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Structural and Conformational Properties of Peptides Interacting with the Glutathione Receptor of Hydra

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SUMMARY

We have made a detailed investigation of structure-activity relationships of the glutathione-induced feeding response in the fresh water coelenterate, *Hydra attenuata*, to map structural and conformational properties of feeding response agonists. We find that the γ-glutamyl residue of glutathione contains essential binding sites for receptor interaction, without which antagonistic as well as agonistic properties are lost. Any structural alteration which perturbs either the α-amino or the α-carboxyl group or their relative spatial orientations within the peptide has yielded an inactive derivative. An absolute requirement for activation of the receptor is a second-residue side chain of the appropriate size; analogues with second-residue side chains too large or too small are inhibitory. On the basis of the activity of conformational analogues of glutathione, torsional angles for the second residue equal to those of a right-handed α-helix are compatible with stimulus generation.

INTRODUCTION

A number of small peptides, amino acids, and related compounds such as dopamine, glutamate, glycine and GABA[6] are known mammalian neurotransmitters (1–3). Similarly, a number of low-molecular weight peptides and amino acids including tyrosine, glutamate and glutathione (GSH) serve as chemical activators of olfaction and gustation in primitive animals (4–8). There are structural and possible evolutionary relationships between receptors for these substances in lower organisms and those for mammalian neurotransmitters.

GSH, in addition to its intracellular roles (9, 10), acts as a chemoexcitant of olfactory and feeding responses in a number of lower animals (5–7), including hagfish, ticks, sea anemone, and hydra. In the simple fresh-water coelenterate hydra, GSH is a natural activator of feeding behaviors (8, 11). Previous studies concerning the specificity of the feeding response for GSH [Glu(Cys-Gly)] in *Hydra littoralis* established the following: (a) analogues with the cysteine sulfhydryl group substituted or replaced, such as *S*-methylglutathione [Glu(Cys(Me)-Gly)] (12) and ophthalmic acid [Glu(Abu-Gly)] (13) are excellent feeding activators; (b) asparthione, a GSH analogue in which aspartic acid, not glutamic acid, is the NH_2-terminal residue (thereby shortening the tripeptide backbone by one carbon atom) is not an activator but an inhibitor of feeding (8, 12); and (c) free glutamate itself is an excellent inhibitor of the feeding response, whereas aspartate is not (12). These studies indicate that the NH_2-terminal γ-glutamyl residue is essential for stimulating feeding behavior, whereas none of the properties of the sulfhydryl group of the cysteine residue are required for biological activity. Described herein is a detailed investigation of structure-activity relationships of the GSH-induced feeding response in a closely related species of fresh-water hydra, *H. attenuata*, in an effort to map structural and conformational properties of feeding response agonists.

MATERIALS AND METHODS

Materials

Norvaline, D-glutamic acid, GABA, glutaric acid, DL-*N*-methylglutamic acid, pyroglutamic acid, GSH, and GSSG were obtained from Sigma Chemical Company (St. Louis, Mo.). Isoglutamine was obtained from

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[6] The abbreviations used are: GABA, γ-aminobutyric acid; GSH, γ-glutamylcysteinylglycine, glutathione; Nva, norvaline; GSSG, oxidized glutathione; Boc, *t*-butyloxycarbonyl; Cha, cyclohexylalanine; Aib, aminoisobutyric acid; TLC, thin-layer chromatography; Phe(*p*Cl), *p*-chloro-phenylalanine; Sar, sarcosine; Cle, cycloleucine; Abu, 2-aminobutyric acid; E_m, maximal response time; C_{50}, peptide concentration which gives a half-maximal response; I_{50}, analogue concentration which gives half-maximal inhibition.

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Bachem (Torrance, Calif.). Merrifield resins were obtained from Bio-Rad Laboratories (Richmond, Calif.) and Pierce Chemical Company (Rockford, Ill.), and benzhydrylamine resin was obtained from Schwarz/Mann (Orangeburg, N. Y.). Available Boc amino acids and derivatives were purchased from Bachem, Vega-Fox Biochemicals (Tucson, Ariz.) or Peninsula Laboratories (San Carlos, Calif.). Boc-cyclohexylalanine, prepared by hydrogenation of Boc-phenylalanine, was the gift of Dr. Everett Flanigan, Revlon Health Care (Tuckahoe, N. Y.), and Boc-aminoisobutyric acid was prepared by a modification of the Schnabel method as previously described (14). Dimethylformamide was distilled from KOH (15).

Analytical Methods

TLC was performed on 0.25-mm silica gel G plates (Analtech, Newark, Del.) in the solvent systems defined in Table 1. Chromatograms were developed by ninhydrin and Clorox-starch sprays (15). Peptides were hydrolyzed for 2–4 hr with 12 N HCl-propionic acid (1:1) (16). Amino acid analyses were performed on a Beckman Model 120C analyzer. Cysteine was determined as cysteic acid (17). Melting points were determined with a capillary melting-point apparatus. Optical rotations were measured with a Cary 60 spectrometer. Elemental analyses were performed by PCR, Inc. (Gainesville, Fla.).

