Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 630-635

PHOTOAFFINITY REAGENTS FOR USE WITH PEPSIN AND OTHER CARBOXYL PROTEASES*

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Received January 27, 1983

SUMMARY: Two compounds have been designed to serve as photoaffinity reagents for use with carboxyl proteases. 1,2-Epoxy-3-(4'-azido-2'-nitrophenoxy)propane has been synthesized and shown to react with porcine pepsin in the same fashion as the traditional inhibitor 1,2-epoxy-3-(p-nitrophenoxy)propane, while p-azidophenacyl bromide is similar to other phenacyl bromides in its reaction with pepsin. In combination with p-azido-α-diazoacetophenone, previously shown to resemble α-diazo carbonyl reagents in its reaction with pepsin, photoaffinity analogs are now available for all three of the widely-used carboxyl protease inhibitors.

Enzymes ranging from the gastric digestive proteases, pepsin and chymosin, of vertebrates to the extracellular fungal proteases from Penicillium janthinellum, Rhizopus chimensis, and Endothia parasitica are now well-documented as belonging to a single class of carboxyl proteases. These enzymes require two active site aspartate residues for their function and have much structural homology to porcine pepsin (1,2). However, speculation continues about the common mechanistic and structural features of a large number of other, less well-studied, carboxyl proteases (1,3,4) and, in some cases, about the physiological roles of the proteins (1).

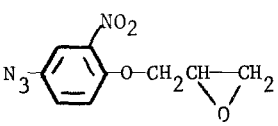
We have developed a series of bifunctional photoaffinity reagents which will allow application of the powerful photoaffinity technique (5,6,7) to investigating questions of structure and function for carboxyl proteases. An earlier report (8) on photoaffinity reagents for use with pepsin noted discouraging results with p-diazobenzenesulfonic acid and with photolabile pep-

*Supported by grants from the Petroleum Research Fund, administered by the American Chemical Society, the Research Corporation, and the National Science Foundation (PRM-7911489).

0006-291X/83 $1.50

tide analogs. Our reagents, on the other hand, were designed to be comparable to the three widely-used, traditional, carboxyl protease inhibitors (1,9,10,11) and to contain the aryl azide chromophore which is known to give good levels of insertion into protein upon photolysis (5,12,13).

The first inhibitor, 1,2-epoxy-3-(4'-azido-2'-nitrophenoxy)propane, 1,[1] is a photoaffinity analog of 1,2-epoxy-3-(p-nitrophenoxy)propane (EPNP), while p-azidophenacyl bromide, 2, serves as an analog of the p-substituted phenacyl bromides. Finally, p-azido-α-diazoacetophenone, 3, is an analog of the α-diazo carbonyl inhibitors. We report here the synthesis of 1 and the results of the incubation of porcine pepsin with 1 and 2. The reaction of 3 with porcine pepsin has been described earlier (13).



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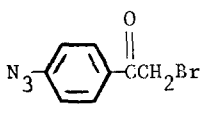

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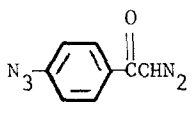

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MATERIALS AND METHODS

Inhibitors. [carbonyl-^{14}C]p-Azidophenacyl bromide, ^{14}C-2, was synthesized as described previously (14).

