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Biochemical Pharmacology, Vol. 27, pp. 824–826. Pergamon Press, 1978. Printed in Great Britain.

Maleimide II—interaction with L-asparaginase from *Escherichia coli*

(*Received* 8 *February* 1977; *accepted* 8 *June* 1977)

In functional terms, maleimide, like *N*-ethyl maleimide, is a reagent well known for its ability to react covalently with sulfhydryl functions; on structural grounds this compound also bears a strong resemblance to the hypothetical cyclic anhydride of L-asparagine [1–3]. As a consequence of this resemblance, it might be postulated that maleimide could serve as a substrate for the amido hydrolytic action of L-asparaginase (EC 3.5.1.1). By similar reasoning, maleimide might be expected to compete with L-asparagine as well as with other alternate substrates for the active site on the enzyme. To test these hypotheses, we have examined the interaction of maleimide with crystalline L-asparaginase from *Escherichia coli*.

L-Asparaginase from *E. coli* (EC 3.5.1.1; sp. act. 300 I.U./mg of protein) was a gift to the National Cancer Institute from Merck, Sharpe & Dohme, Rahway, NJ, U.S.A. L-Asparaginase inactivated with L-DONV (>90 per cent inactivated) was kindly donated by Dr. Robert L. Handschumacher of the Yale University School of Medicine, New Haven, CT, U.S.A. L-Glutamate-oxaloacetate transaminase (GOT) (EC 2.5.1.1; sp. act. 180 I.U./mg of protein) was purchased from Boehringer-Mannheim, New York, NY, U.S.A. Maleimide, *N*-ethyl maleimide (NEM), maleamic acid, maleic acid, succinamic acid and succinimide were the products of Aldrich Chemical, Cedar Knolls, NH, U.S.A. β-Cyano-L-alanine and α-ketoglutaric acid were procured from CalBiochem, Gaithersburg, MD, U.S.A. L-[U-^{14}C]asparagine (sp. act. 151 μCi/μmole) was the product of Amersham Searle Corp., Silver Spring, MD, U.S.A. Purity was assessed as previously described [4]. Eppendorf 1500-μl plastic centrifuge vessels, procured from the Brinkman Instrument Co., Silver Spring, MD, U.S.A., were used throughout.

Electrophoretic separation of maleimide, maleamic acid and maleic acid was carried out for 1 hr at 3000 V on Whatman 3MM paper (96 cm) saturated with 0.1 M potassium phosphate buffer, pH 7.0. The spots were identified under ultraviolet (u.v.) light and excised, and their content of the amide or imide was measured by u.v. spectrometry. Under these electrophoretic conditions, maleimide remained at the origin, whereas maleamic acid and maleic acid migrated 3.1 and 5.2 cm respectively.

The hydrolysis of β-cyano-L-alanine and L-asparagine by L-asparaginase from *E. coli*, in the presence and absence of maleimide, was quantified by a coupled spectrophotometric technique for the measurement of L-aspartic acid [5]. Hydrolysis of the other substrates was quantitated with an enzymatic assay for ammonia [6]. In no case was sufficient maleimide sampled to interfere with the stoichiometrics of the ammonia analysis. In cases where the concentration of maleimide in the cuvette would be likely to interfere with the spectrophotometric analysis, an equimolar quantity of mercaptoethanol was added to the incubation mixture prior to sampling for the spectrophotometric assay.

When necessary, a radiometric technique was used to quantify the hydrolysis of L-asparagine, as follows: 5 μl of a freshly prepared maleimide solution of the requisite molarity in 0.1 M Tris–HCl buffer, pH 8.4, or 5 μl of 0.1 M Tris–HCl buffer, pH 8.4, and 5 μl of L-asparaginase solution were driven onto a 5-μl droplet (0.25 μCi; 0.0016 μmole) of L-[U-^{14}C]asparagine contained in the bottom of an Eppendorf 1500-μl centrifuge vessel by a 5-sec acceleration to 12,000 *g*. After a 30-min incubation at 25°, 10 μl of 1 M HCl was added to each vessel and the mixture heated to 95° for 5 min. Then 10 μl of 1 M NaOH was added to each vessel followed by 20 μl of a decarboxylation mixture consisting of 6.8 mM α-ketoglutaric acid, 8.5 mM $ZnSO_4$ in 0.66 M sodium acetate buffer, pH 5.0, containing 20 I.U. GOT/ml. The vessels were incubated for 60 min at 50°. [^{14}C]O_2 arising from the β-decarboxylation of [U-^{14}C]oxaloacetic acid produced by the GOT-catalyzed transamination with α-ketoglutaric acid of any L-[U-^{14}C]aspartic acid liberated was trapped in a 5-μl droplet of 40% (w/v) KOH deposited on the lid of the closed vessels, then counted as previously described [7].