Trifluoroacetyl-DL-Phe(pCl). DL-Phe(*p*Cl) (40 g) was dissolved in 130 ml of trifluoracetic acid. Trifluoroacetic anhydride (50.66 g) was added over 10 min at −10°. After 1 hr the solution was evaporated and dissolved in 400 ml of ethyl acetate. Addition of 350 ml of petroleum ether induced crystallization of 50.7 g (86%) with a melting point of 150–151°. The presence of the trifluoroacetyl group was confirmed by the appearance of bands at 1730 and 1100 cm^{-1} in the IR spectrum. The derivative gave a single ninhydrin-negative, Clorox-starch-positive, and UV-positive spot by TLC with R_FC = 0.82 compared with free amino acid with R_FC = 0.74. Expected elemental analysis:

Calculated: C 44.68, H 3.07, N 4.74
Found C 44.51, H 2.90, N 4.71

L-Phe(pCl). Trifluoroacetyl-DL-Phe(*p*Cl) (50.32 g) was dissolved in 1450 ml of water maintained at pH 7.2 with NaOH in a Radiometer pHStat. Digestion with 1500 units (30 mg) of diisopropylfluorophosphate-treated carboxypeptidase A (18, 19) proceeded at 37° for 48 hr. A portion (700 ml) of the solution was adjusted to pH 3 and filtered, and 500 ml of ethyl acetate were added. Material recovered from the aqueous phase by recrystallization from a hot water-ethanol mixture weighed 7.22 g (88%) and contained one ninhydrin-, Clorox-starch-, UV-positive component by TLC, R_FC = 0.57, R_FB = 0.65. The melting point was 210° with decomposition. The putative L-Phe(*p*Cl) was characterized by an optical rotation of $[\alpha]_{23}^{D} = -23.0 \pm 0.5°$, c = 0.52, H_2O, in excellent agreement with the literature value of −23 ± 1°, c = 0.5, H_2O. Expected elemental analysis for $C_{18}H_{22}N_2O_5Cl_2$ [Phe(pCl)·½ H_2O] × 2:

Calculated: C 51.79, H 5.27, N 6.72
Found: C 51.37, H 5.03, N 6.56

The acetate phase yielded 3.95 g (66%) of trifluoroacetyl-D-Phe(*p*Cl), which had a melting point of 146–147° and appeared as one ninhydrin-negative, UV-positive, Clorox-starch-positive spot by TLC. Expected elemental analysis:

Calculated: C 44.68, H 3.07, N 4.74
Found: C 44.21, H 2.81, N 4.60

Boc-L-Phe(pCl). *N,N,N′,N′*-tetramethylguanidine (4.6 g) and Boc-azide (3.0 g) were added to 5.0 g of L-Phe(*p*Cl) in 50 ml of dimethyl sulfoxide. Twenty-four hours later 1 extra Eq of Boc-azide was added. After adding 22 ml of water and 18 ml of ether, the aqueous phase (pH 7–8) was washed with 5–10 ml of ether to remove unreacted azide. Following acidification to pH 3 with concentrated $NaHSO_4$, the solution was extracted three times with 60-ml portions of ethyl acetate/ether (1:1). The ethyl acetate phase was dried with anhydrous Na_2SO_4, filtered, and evaporated. The Boc-amino acid was recrystallized from ethyl acetate and petroleum ether yielding 5.34 g (89%) with a melting point of 94.5–95°. TLC revealed one spot, ninhydrin-positive, only after prolonged heating; R_FC = 0.64 compared with free amino acid (R_FC = 0.53). Expected elemental analysis for $C_{16}H_{14}NO_5ClS$ [Boc-Phe(*p*Cl)·dimethyl sulfoxide; stoichiometric ratio of dimethyl sulfoxide confirmed by NMR, see also ref. 18]

Calculated: C 50.83, H 3.80, N 6.40
Found: C 51.27, H 3.83, N 6.47

Boc-GABA. Tetramethylguanidine (5.75 g) and *t*-butylphenylcarbonate (9.7 g) were added to 5.15 g of GABA in 150 ml of dimethyl sulfoxide, according to the method of Ragnarsson *et al.* (20). After overnight reaction, Boc-GABA was isolated and recrystallized as described for Boc-Phe(*p*Cl), yielding 4.56 g (46%), melting range 55–58°; it was homogeneous in two TLC systems: R_FC = 0.82, R_FH = 0.09.

Calculated: C 53.18, H 8.43, N 6.89
Found: C 53.06, H 8.64, N 6.83

Peptide Syntheses

Peptides were synthesized by the Merrifield solid-phase technique (21, 22) with the modifications described by Hancock *et al.* (23). Boc-amino acid polymers were prepared by the cesium salt method (24), by the Merrifield method (15), or by reaction of the polymer with the appropriate Boc-amino acid and an equimolar amount of Na_2CO_3 in a 1:1 mixture of dimethylformamide and dimethyl sulfoxide for 24 hr at 70°. The substitution range was 0.39–0.80 mmole/g. Peptide amides were synthesized using benzhydrylamine polymer (0.51 mEq/g) (25). The following protecting groups were used: side-chain carboxyl, benzyl ester; serine hydroxyl, benzyl ether; sulfhydryl, *t*-butyl thiol; amino, Boc. In some syntheses *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was the coupling agent with coupling times extended to 12 hr (26). Completeness of coupling was monitored with ninhydrin (27). Peptides were cleaved from the resin by anhydrous HF with a 20–100 times molar excess of anisole at 0° for 50 min (28), and extracted with 1% acetic acid. Purification was effected by Bio-Gel P-2 chromatography in 0.1 N acetic acid, 0.1 N or 1 N ammonium bicarbonate, and/or by countercurrent distribution in 1-

