are: $3 \cdot 10^{-4}$M (**1**); $10 \cdot 10^{-4}$M (**1a**), and $8.6 \cdot 10^{-4}$M (**2**). The value found for Z · Ala-Ala-Phe · OH (**1a**) is in excellent agreement with values determined by the titration method [3] [4] at the same pH. The two Car derivatives are even better inhibitors of chymotrypsin than **1a**.

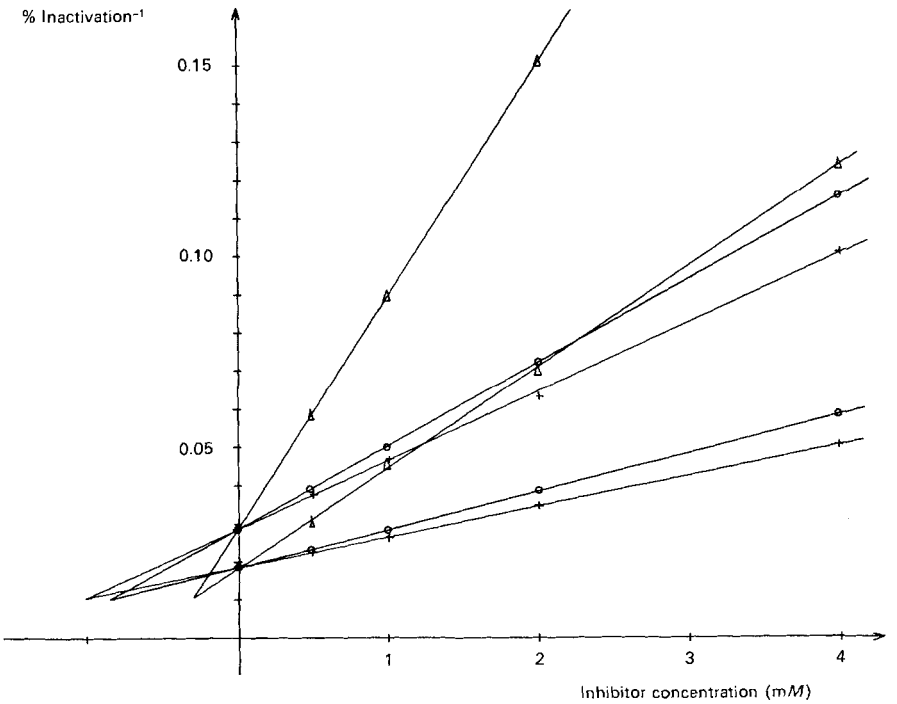

Fig. 1. *Dixon plot of the inhibition of photolytic inactivation of α-chymotrypsin.* Photoaffinity ligand: Z · Ala-Ala-Pap · OH (**3**), 1 and 2 mM (upper and lower regression lines of an experiment, respectively). Inhibitors: Z · Ala-Ala-Car · OH (**1**) (△); Z · Ala-Ala-Phe · OH (**1a**) (+); Ac · Car · OEt (**2**) (○).

In our as yet unpublished investigation [6] of the chemical details of chymotrypsin photoaffinity labelling with Z · Ala-Ala-Pap · OH (**3**) [4], we find that the reaction of **3** in the presence of *p*-aminophenylalanine as a radical scavenger is highly specific for the 'aromatic recognition site' [5] of the enzyme. Furthermore, *Bosshard & Berger* [3] concluded that K(i) differences found for various Z · Ala-Ala-P · OH analogues

(P = different aromatic amino acids) are to be explained by variations of P-residue affinity for the recognition site. In the dark, L is also a reversible inhibitor of the *Bosshard-Berger* type [4]. We can therefore be quite sure that **1** and **2** react with chymotrypsin by insertion of the carboranylalanine side chain into the aromatic recognition site.

This conclusion is further substantiated by the stoichiometry of inhibitor binding. *Figure 2* indicates a 1:1 molar ratio of inhibitor to enzyme for the two tripeptides **1** and **1a**. Ac · Car · OEt (**2**) displays a lower ratio; this is understandable because the compound is a racemate and the D-enantiomer is expected to have a higher $K(i)$ than the L-enantiomer (similar lowering of the ratio is observed with isoenzyme mixtures containing enzymes with different inhibitor affinities [8]).