With L-asparagine as substrate, maleimide inhibited L-asparaginase from *E. coli* in a noncompetitive manner* (K_i = 0.05 M) (Fig. 1B), but inhibition was apparent only at relatively high concentrations of inhibitor (0.025 to 0.20 M); no inhibition whatsoever could be detected with

* Inhibition of L-asparaginase by maleimide can possibly be explained by the known proclivity of this agent to react covalently with α-amino acids [8]. Some support for the notion that maleimide can react with L-asparagine was provided by experiments in which the two compounds were incubated together each at a concentration of 0.05 M for 48 hr in sodium phosphate buffer, pH 7.0. Ascending chromatography of the reaction mixture in the systems described by Smyth *et al.* [8] revealed the formation of a new entity yielding a yellow chromogen with ninhydrin and with an R_f of 0.1. However, determination of the rate of disappearance of L-asparagine at 0, 24 and 48 hr in the incubation mixtures of the composition given above revealed a diminution of, at most, 5 per cent. Moreover, this decrement was not progressive with increasing time of incubation. The quantitatively minor extent of the reaction suggests that the covalent interaction of maleimide with L-asparagine is not causative of the inhibitory action of maleimide vs L-asparaginase from *E. coli*.

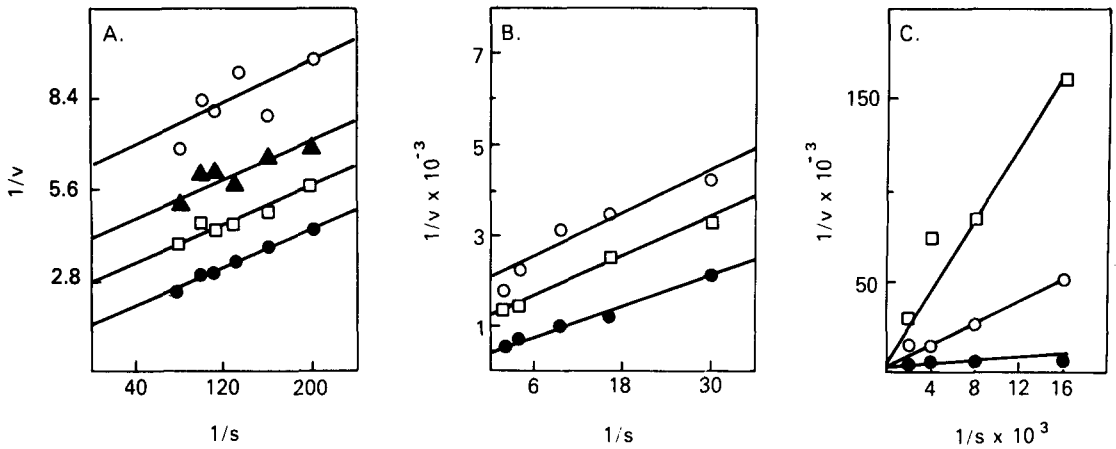

Fig. 1. Inhibition of the enzymic hydrolysis of β-cyano-L-alanine and L-asparagine by maleimide and maleamic acid. (A) Five hundred μl of β-cyano-L-alanine solution (0.005 to 0.0175 M) in 0.5 M Tris–HCl buffer, pH 8.4, containing maleimide (0 to 0.1 M) was dispensed in triplicate into Eppendorf 1500-μl plastic centrifuge vessels and incubated with 0.6 I.U. of L-asparaginase from *E. coli* for 60 min at 25°. The reaction was terminated by the addition of 200 μl of 2 M HCl, then neutralized 10 min later with 200 μl of 2 M NaOH. Fifty μl mercaptoethanol was added to all the vessels and 500 μl of the resulting mixture then was assayed spectrophotometrically for L-aspartic acid as described earlier. Key: (●) control, (○) 0.1 M maleimide, (▲) 0.075 M maleimide, and (□) 0.050 M maleimide. S = molarity, and V = nmoles/min. (B) The hydrolysis of L-[U-^{14}C]asparagine by L-asparaginase from *E. coli* was conducted radiometrically in the presence and absence of maleimide as described earlier. The reaction was terminated with HCl, then neutralized with NaOH. Subsequent recovery of [^{14}C]O_2 representing the fourth carbon of the resulting L-[U-^{14}C]aspartic acid was conducted as described earlier. Key: (●) control, (○) 0.2 M maleimide, and (□) 0.025 M maleimide. S = molarity, and V = pmoles/hr. (C) The hydrolysis of L-[U-^{14}C]asparagine by L-asparaginase from *E. coli* was conducted radiometrically in the presence and absence of maleamic acid as described under part B. Key: (●) control, (□) 0.08 M maleamic acid, and (○) 0.06 M maleamic acid. S = molarity, and V = pmoles/hr. In no case did the spontaneous hydrolysis of maleimide to maleamic acid amount to greater than 5 per cent of its initial concentration.