butanol/acetic acid/water (4:1:5) or 1-butanol/pyridine/acetic acid/water (9:3:1:12). The amino acid analyses, TLC mobilities, optical rotations, and yields of peptides are presented in Table 1. Peptides ranged from 60% to 80% peptide by weight.

Conformational Analogues

An indirect approach to the elucidation of the receptor-bound conformation of a peptide is to generate peptide analogues with restricted, partially defined conformations. Their biological activities may be used as an index of how closely the introduced conformational constraints approximate the binding constraints imposed by the receptor. Marshall (29), Marshall and Bosshard (30), and subsequently others (31) have shown from theoretical studies on the allowed torsional angles of model peptides that the replacement of the α-proton of an amino acid residue with a methyl group reduces the conformational space available to the peptide backbone at that residue; that is, the steric hindrance introduced by the methyl group prevents that residue from attaining certain conformations that can be achieved by the peptide backbone containing an unmodified amino acid. Similarly, replacement of the amide proton of an amino acid residue with a methyl group reduces the conformational space available to the peptide backbone not only at that residue but also at the preceding one. The conformational restrictions predicted by these theoretical studies have been substantiated by comparison with experimentally determined data from protein and peptide crystals (See especially refs. 32 and 33).

Peptide hormone analogues containing α-methyl or *N*-methylamino acids should correspond closely to conformational analogues of the hormone, i.e., analogues which have a primary structure essentially identical with that of the native hormone but which are capable of existing only in a more restricted set of conformations (34). If analogues with conformational constraints are active, then clearly the conformational constraints must be compatible with binding restrictions imposed by the receptor as well as with requirements for stimulus generation. On the other hand, if such analogues are competitive inhib-

TABLE 1

Physical characteristics of synthetic GSH analogues

The amino acid ratios are listed in order of their sequence in the peptide. The R_F values are listed with solvent systems defined as follows: A = 1-butanol/acetic acid/water (1:1:1); B = 1-butanol/acetic acid/water (4:1:1); C = 1-butanol/pyridine/acetic acid/water (15:10:3:12); D = 1 butanol/pyridine/acetic acid/water (3:1:1:3); E = pyridine/water (65:35); F = isopropanol/acetic acid/water (4:1:1); G = 1-butanol/pyridine/water (1:1:1); H = chloroform/methanol/acetic acid (85:10:5). Cyclohexylalanine and Phe(*p*Cl) emerged after the acidic and neutral amino acids on the short column, both with constants equal to phenylalanine. Sarcosine eluted before glutamic acid with a constant 8% of glutamic acid. Cycloleucine and norvaline co-chromatographed with leucine with respective constants 32% and 106% of glycine. Aminoisobutyric acid, with a color constant about 10% of glycine was retained 10 min longer than alanine and appeared before 2-aminobutyric acid; the 2-aminobutyric acid color constant was 98% of glutamic acid.

