J. exp. Biol. (1982), 101, 287–293
With 1 figure
Printed in Great Britain

AZASERINE AFFINITY LABELLING OF γ-GLUTAMYL TRANSFERASE OF *HYDRA ATTENUATA* WITHOUT INACTIVATION OF THE GLUTATHIONE RECEPTOR

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(*Received* 19 *April* 1982 – *Accepted* 6 *May* 1982)

SUMMARY

Intrigued by similar specificities of the hydra feeding receptor and γ-glutamyl transferase activity toward GSH, we examined the possibility that these two GSH-binding activities might reside in the same protein. We find that the two activities differ in specificity toward the γ-glutamyl moiety of GSH. The hydra transferase recognizes L-azaserine, L-Glu, D-Glu and L-Gln. The feeding receptor recognizes only L-Glu and L-Gln; L-azaserine and D-Glu have no effect. L-azaserine, known to bind covalently to the γ-glutamyl donor site of mammalian transferase, irreversibly inactivates hydra transferase activity. The transferase affinity label, however, has no effect on the GSH-stimulated feeding response, permitting us to demonstrate that these two activities have different GSH recognition sites and appear to reside in different proteins.

INTRODUCTION

The ubiquitous tripeptide, reduced glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH), plays a role in a wide variety of intracellular reactions (Meister, 1975; Meister & Tate, 1976). In addition to the intracellular roles of GSH, its release from living cells signals olfactory and feeding responses in lower animals including the snail *Helisoma trivolvis* (Kater & Rowell, 1973), certain ticks (Galun, 1974), seastars (Reimer

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& Reimer, 1974), and many cnidarians, including the Portuguese man-of-war, sea anemones, corals and hydrozoans (see Lenhoff, Heagy & Danner, 1976; Lenhoff & Heagy, 1977).

The hydra GSH-feeding receptor, which has been extensively studied (Lenhoff *et al.* 1976; Lenhoff & Heagy, 1977), recognizes the γ-glutamyl linkage but not the thiol of GSH (Cliffe & Waley, 1958; Lenhoff & Bovaird, 1961). Discovery in hydra of γ-glutamyl transferase (E.C.2.3.2.2), an enzyme with a similar specificity toward the γ-glutamyl linkage and thiol of GSH (see Tate & Meister, 1976; Danner *et al.* 1976, 1978), led Tate & Meister (1976) to suggest that γ-glutamyl transferase functions as the hydra feeding receptor.

Although its *in vivo* functions are not fully understood, γ-glutamyl transferase is thought to catalyse the intracellular degradation of GSH by transferring the γ-glutamyl residue to an acceptor amino acid or peptide (Meister, 1973, 1974). Evidence led to the proposal that the enzyme is membrane-bound (Meister, 1974; Novogrodsky, Tate & Meister, 1976) and functions in the transport of amino acids (Meister, 1973, 1974; Orlowski & Meister, 1970, 1973).

To ascertain if γ-glutamyl transferase and the GSH feeding receptor are the same molecule, previous studies analysed their relative specificities with GSH analogues having glycyl-substitutions (Danner *et al.* 1976, 1978). Relative to GSH, the feeding receptor was activated equally well by analogues with bulky glycyl-substitutions, such as Leu and Tyr, whereas transferase activity was reduced. The transferase, however, is capable of catalysing the transfer of Glu in γ-glutamyl dipeptides which lack a 3-position residue (Tate & Meister, 1974). The decreased activity observed with tripeptides having bulky 3-position residues could, therefore, result from either weaker binding to the enzyme or from steric hindrance at the enzyme acceptor site.

To determine whether the hydra feeding receptor, which has not been isolated, and γ-glutamyl transferase are the same molecule we investigated the effects of L-azaserine (O-diazoacetyl-L-serine), L- and D-Glu, and L-Gln on these two activities in *Hydra attenuata*. L-azaserine, a glutamic acid analogue, is an affinity label which rapidly binds to and inactivates the donor site of mammalian γ-glutamyl transferases (Tate & Meister, 1977).