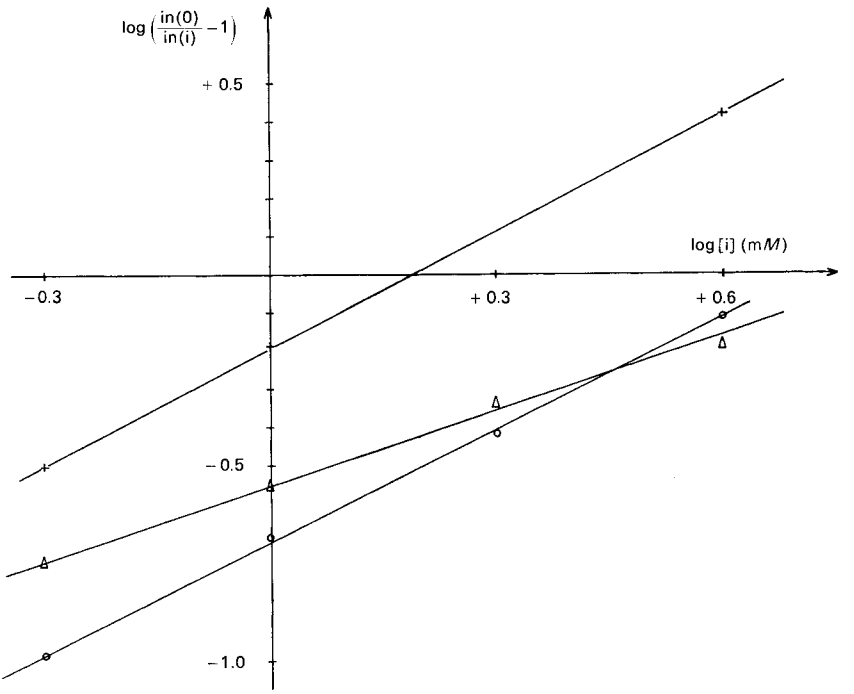

Fig. 2. *Stoichiometry of the inhibitor: enzyme interaction.* The basic equation is [8]: $\log(v/v(i)-1) = \log(\overline{K}(i)/1 + \overline{K}(p)[p]) + \log[i]$, wherein v and v(i) are the initial velocities without and in the presence of the inhibitor; $\overline{K} = 1/K$; [p] is the concentration of **3**, and $K(p)$ that [p] giving a 50% inactivation of the enzyme. For our system, the expression $\log(in(0)/in(i)-1)$ was used instead of $\log(v/v(i)-1)$; in(0) is the photolytic inactivation of the enzyme by **3** without added inhibitor, in(i) in the presence of the competitive inhibitor; $K(p) = 2.9 \cdot 10^{-3}$ M.
A one-to-one molar interaction of inhibitor to enzyme is indicated by a slope of 1 as shown by the tripeptides **1** (○) and **1a** (+); an interaction ratio of 0.66 is shown by **2** (△).

The aromatic recognition site of α-chymotrypsin is usually described as a hydrophobic pocket in the surface of the enzyme molecule [3] [5] [9]. The aromatic rings of the natural amino acids Phe, Tyr, and Trp, and of certain artificial ones [3] can slip into the slot between the enzyme peptide bonds 190–191–192 on one side of the

pocket and 215–216 on the other side [5]. The α-NH and α-carbonyl groups of the aromatic amino acid substrate are held in position by hydrogen bonds to other parts of the enzyme. However, in order to accommodate the carboranyl icosahedron with a *van der Waals* diameter of roughly 5 Å, the opening of the pocket (approximately 4–5 Å) must be widened. This process is not without precedent, because it appears that the observed rotation of phenyl rings in proteins is also accompanied by deformations of the molecular surroundings [2].

If we accept that Ac · Car · OEt interacts well with the aromatic recognition site, then the failure of ester hydrolysis must be explained. It could be due to either one of two factors or to both in conjunction: (i) the position of the ester carbonyl group relative to the enzyme mechanistic site (especially Ser 195) is non-functional, (ii) the deformation of the pocket by the carboranyl group is propagated to the mechanistic site and disrupts the proper spatial relationships that are necessary for catalysis.

In view of the planned replacement of aromatic amino acids by carboranylalanine in other biologically active peptides, such as hormones, we might expect the replacement to produce compounds that bind to receptor sites but do not necessarily stimulate them (competitive non-agonistic inhibitors).

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