Analogue	Amino acid analysis	(TLC R_F)	$[\alpha]_{23}^{D}(c)$	% Yield
Glu-Cys-Gly[a]	1.00, 0.90, 1.04	A = 0.50, B = 0.10, C = 0.08, D = 0.18	−85.8 (0.62)	90
Glu-Abu-Gly	1.00, 1.01, 0.99	A = 0.61, B = 0.27, C = 0.33, D = 0.47	−5.9 (1.01)	57
Asp(Abu-Gly)	1.02, 1.01, 0.95	A = 0.12, B = 0.42, C = 0.29, E = 0.68	−15.1 (1.17)	62
GABA(Abu-Gly)[b]	—, 1.04, 1.00	A = 0.60, B = 0.17, C = 0.15	−48.1 (1.33)	53
AcGlu(Abu-Gly)	0.97, 1.02, 1.00	A = 0.36, B = 0.30, C = 0.28, G = 0.72	41.5 (1.03)	67
D-Glu(Abu-Gly)	0.96, 1.03, 1.00	A = 0.66, B = 0.15, C = 0.12	−64.6 (0.70)	70
Glu(Gly-Gly)	1.00, 1.06, 1.06	A = 0.44, B = 0.11, C = 0.12, D = 0.23	+2.4 (0.93)	94
Glu(Ala-Gly)	1.00, 1.00, 1.04	A = 0.47, B = 0.13, C = 0.18, D = 0.25	−33.3 (0.80)	51
Glu(Aib-Gly)	1.00, 0.91, 1.01	A = 0.11, B = 0.40, C = 0.29, E = 0.63	+7.5 (1.45)	63
Glu(Ser-Gly)	1.01, 0.95, 1.00	A = 0.38, B = 0.07, C = 0.26, E = 0.75	−12.2 (1.03)	51
Glu(Abu-Gly)	1.00, 0.97, 1.03	A = 0.52, B = 0.18, C = 0.20, D = 0.28	−29.9 (0.73)	74
Glu(Val-Gly)	1.08, 1.00, 0.97	A = 0.63, B = 0.27, C = 0.35, E = 0.72	−23.6 (0.75)	46
Glu(Nva-Gly)	1.02, 0.96, 1.00	A = 0.63, B = 0.27, C = 0.43, D = 0.47	−21.0 (0.86)	74
Glu(Leu-Gly)	1.05, 0.97, 1.00	B = 0.08, C = 0.47, F = 0.65	−24.2 (0.66)	52
Glu(Cle-Gly)	1.00, 0.96, 1.06	A = 0.65, B = 0.30, C = 0.42, D = 0.39	+6.0 (0.99)	66
Glu(Pro-Gly)	1.01, 0.98, 1.00	A = 0.40, B = 0.11, C = 0.13, D = 0.23	−69.2 (0.98)	55
Glu(Cha-Gly)	1.00, 0.98, 1.06	A = 0.30, B = 0.20, C = 0.54	−12.9 (0.33)	35
Glu(Phe-Gly)	1.01, 1.00, 0.98	A = 0.70, B = 0.42, C = 0.18	−11.0 (0.45)	44
Glu(Tyr-Gly)	1.05, 1.00, 0.93	A = 0.27, B = 0.27, C = 0.40, G = 0.75	+21.3 (0.88)	80
Glu(Abu-Sar)	1.00, 1.01, 0.85	A = 0.50, B = 0.17, C = 0.22, D = 0.29	−29.8 (0.59)	15
Glu(Abu-Ala)	1.09, 1.00, 0.99	A = 0.60, B = 0.27, C = 0.90, D = 0.47	−49.9 (1.55)	65
Glu(Abu-Aib)	1.00, 1.03, 0.88	A = 0.24, B = 0.30, C = 0.40, G = 0.71		60
Glu(Abu-Pro)	1.00, 0.99, 1.06	A = 0.64, B = 0.31, C = 0.41, D = 0.25	−79.2 (1.02)	74
Glu(Abu-Tyr)	1.02, 1.00, 0.86	A = 0.71, B = 0.43, C = 0.53, D = 0.60	−14.7 (0.78)	50
Glu(Abu-Leu)	1.05, 1.00, 0.99	A = 0.68, B = 0.46, C = 0.48, D = 0.50	−48.0 (0.58)	72
Glu(Abu-Phe(*p*Cl))	1.07, 0.99, 1.00	A = 0.33, B = 0.40, C = 0.57, G = 0.77	−10.5 (0.49)	54
Glu(Abu-Gly-NH_2)	0.99, 1.05, 1.00	A = 0.49, B = 0.14, C = 0.47, D = 0.60	−21.4 (0.78)	60
Glu(Cys-Gly-NH_2)[a]	1.06, 0.96, 1.00	A = 0.29, B = 0.04, C = 0.25, E = 0.75	−86.7 (2.20)	51
Glu(Abu-Gly-Ala)	0.98, 1.00, 1.00, 1.06	A = 0.13, B = 0.15, C = 0.28, G = 0.70	−40.7 (0.98)	54
Glu(Abu-NH_2)	0.93, 1.00	A = 0.14, B = 0.17, C = 0.28, G = 0.63	−10.7 (0.50)	87
Glu(Nva)	1.00, 1.01	A = 0.28, B = 0.32, C = 0.39, G = 0.71	−11.1 (1.80)	43

[a] Oxidized.

[b] Structure confirmed by mass spectroscopy.

itors, then the constraints, although compatible with binding restrictions, do not fit the requirements for stimulus generation.

Biological Assay

Specimens of fasted, asexual *H. attenuata*, reared in mass culture (35), were transferred 1–3 days prior to bioassay into 1 mM histidine buffer (pH 6.2), 1 mM NaCl, and 1 mM $CaCl_2$. The stimulatory activity of GSH and its analogues was quantified essentially by the method of Lenhoff (11). Several parameters were evaluated to quantify the feeding response, including initial time of mouth opening, proportion of animals responding, and response duration. Results obtained with these methods were similar, and the orders of activity with analogues were identical. As previously shown with *H. littoralis* (11), monitoring response duration is the method of choice because it requires fewer animals and gives less variability within any single group of animals than do other methods. The duration of the response of *H. attenuata* to GSH increased with increasing concentrations until a maximum was reached at about 5 μM GSH (Fig. 1). In general the standard deviation of the mean response time for five animals was no greater than 10–15% of the mean. The maximal response (E_m) and the concentration producing the half-maximal response (C_{50}) were determined by a Hanes-Woolf reciprocal transformation used for analysis of enzymes (ref. 36; see also ref. 37, pp. 208–210). Although the data fit the usual format of an enzyme-substrate velocity curve (Fig. 1), these results do not necessarily imply that the response duration is directly proportional to receptor occupancy. During the 3-year course of our studies the C_{50} and the E_m values obtained from GSH assays ranged from 0.3 to 1.5 μM and from 18 to 47 min, respectively. The interexperimental variation of the C_{50} and E_m values reflected differences in the mean response times and were found to be small when experiments were conducted 1–2 days apart. Thus, we have reported the activity of an analogue with that of GSH on the same day for comparison. Peptides active only at 100- to 1000-fold higher concentrations than GSH frequently caused stress and death of hydra. The E_m values obtained with some agonists were lower than those obtained for GSH, perhaps owing to the inability to assay saturating peptide concentrations without stressing animals.