RESULTS AND DISCUSSION

The effects of L-azaserine on γ-glutamyl transferase activity of *H. attenuata* were investigated by measuring the L-azaserine inhibition of *p*-nitroaniline release from the synthetic substrate γ-glutamyl-*p*-nitroanilide. The enzyme can either hydrolyse γ-glutamyl-*p*-nitroanilide or transfer the glutamyl moiety to an acceptor such as glycyl-glycine (Orlowski & Meister, 1963, 1965; Thompson & Meister, 1976). Compounds which bind to the γ-glutamyl donor site of the transferase compete with γ-glutamyl-*p*-nitroanilide and therefore inhibit the release of *p*-nitroaniline. The effects of L-azaserine on the enzyme catalysed release of *p*-nitroaniline are shown in Table 1. In the presence of the added acceptor glycyl-glycine, 4 mM azaserine inhibited the transferase activity by 34%, whereas 20 mM azaserine inhibited the activity by 85%. On an equimolar basis, L-azaserine was a better inhibitor of transferase activity than L-Glu or two other Glu analogues, D-Glu and L-Gln (Table 1)

Table 1. *Effects of* L-*azaserine,* L-*Glu,* D-*Glu and* L-*Gln on the release of p-nitroaniline*

Amino acid	Concentration (mM)	Percent inhibition
L-Azaserine	4	34
L-Azaserine	20	85
L-Glu	4	26
L-Glu	20	56
L-Gln	4	10
L-Gln	20	50
D-Glu	20	21

γ-Glutamyl transferase activity in *H. attenuata* was assayed as described (Danner *et al.* 1976, 1978; Orlowski & Meister, 1963). Homogenates were prepared from laboratory reared specimens of *H. attenuata* which had not been fed for 1–2 days and then treated with rifampicin, 50 μg/ml in 'M' solution (Lenhoff & Brown, 1970), for an additional 1–2 days. Animals were washed and then homogenized in 10 mM-Tris-HCl, pH 9·0, containing 10 mM-$MgCl_2$. After a 3 min centrifugation at 100 *g*, the supernatant was either dialysed at 4° against homogenization buffer for 18–24 h (S_1) or centrifuged at 12000 *g* for 20 min and the pellet resuspended in buffer (P_2). Reaction mixtures in 1 ml 0·1 M-Tris-HCl, pH 9·0, contained 0·02–0·05 mg hydra protein (Lowry *et al.* 1951), 2·5 mM γ-glutamyl-*p*-nitroanilide, 10 mM-$MgCl_2$ and 20 mM-glycyl-glycine. Following a 20 min incubation at 38 °C, reactions were terminated by adding 0·9 ml of 1 N-acetic acid and 0·1 ml 2 % sodium dodecyl sulphate. Release of *p*-nitroaniline was determined by measuring absorbance at 410 nm; activities were determined after correction for absorbance measurements with both no enzyme and no substrate controls. Each amino acid was tested with at least two separate homogenates. No distinction is made between the data from S_1 and P_2 as no differences were noted between specific or relative activities. For example, specific activity, nmol *p*-nitroaniline/min·mg protein, with 2 S_1 preparations was 93 and 116 and with 3 P_2 preparations was 98, 116 and 106; in the presence of 20 mM-L-Glu an S_1 preparation was 56 % inhibited and a P_2 52 % inhibited.

