α-Amanitin: Inactivation by bovine lactoperoxidase[1]

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Summary. The principle amatoxin, α-amanitin, is found to be extremely sensitive toward lactoperoxidase catalyzed degradation, rather than iodination, of the indole nucleus. Extensive attenuation of inhibitor potency against eukaryotic DNA-dependent RNA polymerase II accompanies the treatment of α-amanitin with lactoperoxidase, iodide and hydrogen peroxide.

The frankly toxic members of the genus *Amanita* contain several cyclopeptide families[2], one of which, the amatoxins, is largely responsible for the fatal intoxications resultant from ingestion of these mushrooms. Amatoxins are not solely restricted to *Amanita* but also occur in toxic levels in *Galerina marginata*[3-5] and in nontoxic levels in several mushrooms including the edible *Agaricus silvaticus* and *Boletus edulis*[6], where the amatoxins are $\sim 10^4$ times lower in concentrations. In addition to the amatoxins, *Amanita phalloides* also contains phallotoxins, antamanide and cycloaminides[2,6]; each is a distinct cyclopeptide family. The monocyclic decapeptide, antamanide, exhibits Na^+ and Ca^{+2} chelation[8] and is an antagonist to phallotoxin poisoning[9,10]. The several phallotoxins are all bicyclic heptapeptides[2] effecting the recruitment of G-actin to F-actin[11-13] with consequent cellular disruption; the LD_{50} (white mouse bioassay) for the phallotoxins is rather high at 2.5 mg/kg b.wt[2].

Most toxic among the mushroom poisons are the bicyclic octapeptides, the amatoxins (LD_{50} = 0.3 mg/kg in the white mouse)[2]. The amatoxins are known to be potent inhibitors of RNA polymerase II (E.C. 2.7.7.6) in higher eukaryotes[14,15]. The class III RNA polymerase also is susceptible to amatoxin inhibition but only at toxin concentrations some 10^4 times greater than required for the class II enzyme. Experimental intoxications with the amatoxins reveal diminished RNA synthesis in liver and kidney with the development of necrosis in these organs and the intestine[16-18]. It is probable that the toxicity of the amatoxins results from RNA polymerase II inhibition in the affected organs.

During experiments leading to the synthesis of a stable iodine substituted α-amanitin, we observed a facile inactivation of α-amanitin catalyzed by bovine lactoperoxidase. Lactoperoxidase with H_2O_2 and I^- reactants has become the basis for a protein iodination procedure that is gentle and selective[19], often catalyzing iodination without inactivation of the recipient enzyme. Submission of α-amanitin to lactoperoxidase catalyzed iodination surprisingly results in the destruction of the amatoxin. 7-Iodo-α-amanitin[20] is a known compound, stable in aqueous solutions at pHs on either side (e.g. pH 7.5 and 9.8) of the pK_A (7.94) for the 6-hydroxy indole moiety, and further, is an established inhibitor of RNA polymerase II from sea urchin, *Drosophila*, and MOPC 315 plasmacytoma. Figure 1 demonstrates the consequences of lactoperoxidase catalyzed and uncatalyzed iodination upon the electronic spectrum of α-amanitin; the characteristic λ_{max} at 304 nm (figure 1, spectrum A) decreases in intensity as the reaction proceeds until there remains neither evidence of the former λ_{max} nor the λ_{min}. The ultimate spectrum from such reactions (figure 1, spectrum C) is stable and persists up to 1 day in the presence of excess H_2O_2, I^- and active bovine lactoperoxidase; as such, it probably is due to a mixture of oxidation products of the α-amanitin. The aqueous 7-iodo-α-amanitin spectrum is