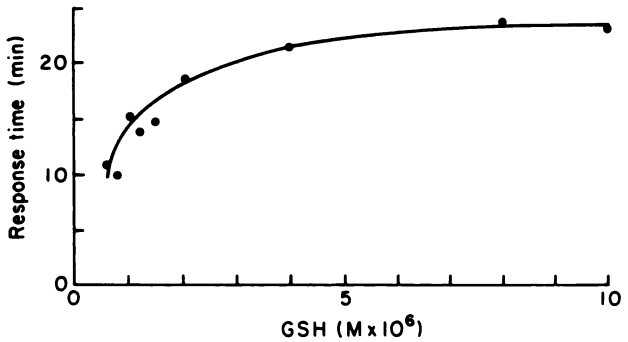

FIG. 1. *Concentration dependence of the GSH-induced feeding response of Hydra attenuata*

Test substances (2 ml) in standard solution (pH 6.1 ± 0.2) were added to 2.5-cm diameter Plexiglas wells surrounded by a closed circulating water system maintained at 28 ± 2° by a Haake Model F constant-temperature unit. The feeding response, monitored at ×20 magnification with a dissection microscope, was assayed by noting the times at which the mouths of five animals opened and closed. Duration of the response, expressed as the average response time for the five animals, gave results similar to those obtained using initial mouth opening time or proportion of animals responding as the assay. Each analogue was tested in at least two series of experiments.

The inhibitory effect of each possible antagonist was determined by measuring its ability to decrease the response time caused by a solution of 2 μM GSH. To determine the concentration of an inhibitor which gave 50% inhibition (I_{50}; see p. 106 of ref. 37), the log of the concentration ratios (inhibitor/GSH) was plotted on the abscissa and the percentage response on the ordinate. A linear plot was obtained using the least-squares method of best fit, and the I_{50} was read from this plot. The I_{50} is not a binding constant for the inhibitor, but it permitted a numerical comparison of inhibitors. The ability of GSH to overcome glutamic acid inhibition at a number of GSH and glutamic acid concentrations was measured. Addition of 10 μM GSH was sufficient to overcome the 50% inhibition by 10 μM glutamic acid of the feeding response induced by 2 μM GSH. Reciprocal plots of inhibitor data indicated that the inhibition was competitive.

RESULTS

Activities of position 3 analogues. All position 3 tripeptide analogues tested were active (Table 2). Supporting the findings of Loomis (8) in *H. littoralis*, GSSG was an excellent feeding inhibitor. Similar to GSSG, oxidized Glu(Cys-Gly-NH_2) also inhibited. Since oxidized peptides were inhibitory and because the —SH group of cysteine was not needed for biological activity, we avoided problems with auto-oxidation by incorporating 2-aminobutyric acid, which is isosteric with cysteine, rather than cysteine into the majority of position 3 analogues. Replacement of glycine by alanine, leucine, tyrosine, phenylalanine, or Phe(*p*Cl) yielded tripeptides that were slightly more active than the parent molecule [Glu(Abu-Gly)]. The tetrapeptide Glu(Abu-Gly-Ala) was active at

TABLE 2

Activities of GSH analogues with position 3 modifications

Assays were as described in the legend to Fig. 1.

Analogue	Activity			
	GSH		Analogue	
	C_{50}	E_m	C_{50}	E_m
	μM	min	μM	min
Glu(Abu-Ala)	0.7	19.4	0.3	12.3
Glu(Abu-Tyr)	1.6	21	2.0	16.5
Glu(Abu-Sar)	0.7	19.4	0.5	12.8
Glu(Abu-Leu)	1.0	22.4	1.9	14.9
Glu(Nva-Phe)	0.5	25	0.9	15.5
Glu(Abu-Phe(*p*Cl))	0.5	25	2.1	13.9
Glu(Abu-Pro)	0.3	15.4	4.5	17.4
Glu(Abu-Gly-Ala)	1.9	53.5	12.0	19.5
Glu(Abu-Gly-NH_2)	0.6	24.4	70.0	17.4
Glu(Abu-Aib)	0.2	14.4	50.0	17.4
Glu(Cys-Gly-NH_2)			$I_{50} = 1 \times 10^{-4}$ M	

concentrations lower than that of the tripeptide amide (C_{50} = 10 times that of GSH).