Reagent 1 was synthesized by the reaction of 4-azido-2-nitrophenol, prepared through the 2-nitroquinone diazide, with epichlorohydrin. The method of Ried and Appel (15) was used to prepare 2-nitroquinone diazide which was obtained as a brown solid that detonated on heating to 164°C. The procedure of Zhmurova et al. (16) was used to convert the diazide to 4-azido-2-nitrophenol in an overall yield of 81% from the 4-amino-2-nitrophenol (Aldrich) starting material. Recrystallization of the 4-azido-2-nitrophenol from 95% ethanol gave yellow needles, mp 88-88.5°C, R_f on silica gel of 0.58 in 1:2 (v/v) acetone/ligroin, R_f of 0.54 in 1:2 (v/v) ether hexane. Lauguin et al. describe an alternate synthesis and provide other physical data for 4-azido-2-nitrophenol (17). To synthesize 1, a 2.7-ml (3.2 g, 34 mmole) portion of epichlorohydrin was added to a solution containing 4.0 g (22 mmole) of 4-azido-2-nitrophenol and 1.2 g (30 mmole) of sodium hydroxide in 200 ml of water. The reaction was stirred at room temperature for 17 h. An additional 2.7 ml of epichlorohydrin was added to the reaction and stirring was continued for 24 h. The reaction mixture was diluted with water and extracted with ether. After washing with base and water, the ether layer was dried and evaporated to an oil which was crystallized from 95% ethanol to give 0.73 g (corrected yield, 19%) of 1. Unreacted phenol starting material was recovered (0.99 g, 5.5 mmole) by acidifying the basic extracts and collecting the precipitate. Recrystallization of the epoxy product from 95% ethanol gave yellow crystals: mp 97.5-98.5°C; R_f on silica gel of 0.09 in 1:2 (v/v) ether/hexane; nmr ($CDCl_3$, Varian T-60 Spectrophotometer) δ 2.80-3.03 (m,2,CH_2 epoxide), 3.23-

[1] Abbreviations used: 1, 1,2-epoxy-3-(4'-azido-2'-nitrophenoxy)propane; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; 2, p-azidophenacyl bromide; 3, p-azido-α-diazoacetophenone.

3.53 (m,1,CH epoxide), 3.93-4.57 (m,2,CH_2), 7.10-7.57 ppm (m,3,aromatic); uv (0.4-2% methanol in 0.005 M acetic acid) λ_{max} 249 nm (ε 2.05 x 10^4 cm^{-1} M^{-1}) and 363 nm (ε 2.14 x 10^3 cm^{-1} M^{-1}). The glycol prepared (18) from 1 showed similar uv characteristics in 0.005 M acetic acid: λ_{max} 251 nm (ε 1.95 x 10^4 cm^{-1} M^{-1}) and λ_{max} 365 nm (ε 2.11 x 10^3 cm^{-1} M^{-1}) with an ε_{280} of 4.89 x 10^3 cm^{-1} M^{-1}.

Pepsin purification, assay, and oxidation. Porcine pepsin (Worthington PM) either was used directly or was purified further by passing it through a Sephadex G-25 (fine) column. An ε_{280} of 5.14 x 10^4 cm^{-1} M^{-1} (19) was used for the enzyme in citrate buffer. Enzyme activity was assayed against hemoglobin as substrate (19).

Pepsin was oxidized by the method of Kido and Kassel (20). Following treatment with catalase, the pepsin solution was dialyzed against two changes of water at 5°C for a total of 19 h. For the nonoxidized control samples of pepsin, water was added to the enzyme in place of 30% hydrogen peroxide. Oxidized and nonoxidized preparations were adjusted to identical concentrations in the range of 6-7 x 10^{-5} M. Oxidized pepsin retained 74-80% of the activity of the nonoxidized enzyme.