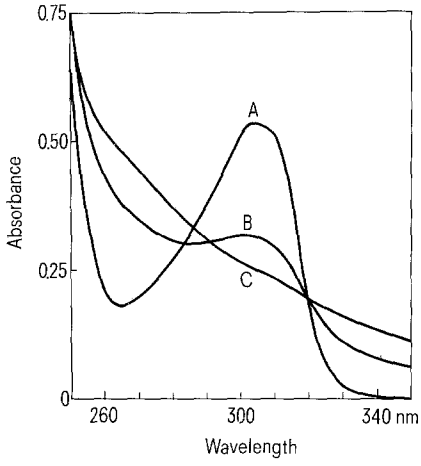

Fig. 1. Lactoperoxidase catalyzed degradation of the hydroxyindole in α-amanitin. The toxin at 4.3×10^{-5} M in 18 mM tris · HCl (pH 7.9) is reacted with 3.6×10^{-4} M H_2O_2, 3×10^{-4} M KI and 20 µg/ml bovine lactoperoxidase (P.L. Biochemicals) at 30 °C. Spectrum A: α-amanitin in tris buffer alone. Spectrum B: α-amanitin, 35 min after admixture with KI and H_2O_2 but no lactoperoxidase. A similar spectrum is obtained at 2 min after admixture of the complete reaction including lactoperoxidase. Spectrum C: α-amanitin, 8.5 min after admixture with KI, H_2O_2 and lactoperoxidase; a similar spectrum is obtained after 80 min reaction with KI and H_2O_2 but no lactoperoxidase. These spectra were obtained by blanking against tris buffer only; the KI, H_2O_2, and lactoperoxidase contribute little to the spectra in the region of 350-260 nm. All spectra were recorded on the Cary 15.

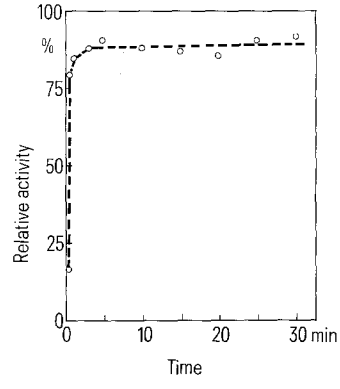

Fig. 2. Attenuation of α-amanitin inhibition on RNA polymerase II. α-Amanitin (10^{-5} M) is treated at 30 °C with 10^{-4} M each of KI and H_2O_2 plus 40 µg/ml lactoperoxidase. At the times indicated, 5 µl aliquots were withdrawn and added to 25 µl of RNA polymerase reaction mixture (15) containing 5 mM dithiothreitol to reduce H_2O_2 and I_2. RNA polymerase II (20 µl, *Drosophila melanogaster*, Oregon R strain) was added, incubated for 20 min at 30 °C, and then processed to quantitate the RNA synthesis (15). Uninhibited RNA polymerase II incorporated 22,000 cpm of [^{3}H]-uridine monophosphate (22 pmoles) corresponding to a relative activity of 100%. The lactoperoxidase, KI and H_2O_2, together or singly, exerted no inhibition on the RNA polymerase II. The plateau in the reactions results from lactoperoxidase inactivation; addition of fresh lactoperoxidase further reduces the α-amanitin to insignificant concentrations.

distinctly unlike spectrum C and, in fact, closely resembles the original α-amanitin in λ_{max}, λ_{min}, and overall shape[20].

The degradation of the electronic spectrum accompanies an attenuation of the α-amanitin potency against RNA polymerase II (E.C. 2.7.7.6) as shown in figure 2 for the *Drosophila* enzyme; similar results are found with the sea urchin and calf thymus enzyme. With relatively large ratios of lactoperoxidase to α-amanitin the attenuation proceeds rapidly ($t_{1/2} < 1$ min, figure 2) from the initial α-amanitin concentration of 10 μM to the final at < 0.1 μM. The residual level of α-amanitin is estimated by comparison of the polymerase II inhibition achieved by the degraded α-amanitin to the inhibition achieved by known concentrations of α-amanitin (figure 3). Further degradation of the α-amanitin to a final concentration $\sim 10^4$-fold less than the original can be achieved by the addition (e.g. at 15 min in figure 2) of fresh lactoperoxidase.