Activities of position 2 analogues. Glu(Gly-Gly), with the cysteine side chain replaced by a proton, was a weakly inhibitory peptide (Table 3). Incorporation of amino acids with large side chains as the middle residue, such as phenylalanine and tyrosine (both of which are γ-branched), also generated antagonists. The peptide with cysteine replaced by alanine was an agonist with a C_{50} 30 times that of GSH. The isosteric replacement of 2-aminobutyric acid for cysteine yielded an analogue with activity over the same concentration range as GSH. Making another nearly isosteric replacement of serine for cysteine, which replaced sulfur with oxygen at this residue, resulted in a peptide with maximal activity at 50 times the GSH concentration, similar to the characteristics of Glu(Ala-Gly). Incorporation of leucine, containing a γ-branch, as the second residue produced a feeding activator with a C_{50} 6 times greater than GSH. *S*-Methylglutathione and its isosteric analogue, Glu(Nva-Gly), as well as Glu(Val-Gly), with a β-branched second residue, activated over the same range as GSH. The β-branch in Glu(Val-Gly) did not increase the C_{50} as did the γ-branch found in Glu(Leu-Gly). The plot of π (a measure of lipophilicity derived from ethanol/water partition) using the constants of Pliška and Fauchère (38) of the second residue side chain versus peptide activity is given in Fig. 2. Assuming that differences in C_{50} of 50% between analogues are insignificant, there was a good correlation between the lipophilicity of the second residue side chain and apparent affinity indicated by C_{50}, until the side chain contained a γ-branch.

Activities of position 1 analogues. The tripeptide Asp(Abu-Gly), containing a β-aspartyl peptide bond and one less methylene group in its backbone than GSH, was neither active nor inhibitory. Neither Glu-Abu-Gly nor Glu-Cys-Gly, with α-glutamyl rather than γ-glutamyl bonds, nor D-Glu(Abu-Gly) had any antagonistic or agonistic properties. These results clearly demonstrate that the γ-glutamyl linkage is required for activity as previously reported for *H. littoralis*. Analogues without a free α-carboxyl [GABA(Abu-Gly)] or α-amino group [AcGlu(Abu-Gly)] were inactive. Incorporation of non-inhibitory amino acids at position 1 resulted in inactive peptides. Since inhibition by an amino acid paralleled activity of a peptide containing that amino acid in position 1, amino acids were screened for inhibition as described in detail elsewhere.[7]

Activities of dipeptides and constituent amino acids. Shortening the tripeptide by omitting the third residue greatly decreased activity and in almost all cases produced inhibitors. Neither cysteine nor glycine alone inhibited the response, nor did the combination of glutamic acid + cysteine + glycine activate.

Inhibitory activities of glutamic acid analogues. Neither *N*-methyl-DL-glutamic acid nor pyrrolidone carboxylic acid nor glutaric acid, all of which have modified NH_2-terminals, was inhibitory. β-Aminoglutaric acid, with the amino group on the β-carbon rather than α-carbon, was also not an inhibitor. Isoglutamine, having a blocked α-COOH, was a very weak inhibitor with an I_{50} greater than 1 mM, whereas GABA, with no α-COOH,

TABLE 3

Activities of GSH analogues with position 2 modifications

Assays were as described in the legend to Fig. 1.

Analogue	Activity			
	GSH		Analogue	
	C_{50}	E_m	C_{50}	E_m
	μM	min	μM	min
GSH	0.3–1.5	18–47		
GS-Me	0.7	30.0	1.1	31.2
Glu(Nva-Gly)	0.6	21.8	1.3	25.8
Glu(Val-Gly)	0.7	41	1.3	38.9
Glu(Abu-Gly)	1.5	46.7	2.5	24.8
Glu(Leu-Gly)	0.9	18.7	5	13.6
Glu(Ala-Gly)	0.3	17.6	8	12.6
Glu(Ser-Gly)	0.8	25.8	56	17.9
Glu(Aib-Gly)	0.6	21.3	48	11.8
Glu(Cle-Gly)	0.4	20.6	38	13.8
	I_{50} (10^{-5} M)			
GSSG	5			
Glu(Cha-Gly)	5			
Glu(Tyr-Gly)	5			
Glu(Phe-Gly)	16			
Glu(Gly-Gly)	100			
Glu(Pro-Gly)	Lethal			

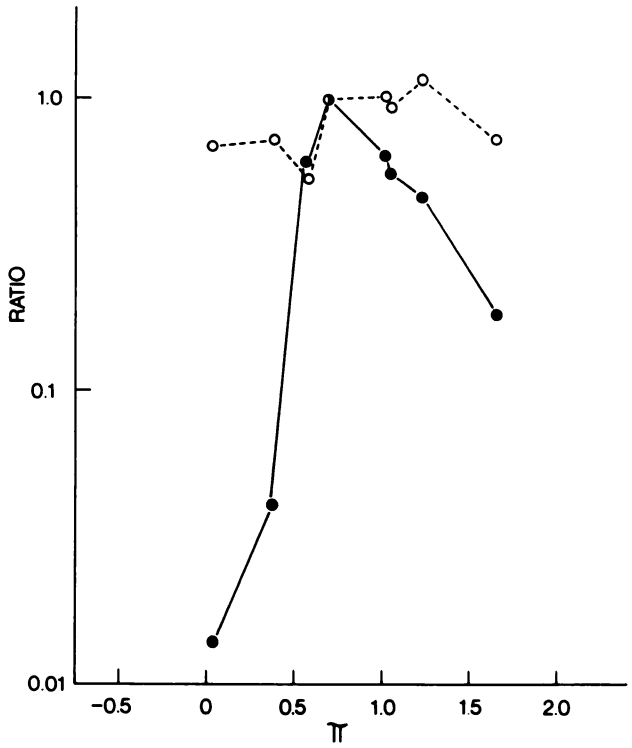

FIG. 2. *Effect of lipophilicity of the second-residue side chain on analogue E_m and C_{50}*

