p-NITROPHENYLALANINE, *p*-AZIDOPHENYLALANINE, *m*-AZIDOPHENYLALANINE, AND *o*-NITRO-*p*-AZIDO-PHENYLALANINE AS PHOTOAFFINITY LABELS

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1. Introduction

Earlier this year, we have reported the synthesis of peptides containing the potential photoaffinity-labelling amino acid *p*-azido-L-phenylalanine. The compounds were substrates and reversible inhibitors of enzymes like chymotrypsin and aminopeptidases [1]. Illumination of the inhibitor-enzyme mixtures impaired the reversibility rather strongly. We did not, however, accept this as evidence for covalent attachment, because control experiments with compounds containing the *p*-nitro in place of the *p*-azido group also irreversibly decreased enzyme activity when irradiated (unpublished results). A possible explanation was excitation energy transfer from the substituted aromatic system to an appropriate acceptor (tryptophane?) in the enzyme, damaging catalytic function. We therefore made experiments designed to correlate incorporation of a radioactive photoaffinity ligand into chymotrypsin with its capacity to destroy proteolytic activity. To our astonishment, it turned out that not only phenylalanine substituted with azido groups but also *p*-nitro-L-phenylalanine was photolytically activated and (covalently) incorporated into the enzyme. As far as we are aware, the aromatic nitro group has never before been shown to be useful as a photoaffinity label. Indeed, very little is known about the ability of the nitro group to produce chemically reactive species (radicals?) upon illumination. The use of this group instead of aromatic azides represents a simplification of preparative procedures and opens new possibilities.

2. Materials and methods

The preparation of compounds 1–5 (table 1) shall be reported elsewhere [2]. α-Chymotrypsin (Sigma Chemicals) and its substrate, *N*-acetyl tyrosine ethyl ester (Fluka Ltd.) were commercially available materials and were found to be of high purity (the enzyme showed at least 93% of the activity of the best reported samples (see [3]).

Kinetic studies were carried out with a pH-Stat (Radiometer) at 25°C with titration end-point pH 8.0. Stock solutions were: (i) 0.01 mM α-chymotrypsin in H_2O, (ii) 10 mM Ac·Tyr·OEt** in H_2O, (iii) 10 mM phosphate buffer, pH 7.7, (iv) 2M NaCl, and (v) 0.1 M NaOH. For each experiment, 20 μl I, 0.5–5 ml ii, and 0.5 ml each of iii and iv in a total volume of 10 ml (dilution with H_2O and appropriate amounts of the inhibitor). The inhibition constants were determined graphically using a double reciprocal plot (I/V_0 versus $1/S$), in a manner similar to that in [3] and [4].

Photoinactivation experiments were performed similarly after irradiation of an enzyme-inhibitor mixture (with or without added scavenger *p*-amino-phenylalanine) for 30 min at 25°C under argon with light of λ = 365 nm (200 W mercury lamp fitted with an interference filter [2]). The irradiation mixture contained 5 mg of α-chymotrypsin in 0.75 ml of H_2O plus 1 ml of 4 mM inhibitor (1–5) in 50 μM Tris-

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** Abbreviations according to the IUPAC-IUB recommendations, see e.g. J. Biol. Chem. 247 (1972) 977.

Table 1
Per cent incorporation of radioactivity into enzyme protein*

		Inactivation %			Incorporation %
	$K_i \times 10^4$ M	A	B	C	
1 Z·Ala–Ala–Phe·OH	9.10 ± 0.3	0	0	0	0.6
2 Z·Ala–Ala–Phe(pNO$_2$)·OH	1.52 ± 0.05	94	37	6.5	25.0
3 Z·Ala–Ala–Phe(pN$_3$)·OH	8.54 ± 0.4	93	47	8	38.2
4 Z·Ala–Ala–Phe(mN$_3$)·OH	2.21 ± 0.04	48	43	8	9.2
5 Z·Ala–Ala–Phe(oNO$_2$,pN$_3$)·OH	3.01 ± 0.3	79	56	8	35.0

Inhibition constants, K_i, for reversible inhibition of α-chymotrypsin by the benzyloxy carbonyl tripeptides, 1–5, in the dark. Percent irreversible inactivation by photolysis without (A) and with (B) added scavenger, and after pre-illumination of the compounds, 1–5 (C).