Examination of the lactoperoxidase reaction with $^{125}I^-$ fails to demonstrate any synthesis of 7-iodo-α-amanitin or other iodinated toxin. Under limiting reaction conditions (e.g. 1 μg/ml lactoperoxidase, 0.2 mM KI, 0.36 mM H_2O_2 and 0.06 mM α-amanitin) insignificant degradation of the α-amanitin spectrum can occur within a limited time. If an iodinated amanitin intermediate exists, such limited reactions favor its identification. Fractionation of these reactions by thin layer (Eastman Kodak cellulose/butanone:acetone:H_2O; 8:3:0.5 v/v) or partition (sephadex LH-20/methanol:H_2O; 1:1, v/v) chromatography results in the fortuitous co-elution of ^{125}I and α-amanitin. However, fractionation on polyamide plates (Cheng Chin; 1-butanol:H_2O:acetone; 8.9:0.8:0.3, v/v) cleanly separates the amanitin from all ^{125}I. We have been unable to detect any ^{125}I-labeled amanitin intermediate in the lactoperoxidase catalyzed degradation.

Inspection of the requirements for perturbation of the electronic spectra and attenuation of the RNA polymerase II inhibition shows that the lactoperoxidase performs a catalytic role only and is not required for the reaction. The spectral perturbation rate (ΔA_{304}/min) in the absence of lactoperoxidase is second-order in $[I^-]$ within the limits of [α-amanitin] $= 4.3 \times 10^{-5}$, $[H_2O_2] = 36 \times 10^{-5}$ M, and $[I^-]$ varied between 6 and 30×10^{-5} M. This second order dependency in iodide is consistent with the oxidation reaction, $2\ I^- \rightarrow I_2 + 2\ e^-$, being the rate limiting step. At concentrations of $I^- <$ α-amanitin the reaction rate is mixed order with respect to I^-; nonetheless, the decrease in absorbancy at 304 nm continues until residual α-amanitin is not detectable. Deletion of either I^- or H_2O_2 from the reaction yields no decrease in A_{304} over several h of observations; thus both components are required for the reaction upon α-amanitin and the oxidant, H_2O_2, alone does not suffice. Other electropositive halogens (e.g. ICl in tris · HCl, pH 7.5) also are effective in α-amanitin degradation. The second order dependency upon iodide, the persistence of the reaction with iodide less than equimolar to α-amanitin, and the reaction of N-iodoamines (unpublished, P.W.M.) with α-amanitin supports the hypothesis that I_2 catalyzes phenol oxidation to yield a putative amanitin quinone and I^-. In parallel with known reactions[21], the amanitin quinone in aqueous solution is probably susceptible to attack by hydroxide with ultimate cleavage of the aromatic ring. Oxidative coupling of 2 phenol moieties[22] to yield an amanitin dimer appears less likely since we find no apparent extension of electronic conjugation. Due to limitation on α-amanitin availability and the mixture of products obtained, the precise fate of the hydroxy-indole following the oxidation reaction is unknown. However, it does seem unlikely that the final product is capable of reactivation to restore the RNA polymerase inhibitor.

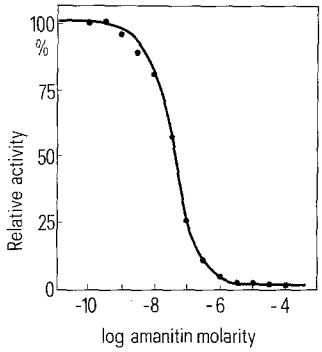

Fig. 3. Sensitivity of *Drosophila melanogaster* RNA polymerase II to α-amanitin. Aliquots of RNA polymerase II were assayed with a range of α-amanitin concentrations and then processed for [^{3}H]-uridine monophosphate incorporation into RNA (15). With this titration curve, one can estimate that the α-amanitin concentration in those RNA polymerase assays within the plateau region of figure 2 approximates 3×10^{-9} M. Allowing for 1:10 dilution between the lactoperoxidase treatment and the polymerase assay, this is a 330-fold reduction of the effective α-amanitin concentration.