The lipophilicity of the second-residue side chain is plotted versus: ●, the ratio C_{50} GSH/C_{50} analogue; ○, the ratio E_m analog/E_m GSH. The π values, taken from ref. 38, are as follows: cysteine, 0.69; alanine, 0.38; 2-aminobutyric acid, 0.58; serine, 0.04; valine, 1.06; norvaline, 1.24; leucine 1.66; CysMe, 1.03.

[7] M. H. Cobb, W. Heagy, J. Danner, H. M. Lenhoff, and G. R. Marshall, manuscript in preparation.

was inactive. Norvaline, which contains the same carbon backbone as glutamic acid (with the γ-COOH replaced by a methyl group), did not inhibit. Glutamine, containing a γ-carboxamide, was a very weak inhibitor, like isoglutamine, with an I_{50} greater than 1 mM.

Activities of conformational analogues. Replacement of both glycine protons with methyl groups in Glu(Abu-Aib) resulted in a peptide producing an E_m up to 20% longer with a C_{50} 250 times that of GSH. *N*-Methylglycine in position 3 yielded a peptide [Glu(Abu-Sar)] reaching maximal activity near the same concentration as GSH. Proline, cyclic as well as *N*-methylated, in position 3 resulted in an analogue with a C_{50} 10 times that of GSH. The Pro-3 and Aib-3 peptides were the only position 3 analogues that gave a response duration equal to or greater than that of GSH (Table 2).

A peptide with the second residue α-proton exchanged for a methyl group, Glu(Aib-Gly), was agonistic (Table 3). The C_{50} was near that of the parent compound Glu(Ala-Gly), suggesting that the α-methyl substitution was well tolerated. This contention is supported by the activity of γ-glutamyl-cyclo-leucyl-glycine [Glu(Cle-Gly)], having a cyclic α-methyl amino acid at position 2.

Incorporation of proline into position 2 produced a peptide lethal to *H. attenuata* at 0.1 mM. This effect was observed with two different peptide preparations. Glu(Pro-Gly)(20 μM) caused a 50% reduction in the GSH response duration independent of incubation time. However, the inhibition was not competitive (data not shown); it could not be overcome by increasing the GSH concentration. The addition of 0.1 mM GSH to the medium containing 20 μM Glu(Pro-Gly) did not protect hydra against the lethal effects of the analogue. Although the mechanism of this peptide's action is not understood, it is not lethal to several other GSH-sensitive coelenterates.[8]

DISCUSSION

In this study we have analyzed structural and conformational features of GSH, thereby elucidating certain requirements for biological activity of compounds that elicit a feeding response in *H. attenuata*. The activation and inhibition constants derived from these measurements were consistent with direct measurements of binding in preliminary experiments.[9] Thus analysis of structure-activity data most likely describes binding characteristics of GSH receptors.

Modification of the side chain of the COOH-terminal residue of GSH causes no reduction in activity and, in fact, a bulky third-residue side chain enhances activity. Both a tripeptide amide and a tetrapeptide, which retain the third residue carbonyl group, are active, whereas dipeptides are generally inhibitory, suggesting that the presence of the third residue carbonyl contributes to efficacy. The free carboxyl-terminal may enhance binding, since the tripeptide amides are required in higher concentrations to achieve the same biological effect relative to analogues containing free carboxyl groups.

The bioassay results of position 2 analogues are consistent with previous findings in the closely related hydra species of *H. littoralis*: cysteine may be replaced without any reduction in agonistic potency, and analogues with bulky side chains at this residue (such as oxidized glutathione) lose intrinsic activity and become inhibitors. A γ-branch in the position 2 side chain found in leucine, as well as in phenylalanine and tyrosine (antagonists), probably interferes with binding in an active orientation. Support for this is derived from the greater activity of the Nva-2 analogue, having an unbranched *n*-propyl side chain of the same length as the leucine isobutyl side chain. Elimination of the second residue side chain as in Glu(Gly-Gly) converts the tripeptide to an antagonist. A plausible explanation is that a hydrophobic cavity in the receptor must be occupied by the second-residue side chain to induce feeding behavior. The weakness of inhibition produced by Glu(Gly-Gly) might be accounted for in one of two ways. (a) Not only an activating requirement but also a binding contact resides in the position 2 side chain, although 100-fold more potent inhibition by glutamic acid makes this questionable, unless we propose that amino acid inhibitors and tripeptides bind differently to the receptor site. (b) Glycine allows much more flexibility in the backbone. Since the population of Glu(Gly-Gly) conformers would be different from the conformer population of GSH, the preferred conformation for receptor interaction might comprise a smaller fraction of the Glu(Gly-Gly) conformer population. The concentration of the preferred conformer would be less than the actual peptide concentration, resulting in reduced activity.