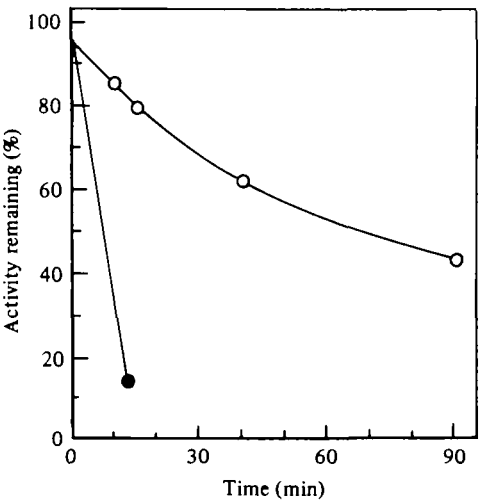

Fig. 1. Inactivation of hydra γ-glutamyl transferase activity by L-azaserine. Transferase activity was assayed as described in Table 1 with the following exceptions. *H. attenuata* were homogenized in 0·1 M-phosphate pH 7·0; an S_1 fraction was obtained by dialyzing the supernatant from a 3 min centrifugation at 1000 *g* against 0·1 M phosphate at 4 °C for 24 h. The 0·15 mg hydra extract protein in 0·1 M phosphate, pH 7, was incubated with L-azaserine at 37 °C. Aliquots of 0·05 ml were withdrawn at intervals up to 90 min and assayed for activity as described for Table 1. At time 0 the specific activity in the absence of azaserine was 53 nmol/min·mg protein. This reduced specific activity relative to that reported in Table 1 (about 50 %) is comparable to that previously observed with phosphate in the reaction mixture (Danner *et al.* 1976, 1978; Orlowski & Meister, 1965). ○—○, 0·2 mM-L-azaserine; ●—●, 20 mM-L-azaserine.

Table 2. *Effects of GSH and glycyl-glycine on the inactivation of hydra transferase activity by* L-*azaserine*

Preincubation period (min)	Additions	Activity (percent)
45	L-azaserine	89
	+GSH	100
	+Gly-gly	48
60	L-azaserine	58
	+GSH	103
	+Gly-gly	46
90	L-azaserine	48
	+GSH	92
	+Gly-gly	43

Transferase activity was assayed as described for Table 1 and Fig. 1. Reaction mixtures were preincubated in the presence of 0·2 mM-L-azaserine and either 0·8 mM-GSH or 0·8 mM-glycyl-glycine as indicated. Activity is referenced to a control without L-azaserine, GSH, or glycyl-glycine.

To determine if L-azaserine irreversibly inhibited the transferase, we incubated the hydra transferase with L-azaserine prior to assay. At 0·2 mM L-azaserine, 50% of the activity was lost after 70 min (Fig. 1). At a 100-fold higher concentration of L-azaserine, 84% of the activity was lost after 15 min (Fig. 1). Thus, L-azaserine both inhibits and inactivates the transferase at concentrations equal to or lower than those required for inhibition by L-Glu. These results are consistent with those of Tate & Meister (1977), who found that L-azaserine covalently bound to the donor site inactivating mammalian kidney transferases. Similarly, as previously reported (Tate & Meister, 1977), the addition of GSH to the incubation mixture prevented the loss of hydra transferase activity; whereas the addition of glycyl-glycine did not (Table 2). Thus, L-azaserine apparently inhibited the hydra transferase by irreversibly inactivating the enzyme donor site.

To investigate the effects of L-azaserine on the feeding receptor we measured the duration of the feeding response in the presence of 2 μM-GSH with and without the addition of L-azaserine. An amino acid which inhibits the response, such as L-Glu (Table 3), decreases the response time elicted by GSH. With 0·2 mM L-Glu almost complete inhibition was observed. In contrast to its effects on transferase activity, L-azaserine did not significantly inhibit the feeding response (Tables 1, 3).

Additionally, we investigated the long term effects of L-azaserine on the feeding response. If the hydra feeding receptor and the transferase activity share a common binding site, then the order of inhibition by L-Glu and Glu analogues should be the same. If the feeding receptor is the same moiety as the enzyme donor site, then as with the enzyme activity, L-azaserine should inhibit and inactivate the feeding response at a concentration lower than L-Glu (see Table 1). We therefore preincubated hydra in 0·2 mM L-azaserine; an equivalent concentration of L-Glu almost totally inhibited the feeding response (Table 3). The duration of the feeding response for animals preincubated in 0·2 mM L-azaserine for 90 min, which does inactivate more than 50% of the transferase activity (Fig. 1, Table 2), was equal to that for untreated controls (Table 3). These results differ from those with the transferase where 20 mM-L-Glu inhibited activity by 56% but only a 15 min incubation in 20 mM-L-azaserine reduced activity to 16% (see above). Thus, L-azaserine which both inhibited and