* Assuming a 1:1 molar ratio of enzyme to inhibitor as 100%.

buffer at pH 8.0. For some experiments (B, table 1), 0.25 ml of a radical scavenger solution (0.1 M p-aminophenylalanine in H_2O) were added. In others (C, table 1), the inhibitor solution (± scavenger) was irradiated for 1 hr without enzyme; enzyme was then added and irradiation continued for 30 min. Enzyme inactivation was then determined by measuring initial velocity (V_0) in a mixture containing 10 μl of the irradiated enzyme solution, 10 ml of 2 mM Ac·Tyr·OEt in H_2O, 10 ml of 0.5 mM phosphate buffer, pH 7.0, and 10 ml of 0.1 M NaCl in H_2O. Inactivation was expressed in percent of maximal V_0 obtained without irradiation.

Incorporation experiments were carried out with the radioactive species of 1–5, Z·Ala–Ala (β^3H)–Xxx·OH (with Xxx a substituted phenylalanine), prepared by chemical synthesis [2]. The respective specific activities were: 2.80 (1), 2.74 (2), 2.72 (3), 2.76 (4), and 2.72 (5) mCi/m mole. Samples containing enzyme, scavenger, and radioactive inhibitor were prepared and irradiated as described above and tested for enzyme inactivation (above) and incorporation. In the latter case, 1 ml aliquots of the irradiated solutions were dialyzed for 12 hr in a rotating cell [2] against 0.2 M acetic acid at 4°C. The membranes were Spectropor No. 2 with an exclusion limit of between 12 000–14 000 daltons. The wash fluid was used in about 1000-fold excess and replaced once. The dialyzed solutions were then diluted to 0.05 mM enzyme (=2 ml) with H_2O. Aliquots of 0.4 ml (containing 0.02 μ mole of α-chymotrypsin) were subjected to gel filtration over 100 ml of Sephadex G25 in a 1.5 cm diameter tube. The column was eluted with 0.2 M acetic acid at 4°C with a speed of 0.2 ml/min. 5.6 ml fractions were collected, and a 0.5 ml aliquot of each counted in 10 ml of Aquasol scintillant. The resulting elution profile is shown in the fig. 1. Fractions 10 and 11, containing the bulk of the enzyme gave the following results (added counts, without deduction of the background in the range of 500 dpm): 500 (1), 30 700 (2), 45 830 (3), 12 750 (4), and 43 500 (5) dpm, calculated for the total 5.6 ml fraction. Fractions 10 and 11 (the remainder of 5.1 ml each) were then com-

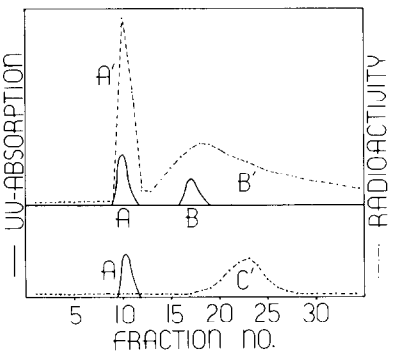

Fig. 1. Elution profiles of photolytic incorporation experiments of 1 (a) and 5 (b) into α-chymotrypsin. A: UV absorption due to chymotrypsin (ordinate: arbitrary units, but to the same scale). A': radioactivity of fraction aliquots in enzyme peak. B and B': UV absorption and radioactivity due to photolysis products of 5. C': radioactivity due to 1 (no UV absorption at this low concentration).

bined and lyophilized. The residue was dissolved in 0.5 ml of 0.05 M NaOH and counted as above (total dpm/background dpm): 1400/500 (1), 31 600/1400 (2), 43 500/800 (3), 12 300/1500 (4), and 42 600/600 (5).

3. Results

The figure shows the elution profile of the Sephadex G25 columns for incorporation experiments with compounds 1 and 5 as extreme examples. In all cases, a high molecular weight compound emerged between fractions 9 and 12 as shown by its UV absorbance at 280 nm. Controls (and profile 1) indicated that this was α-chymotrypsin. This peak was, except in the case of compound 1, always accompanied by radioactivity that had not been removed by dialysis and gel filtration. The later peaks were radioactive and appeared to consist of the tripeptides and their photolysis products (broader peaks with some UV absorbance for compounds 2–5).