The activity of Glu(Ala-Gly) demonstrates that the alanine side chain retains minimal activating requirements not present in Glu(Gly-Gly). Although Glu(Abu-Gly) activates near the same concentrations as GSH, Glu(Ala-Gly) and Glu(Ser-Gly) require elevated concentrations, suggesting that lipophilicity of the second-residue side chain enhances binding. The methyl side chain of alanine is shorter than the ethyl side chain of 2-aminobutyric acid, whereas the hydroxymethyl side chain of serine is much less lipophilic than the isosteric carbon-containing 2-aminobutyric acid or the sulfur-containing cysteine side chains. A plot of side-chain lipophilicity versus the ratio of GSH C_{50} to analogue C_{50} using the π constants of Pliška and Fauchère (38) bears out this interpretation. For non-γ-branched residues, increasing lipophilicity parallels increased activity.

The activities of several tripeptide analogues with modified γ-glutamyl residues substantiate the conclusion that a γ-glutamyl residue is mandatory for an active peptide. Modification of the α-COOH or the α-NH_2 groups of this residue eliminates activity. In fact, peptides not containing both groups are completely ineffective as inhibitors, clearly indicating that these peptides do not bind to the receptors. The peptide D-Glu(Abu-Gly), in which these groups are switched, is neither an agonist nor an antagonist, demonstrating that there must be a minimum of three points of attachment of the tripeptide to the receptor. Peptides without the γ-glutamyl peptide bond are also neither active nor inhibitory, indicating that the exact alignment as well as the presence of the two charged groups in the HN_2-terminal residue is required for binding. This result differs from that obtained with the closely related species *H. littoralis*, in which

[8] W. Heagy, unpublished observations.

[9] M. H. Cobb, W. Heagy, and J. Danner, unpublished observations.

asparthione [Asp(Cys-Gly)] acts as an inhibitor (8, 12). The additional alteration introduced at the second residue (2-aminobutyric acid for cysteine) might interfere with asparthione binding; alternatively, a species difference in GSH receptor specificity may exist. Since modification of the γ-glutamyl residue eliminates binding to the receptor, it has not been possible to assess which, if any, features of this residue contribute to peptide efficacy.

We conclude that (a) the γ-glutamyl residue of GSH contains fundamental receptor-binding elements, without which activity of any kind is lost; (b) one absolute requirement to activate the receptor is a second-residue side chain of the appropriate size; and (c) the presence of the third residue, especially one with a free carboxyl-terminal, substantially enhances activity.

On the basis of the activities of tripeptide conformational analogues of GSH we can deduce certain features of the conformation of GSH bound to the hydra receptor that is able to elicit a feeding response. Conformational constraint of the backbone around the third residue is well tolerated. Activity of analogues with both aminoisobutyric acid and proline in position 3 suggests that the ϕ and ψ angles (see ref. 39) of the third residue are equal to values for a right-handed α-helix ($\phi = -60°$, $\psi = -50°$), although conformational constraint caused by an α-methyl substitution is less severe at the carboxyl-terminal residue than in an internal residue (34). Nevertheless, because of the cyclic constraint, the ϕ angle of the third residue of Glu(Abu-Pro) must be fixed between $-60°$ and $-80°$ (40). This angle must be compatible with requirements for binding to the hydra GSH receptor and intrinsic activity, since this peptide is active. Both the Pro-3 and Aib-3 analogues cause a slightly increased duration of mouth opening relative to ophthalmic acid and perhaps even GSH. Previous reports indicate that degradation of GSH is not one of the factors responsible for response termination (11). However, we have found that, after a 1-hr incubation of a radioactive GSH analogue with hydra, at least two new components appear (41). These could be formed by the action of a carboxypeptidase or by γ-glutamyl transferase and dipeptidase, activities that have been measured in hydra (42–44). A carboxypeptidase-like enzyme might play a role in terminating the response, since the two position 3 analogues with apparently increased duration of action are the only ones that might be resistant to that kind of enzymatic degradation.

The activity, although reduced, of the peptide containing aminoisobutyric acid for cysteine at the second residue suggests that the dihedral angles, ϕ and ψ, for the second residue may be near values for a right-handed or a left-handed α-helix ($\phi = -60°$, $\psi = -50°$, $\phi = 60°$, $\psi = 50°$). The high activity of the Pro-3 analogue restricts the second-residue torsional angles to those of a right handed α-helix. These angles are the only ones compatible for a residue preceding proline (32, 34).

The response of hydra to GSH may represent a primitive level of neurotransmitter action (45). The GSH receptor of hydra controls a behavioral response and may be similar to internal hormone receptor and neurotransmitter binding sites such as the glutamate receptor of mammalian brain (1). If the hydra receptors do, in fact, represent one of the earliest binding sites for peptides, studies such as the one described here may prove to be useful models for studying the mechanism by which amino acids, peptides, and related compounds function as neurotransmitters or hormones in higher animals.

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