Reaction of 1 with pepsin. In a typical run, solid 1 (0.085 g, 0.36 mmole was added to a 38-ml portion of 2.67 x 10^{-5} M pepsin in 0.1 M sodium citrate buffer pH 3.4. The reaction mixture was protected from light and stirred at room temperature. The percent pepsin activity remaining in the 1-pepsin mixture was calculated by comparing the activity of the reaction mixture to that of a control pepsin sample. To determine the molar ratio of 1 bound per mole of pepsin, a 3.0-ml aliquot of the 1-pepsin reaction mixture was removed, centrifuged to remove solid 1, and chromatographed on a 1.4 x 44 cm Sephadex G-25 (fine) column in the dark with 0.005 M acetic acid used as eluant. Early fractions containing material absorbing at 280 nm were scanned in the uv region on a Cary 17 spectrophotometer and the concentrations of 1 residues and of pepsin in these fractions were calculated in either of two ways. First, assuming that all absorption at 365 nm was due to absorption by residues of 1, the ε_{365} for the glycol of 1 was used to find the concentration of residues of 1. The concentration of pepsin, in turn, was determined by noting the A_{280} and using the ε_{280} for the glycol of 1 and an ε_{280} of 4.6 x 10^4 cm^{-1} M^{-1} (18) for pepsin in 0.005 M acetic acid. Alternatively, the A_{251} and A_{280} values of the fractions were noted and the concentrations of residues of 1 and pepsin were calculated by using the ε_{251} and the ε_{280} for the glycol of 1 and an ε_{251} of 1.58 x 10^4 cm^{-1} M^{-1} and an ε_{280} of 4.6 x 10^4 cm^{-1} M^{-1} for pepsin.

Reaction of 2 with pepsin. Pepsin solutions were prepared by diluting oxidized or nonoxidized pepsin samples in water with equal volumes of 0.2 M citrate buffer of pH 2.5, 2.8, 3.4, or 5.2. Ten molar equivalents of a solution of ^{14}C-2 in methanol (22.7 mM, 0.6 mCi/mmole) was added to aliquots of pepsin in 0.1 M citrate buffer. Control reactions contained similar volumes of methanol but no 2. The reactions were incubated for 20-24 h at 37°C in the dark, then assayed for enzyme activity, diluted with citrate buffer of the appropriate pH, and dialyzed in the dark at 5°C against two portions of citrate buffer. Each sample was treated with an equal volume of 2.0 M hydroxylamine, pH 9.0, overnight in the dark at room temperature, and then was dialyzed overnight in the dark at 5°C against water.

RESULTS AND DISCUSSION

Figure 1 shows the loss in activity of pepsin and the incorporation of residues of 1 into pepsin over time for a typical run. Similar results were obtained when the reaction was run in 0.05 M acetic acid rather than citrate buffer. The binding characteristics are analogous to those found by Tang (18) for EPNP in that essentially two moles of inhibitor become bound per mole of

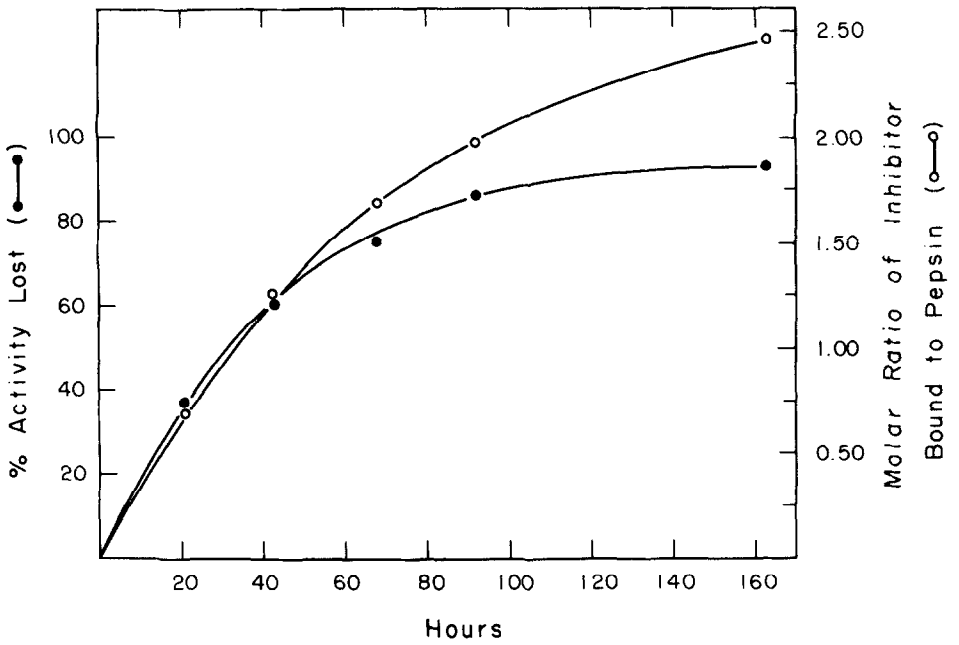

Figure 1. Reaction of 1 with pepsin.