Table 3. *Effects of* L-*azaserine,* L-*Glu,* D-*Glu and* L-*Gln on the feeding response of* H. attenuata

Additions	Average response time + S.D. (min)
GSH	14.3 + 2.9
+ L-Azaserine	12.3 + 2.4
GSH	18.9 + 2.9
*+ L-Azaserine	19·9 + 3·0
GSH	14·4 + 1·0
+ L-Glu	†2·6
GSH	17·0 + 4·5
+ D-Glu	16·3 + 2·6
GSH	11·3 + 1·0
+ L-Gln	6·8 + 2·3

The response was assayed as described (Lenhoff, 1961*a*, *b*). To quantify the response to GSH we measured the duration of mouth opening of 5 animals in the presence of 2 μM-GSH; the duration of the mouth opening response is dependent upon the concentration of GSH in the external environment (Lenhoff, 1961*b*). Inhibition of the response was determined by measuring the response time of 5 animals in the presence of 2 μM-GSH plus 0·2 mM-amino acid. Since the response time depends on a number of factors (Lenhoff, 1965; Lenhoff *et al.* 1976) inhibition was always determined by comparing the response in the presence of 0·2 mM-amino acid to a control group of animals treated with GSH the same day.

* To determine if L-azaserine would inactivate the GSH receptors of hydra the animals were pre-incubated at 30 °C for 2 h in 'M' solution containing 0·2 mM-L-azaserine, then assayed for their ability to respond to 2 μM-GSH plus 0·2 mM-L-azaserine.

† Only 1 of 5 animals responded; the other 4 showed complete inhibition of the feeding response.

inactivated the transferase activity had no discernible effect, either immediately or long term, on the feeding receptor.

Previous studies showed L-Glu to be a competitive inhibitor of the feeding response (Lenhoff & Bovaird, 1961; Cobb *et al.* 1982). No single amino acid could initiate feeding; activation of the feeding response required tripeptides with the γ-glutamyl residue in the first position (Lenhoff & Bovaird, 1961; Cobb *et al.* 1982). As demonstrated by the ability to inhibit the response, only L-Glu and L-Gln bind to the receptors (Table 3).

In contrast, not only L-Glu and L-Gln, but also L-azaserine and D-Glu inhibited the hydra transferase (Table 1). These data with the added acceptor 20 mM glycylglycine are consistent with an earlier report that L-Glu and L-Gln inhibited hydra transferase activity in the absence of acceptors (Danner *et al.* 1978). The inhibition observed with L-Glu, L-Gln, and L-azaserine is similar to the findings for the hog kidney enzyme with which γ-glutamyl amino acids functioned as donors for transferase activity (Orlowski & Meister, 1965). The observation that D-Glu also inhibited hydra transferase activity is consistent with results from hog (Orlowski & Meister, 1965), sheep (Zelazo & Orlowski, 1976) and rat (Thompson & Meister, 1976) kidney transferases, which were active with D-γ-glutamyl-*p*-nitroanilide.

In summary, we find that L-azaserine inactivates the transferase activity of *H. attenuata* in a concentration- and time-dependent manner, but has no discernible effects on the hydra feeding receptor. Additionally, we find that L-Glu, D-Glu, and L-Gln inhibit the transferase activity, whereas only L-Glu and L-Gln inhibit the feeding response. Thus, we conclude that the transferase activity and the feeding

receptor are different proteins. Our results, however, do not exclude the possibility that γ-glutamyl transferase functions in the degradation of GSH serving to terminate feeding behaviour.

As little is known about the molecular structure of invertebrate receptors to chemoexcitants we found especially intriguing the suggestion that γ-glutamyl transferase, a characterized activity, might be the feeding receptor for the simple metazoan hydra. This study, however, indicates that the receptor and the transferase are not the same. The similar specificity of the two activities toward the γ-glutamyl linkage and the thiol of GSH make it tempting to speculate that the two activities evolved from a common ancestral molecule which served as a feeding receptor and/or in amino acid transport in a primitive animal.

This work was supported by USPHS grants NS 11170 (to GRM) and NS 11171 (to HML).

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