concentrations of maleimide below 0.01 M. The hydrolysis of β-cyano-L-alanine, a substrate for the nitrilase activity of L-asparaginase, also was inhibited in a noncompetitive manner by maleimide (K_i = 0.05 M) (Fig. 1A). In contrast, maleamic acid, a straight chain congener of maleimide, inhibited the hydrolysis of L-asparagine competitively (K_i = 0.090 M) (Fig. 1C), and was inhibitory at significantly lower molarities than maleimide.

The relative ineffectiveness of maleimide as an inhibitor suggests that sulfhydryl groups are either absent or recondite at the catalytic site of L-asparaginase. This conclusion concurs with previous studies which established that L-asparaginase from *E. coli* is insensitive to inhibition by sulfhydryl reagents, including *N*-ethyl maleimide [9].

In the course of investigation into the inhibitory potency of maleimide toward the hydrolysis of L-glutamine and L-asparagine by L-asparaginase from *E. coli.* (20 i.u./ml), it was observed that the yield of ammonia was considerably suprastoichiometric in vessels containing maleimide but stoichiometric in vessels lacking the drug. This finding demonstrated that ammonia was being released both from L-asparagine or L-glutamine and from maleimide (or a decomposition product thereof). Using high (0.1 M) concentrations of maleimide alone, it was possible to demonstrate that ammoniagenesis occurred at a constant rate for up to 1000 min in the presence of 25–125 i.u. of L-asparaginase, and that the rate was dependent on the concentration of L-asparaginase. (Such linearity reflects the integrity of the enzyme during the protracted incubations used, and illustrates again the absence of susceptible sulfhydryl groups at the catalytic center.) In addition, it was found that the release of ammonia in these protracted incubations was stoichiometric with the amount of maleimide present at the beginning of the incubation; therefore, ammoniagenesis was not due to an L-asparagine-like contaminant in maleimide.

Since crystalline L-asparaginase with a specific activity of 340 I.U./mg of protein was used, it also seemed unlikely that a contaminating enzyme or substrate was responsible for the effect described here. To test this point, the hydrolytic activity toward maleimide of five separate lots of L-asparaginase was examined. Electrophoretically, typical, geometrically spaced banding patterns were seen in every case, with no evidence of significant protein contaminants. Moreover, the rate of ammoniagenesis from maleimide was found to be roughly comparable with each of the five batches examined. It should be stressed, though, that comparatively large concentrations of enzyme were required for the demonstration of this effect, and that a residual co-crystallized contaminant might have been responsible for this phenomenon. This suggestion is rendered less likely by the observation that L-asparaginase, whose active site had been covalently alkylated with L-DONV, catalyzed ammoniagenesis from maleimide at a negligible rate.

Although these findings appeared to implicate maleimide as a substrate for an "imidohydrolytic" action of L-asparaginase, they were by no means conclusive. The possibility remained that maleimide was non-enzymatically transformed into a straight chain compound before enzymatic attack by L-asparaginase could occur. Indeed, when the pH optimum for the breakdown of maleimide, was examined, ammoniagenesis was detected in vessels receiving no enzyme, especially at pH 6.0; enzymatic ammoniagenesis exhibited two apparent pH optima: the first at pH 7.0, and the second at pH 9.0 (Fig. 2). Attempts, therefore, were made to identify maleamic acid and maleic acid, the sequential hydrolytic products of maleimide, in typical reaction mixtures lacking enzyme. High voltage electro-