Wieland and colleagues have shown that toxicity of the amatoxins is strongly dependent upon integrity of the bicyclic peptide[2]. Both desulfurization with Raney nickel and lactonization at the γ, δ-dihydroxyisoleucyltryptophanyl peptide bond interrupts the bicyclic ring; the resulting dethio- and seco-amatoxins have no detectable toxicity in the white mouse assay and no detectable RNA polymerase II inhibition. In the present work we find that treatment with a source of electropositive iodine in aqueous solution extensively alters the characteristic electronic spectrum of α-amanitin and is accompanied by attenuation of RNA polymerase II inhibition. From additional work it is clear that substitution in the indole nucleus (e.g. at C7 with either morpholine [unpublished, P.W.M.] or iodine[20]) does not decrease the K_i for RNA polymerase II inhibition. On chemical and biochemical grounds it appears most probable that the lactoperoxidase/I^- reaction disrupts the amatoxin bicycle rather than yielding substitution into the indole.

This finding of chemical fragility of α-amanitin holds significance on at least 2 levels. 1. The lactoperoxidase reaction is definitely unsuitable for preparation of radiolabeled iodo-α-amanitin. 2. The rapid degradation of amanitin to a biologically inactive species without any apparent iodinated and toxic intermediate suggests the possibility for an effective degradation of the toxin in the dynamic in vivo state. Amatoxin poisoning in dogs is marked by an apparent enterohepatic recirculation of the toxin[23]; this likewise may be true for human since the vomiting, diarrhea and gastrointestinal pathology induced by the pure α-amanitin in dogs is seen in mushroom poisonings in humans[2,16-18]. Appearance of the toxin in the bile provides a possible approach toward interruption of the enterohepatic recirculation by lactoperoxidase catalyzed attenuation in the intestine. Indeed, this approach need not be restricted to lactoperoxidase since several plant laccases and phenolases catalyze analogous oxidations with O_2, rather than H_2O_2, as the electron sink and thus may obviate the requirement for H_2O_2 and I^-.

1 This work was supported in part by NIH grant 22147 and a grant from the University of Illinois.

2 Th. Wieland, Fortschr. Chem. org. NatStoffe *25*, 214 (1967); Th. Wieland, Naturwissenschaften *59*, 225 (1972); Th. Wieland, Arzneimittel-Forsch. *22*, 2142 (1972); Th. Wieland and O. Wieland, Microb. Toxins *8*, 249 (1972).