inactivated pepsin at room temperature. Hartsuck and Tang (21) went on to show that, although EPNP modified both the catalytically essential asp-32 and the nonessential met-290 at room temperature (22), it became specific for asp-32 at lower temperatures and shorter incubation times. Since the reaction of 1 with pepsin required more than 10 days for the enzyme to lose half its activity at 0-2°C, we did not pursue specificity studies under these conditions.

The phenacyl bromide 2 reacts readily with porcine pepsin. The results of the reaction of ten molar equivalents of 2 with oxidized and non-oxidized pepsin are shown in Table 1. When the experiment was run at pH 2.8 with purified pepsin (nonoxidized), similar results were obtained. Incubation of purified pepsin at pH 2.8 with only five molar equivalents of 2 lowered the incorporation of 2 into enzyme to a 1.1 molar ratio.

Erlanger's work (23) indicated that p-bromophenacyl bromide inactivated porcine pepsin by binding specifically to an aspartate residue, but further work by Tarasova et al. (24) suggested that the modification was actually that of met-290. Tarasova et al. also found that α-bromo-4-amino-3-nitroaceto-

Table 1. Incorporation of 2 into oxidized and nonoxidized pepsin.

Sample	% Enzyme activity remaining	Molar ratio 2 bound to pepsin	% Bound 2 cleaved by NH_2OH
Oxidized pepsin			
pH 2.5	62	0.32	72
pH 2.8	73	0.30	77
pH 3.4	77	0.34	76
pH 5.2	87	0.19	88
Nonoxidized pepsin			
pH 2.5	30	1.19	52
pH 2.8	28	1.26	61
pH 3.4	36	1.31	62
pH 5.2	46	0.94	53

phenone modified only met-290 in pepsin at pH's below 3. The reaction became less specific at higher pH's until up to two molar equivalents of the inhibitor could be bound to pepsin at pH 5.2, with the second mole of inhibitor modifying carboxy groups. Here native pepsin likewise binds 1-1.3 residues of 2 at low pH but does not bind higher quantities at pH 5.2. Oxidized pepsin, in which 2.5 residues of methionine are converted to methionine sulfoxide (20), binds only 0.2-0.3 molar equivalents of 2 suggesting that binding of 2 to native pepsin, even at high 2 to pepsin ratios, high temperatures, and long incubation times, is largely at met-290. Finally, treatment with hydroxylamine of native pepsin modified by 2 releases only about 50-60% of the radioactivity, a result which is reasonable for an alkylated methionine (18,22) but not for an esterified aspartic acid residue where cleavage under these conditions should approach 100% (13). Interestingly, the small amounts of 2 bound by oxidized pepsin are cleaved in high yield (70-90%) by hydroxylamine, a result which is compatible with a high proportion of the residual binding being to carboxy groups.

Our results indicate that 1 and 2 modify porcine pepsin in much the same way that their nonphotoaffinity analogs do, while our earlier work showed that 3 reacts with the catalytically essential asp-215 of pepsin as do other diazo carbonyl reagents. Reagent 1, reactive with at least two amino acid residues

in porcine pepsin, may show greater specificity for the asp-32 equivalent in certain carboxyl proteases; alternatively, 1 may be useful only in studies where the specificity of the original binding of the inhibitor is not a concern in the ultimate photoaffinity work. Because the reaction of 2 appears specific for met-290, its use will be limited to those carboxyl proteases, such as porcine and human (25) pepsins, which have a met-290 equivalent. Reagent 3 should be useful with any carboxyl protease since all members of this enzyme family contain an asp-215 equivalent.

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