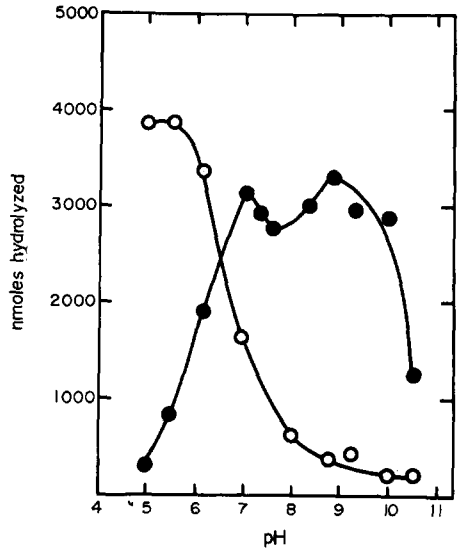

Fig. 2. The pH optimum of ammonia release from maleimide in the presence and absence of L-asparaginase. Maleimide (0.05 M in 0.25 M potassium phosphate buffer at the pH values indicated on the figure) was incubated for 19 hr at 37° in the presence and absence of 40 i.u./ml of L-asparaginase from *E. coli* (*EC*-2). The release of ammonia was quantitated enzymatically using α-ketoglutaric acid and L-glutamic acid dehydrogenase as described earlier. Under the conditions of the ammonia assay, maleimide (final concentration = 1×10^{-3} M) did not interfere with the stoichiometry of the L-glutamic acid dehydrogenase reaction. Values reported for the enzymatic hydrolysis of maleimide were corrected by subtracting the amount of maleimide hydrolyzed non-enzymatically at the appropriate pH values. Key: (●) enzymatic hydrolysis, and (○) non-enzymatic hydrolysis.

phoresis in 0.1 M potassium phosphate buffer, pH 7.0, was found to be capable of separating maleimide, maleamic acid and maleic acid, unambiguously. By this technique, maleamic acid was identified as the proximate product of the non-enzymatic decomposition of maleimide and alkaline pH was found to promote ring opening. These findings confirm the observations by Barradas *et al.* [10] that the hydrolysis of maleimide occurs best in the pH range of 8.5 to 11.5, during which time the neutral maleimide exists in equilibrium with its anion. Both species undergo hydrolysis simultaneously at the appropriate pH. Our findings further suggest that maleamic acid and not maleimide was the proximate substrate for L-asparaginase. Further confirmation of this conclusion came from the observations that the rate of ring opening (maleimide $\xrightarrow{H_2O}$ maleamic acid) did not differ significantly in reaction mixtures having and lacking L-asparaginase.

When the apparent affinity of L-asparaginase for maleimide and maleamic acid was compared, the enzyme was found to exhibit a lower apparent affinity for the cyclic compound (K_m = 0.153 M) than for maleamic acid (K_m = 0.125 M). In order to extend these observations to other congeneric amides and imides, the apparent affinity constants of L-asparaginase from *E. coli* for succinimide and succinamic acid were measured enzymatically. The pattern observed with these two substrates was similar to that found with maleimide and maleamic acid, that is, the amide was a better apparent substrate than the imide.

Previous studies into the mechanism of action of L-asparaginase have suggested that a cyclic intermediate might be involved [1, 2]. However, aspartic anhydride, a compound sterically similar to the postulated cyclic intermediate, is neither a substrate nor an inhibitor of L-asparaginase from *E. coli* [3]; several other structurally related anhydrides and lactams also were incapable of being hydrolyzed [11]. Malemide can now be added to the list of cyclic congeners which are neither substrates nor potent inhibitors of L-asparaginase from *E. coli*.

In summary, the resemblance of maleimide to the hypothetical cyclic anhydride of L-asparagine prompted us to investigate this agent as a substrate for the amidohydrolytic action of L-asparaginase. Ammonia production could, in fact, be demonstrated when maleimide was incubated with high activities of L-asparaginase. This release was determined to occur from maleamic acid, the straight chain congener of maleimide, which is produced by non-enzymatic ring opening at alkaline pH. Maleamic acid and succinamic acid were better apparent substrates for L-asparaginase than their respective imides. Furthermore, maleimide at high concentrations, was found to be a non-competitive inhibitor of L-asparaginase from *E. coli* with L-asparagine as a variable substrate, whereas maleamic acid was a competitive inhibitor under the same conditions. It can be concluded that maleimide is not a substrate for L-asparaginase by virtue of its resemblance to the hypothetical anhydride of L-asparagine, but by virtue of its decomposition to maleamic acid, the ultimate substrate of the enzyme.

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