Table 1 indicates that the benzyloxycarbonyl tripeptides, 1–5, are all inhibitors of α-chymotrypsin (reversible mode). The K_i values range from 10^{-4} to 10^{-5} M at pH 7.7. Upon illumination of inhibitor–enzyme mixtures with light of $\lambda = 365$ nm (with an energy considerably lower than that of the nearest absorption band, except for compound 5), the enzyme is irreversibly inactivated to a high degree by compounds 2, 3 and 5, to a lesser degree by 4, and not at all (as expected) by 1 (column A). Addition of a scavenger, *p*-amino-L-phenylalanine, exhibiting a very weak specific interaction with the enzyme ($K_i \simeq 10^{-1}$ 10^{-2}), is expected to remove all radicals produced in the solvent or near the enzyme surface, but not those produced in the substrate recognition site. This treatment reduces the percentage of inactivation in almost all cases, again with the exception of 4, quite considerably (column B). The prevailing conditions are probably not optimal, and more enzyme could be inactivated with more inhibitor added. If the inhibitor–scavenger mixture is illuminated prior to the addition of the enzyme (see Materials and methods), there is still some irreversible inactivation to be observed (column C). This might be due to unphotolyzed peptides or to unknown, reactive species produced by the process (aziridines, etc.?) with longer lifetimes than the radicals. The incorporation data were calculated from the radioactivity of the enzyme-containing fractions of the gel filtration experiments. 100% incorporation was assumed to be 120,000 dpm, or 1 mole of inhibitor covalently bound to 1 mole of enzyme under the conditions of the experiment (enzyme recovery was indicated by inactivation experiments with compound 1). We assume the difference between the values of columns B and C to represent the inactivation due to specific reaction of the photoaffinity label with the enzyme active site (recognition site). As shown in table 2, these values parallel those obtained for incorporation in a 1:1 molar mode, with the exception of 4 which appears to inactivate more strongly.

4. Discussion

Bosshard [4], and Bosshard and Berger [3] have reported the synthesis of tripeptide inhibitors of chymotrypsin with the general structure Z·Ala–Ala–

Table 2
Correlation of inactivation and incorporation*

Compound	Inactivation %**	Incorporation %	Ratio of inact./incorp.
1	0	0.6	–
2	30.5	25	1.2
3	39	38.2	1.0
4	35	9.2	3.8
5	48	35	1.4

* See footnote of table 1.
** Difference of values B and C of table 1.

Xxx·OH. The C-terminal amino acid residue, Xxx, was aromatic with either L or D configuration. We have confirmed their findings that Z·Ala–Ala–Phe·OH, 1, and Z·Ala–Ala–Phe(pNO$_2$)·OH, 2, are reversible inhibitors and have prepared and examined the new peptides, 3–5 (see table 1), with p-azido, m-azido and o-nitro-p-azido substituents on the aromatic side-chain of Phe. They are also reversible inhibitors, and therefore appear to be suited for photoaffinity-labelling studies. Bosshard and Berger determined the inhibition constants of 1 and 2 at pH 6.5. They found values of 2.2×10^{-4} and 2×10^{-5} M, respectively [3], which are definitely lower than ours, determined at pH 7.7. The difference can largely be accounted for by the variation of K_i with pH. Judging from fig. 1 in [4], an increase of about 4-fold is to be expected, when going from pH 6.5 to 7.7. This reduces the difference between the two sets to acceptable limits.

Our results suggest that peptides 2–5 are all photoaffinity labels of α-chymotrypsin and can bind covalently to the enzyme on illumination, thereby irreversibly inactivating the enzyme. It is generally assumed (see [4]) that the aromatic side chain of such peptides is included into the 'aromatic recognition site' or 'hydrophobic pocket' of chymotrypsins when they form the inhibitor-enzyme complex. Because of the short lifetimes of excited states and free radicals (nitrenes), molecules of 3–5 lying in the recognition site or on the surface of the enzyme during photoactivation are expected to react with a nearby portion of the enzyme. If a scavenger is added in high concentration, it will probably reduce the number of reactions between photo-activated molecules and enzyme at those points to which the scavenger has 'free' access. However, in the recognition site, its interference will be impaired, because this site will be preferentially occupied by the inhibitor, excluding the scavenger. It is therefore assumed that the results of table 1, column B and of table 2 are mainly due to labelling of the recognition site. However, this remains to be proved by degradation experiments. It will be especially interesting to see whether the exceptional properties of the m-azido derivative also lead to labelling of different enzyme amino acids than are labelled by the other compounds.

What these studies do seem to prove is the following: 1) Radioactivity can be covalently incorporated into chymotrypsin by illumination of its complexes with compounds 2–5. 2) Very unexpectedly, the nitro group is also reactive, opening a new class of photoaffinity and photolytic reagents. 3) Activation occurs with a good quantum yield [2], at low energies, where compounds 2–4 have no absorption band, and proteins are not damaged. 4) Photolytic incorporation and enzyme inactivation are sensitive to topographical changes in the disposition of reactive groups (p-azido versus m-azido). 5) The tripeptides are not seriously degraded during or after the process of photoactivation and labelling, because the radioactive marker, situated on alanine 2, remains attached when the substituted phenylalanine 3 is bound to the enzyme.

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