3 S.H.A. Malak, Planta med. *29,* 80 (1976).
4 H. Faulstich, D. Georgopoulos, M. Bloching and Th. Wieland, Z. Naturforsch. *29,* Part C, 86 (1974).
5 R.G. Benedict and L.R. Brady, Lloydia *30,* 372 (1967).
6 H. Faulstich and M. Cochet-Meilhac, FEBS Lett. *64,* 73 (1976).
7 A. Gauhe and Th. Wieland, Justus Liebigs Ann. Chem. 859 (1977).
8 W. Burgermeister, Th. Wieland and R. Winkler, Eur. J. Biochem. *44,* 305 (1974).
9 Th. Wieland, H. Faulstich, W. John, M.V. Govindan, H. Puchinger, Z. Kopitar, H. Schmaus and A. Schmitz, Hoppe Seylers Z. Physiol. Chem. *353,* 1337 (1972).
10 H. Faulstich, Th. Wieland, A. Walli and K. Birkman, Hoppe-Seylers Z. Physiol. Chem. *355,* 1162 (1974).
11 A.M. Lengsfeld, I. Low, Th. Wieland, P. Dancker and W. Hasselback, Proc. nat. Acad. Sci. USA *71,* 2803 (1974).
12 Th. Wieland, Naturwissenschaften *64,* 303 (1977).
13 J. Wehland, M. Osborn and K. Weber, Proc. nat. Acad. Sci. USA *74,* 5613 (1977).
14 P. Chambon, A. Rev. Biochem. *44,* 613 (1975).
15 R.G. Roeder, in: RNA Polymerase, p.285 R. Ed. R. Losick and M. Chamberlin. Cold Spring Harbor Laboratory, 1976.
16 L.G. Hanelin and A.A. Moss, Am. J. Roentg. *125,* 782 (1975).
17 L. Fiume, V. Marinozzi and F. Nardi, Br. J. exp. Path. *50,* 270 (1969).
18 L. Fiume, M. Derenzini, V. Marinozzi, F. Petagzi and A. Testoni, Experientia *29,* 1520 (1973).
19 M. Morrison and G.R. Schonbaum, A. Rev. Biochem. *45,* 861 (1976).
20 P.W. Morris, D.L. Venton and K.M. Kelley, Biochemistry *17,* 690 (1978).
21 H. Musso, in: Oxidative Coupling of Phenols, p.1. Ed. W.I. Taylor and A.R. Battersby. Marcel Dekker, Inc., New York 1967.
22 B.R. Brown, in: Oxidative Coupling of Phenols, p.167. Ed. W.I. Taylor and A.R. Battersby. Marcel Dekker, Inc., New York 1967.
23 U. Fauser and H. Faulstich, Dt. med. Wschr. *98,* 2259 (1973).

Position effect influencing alcohol dehydrogenase activity in *Drosophila melanogaster*[1]

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Summary. A small decrease in alcohol dehydrogenase activity was found in translocation stocks of *Drosophila melanogaster* in which a section of the heterochromatic Y-chromosome is inserted proximal to *Adh.* This small position effect is consistent with our growing knowledge of the control and transcription of the *Adh* locus.

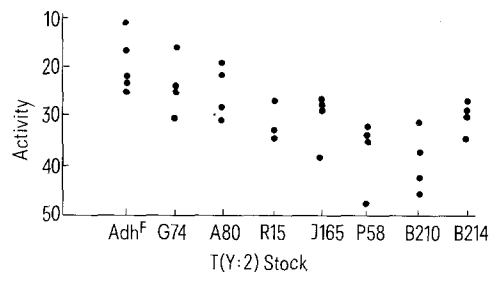
Position effect can be defined as the correlation between a gene's location and its expression, which can be influenced by its proximity to other genes and to heterochromatin. Position effects have been shown for a variety of morphological traits[2] and even for enzyme loci, such as amylase *(Amy)* in *Drosophila melanogaster.* Because of the growing interest in alcohol dehydrogenase as a model genetic system, we were interested in learning whether the activity of the *Adh* locus could be affected by its proximity to heterochromatin.

Alcohol dehydrogenase (ADH) is a small homodimer coded for by a gene on the left arm of the second chromosome (2-50.1) of *D. melanogaster.* It has been localized cytologically by Woodruff and Ashburner[4] to polytene chromosome bands 35B2-3. ADH activity can be measured spectrophotometrically by the rate at which the supernatant from homogenized whole flies breaks down an alcohol substrate. We prepared the homogenates from 1-3-day-old adults by weighing 40 mg of flies or by counting out 40 flies of each sex. Both types of homogenates gave the same results. Activity assays were run by placing 0.1 ml of the homogenized fly supernatant in 2.4 ml of a buffered solution containing 0.072 M tetrasodium pyrophosphate, 0.072 M semicarbazide HCl, 0.021 M glycine and 0.065 M sodium hydroxide in distilled water, with 4 mg β-NAD and 0.03 ml of isopropanol as the substrate. Activity was determined from a plot of the change in absorbance at 340 nm versus time.

In order to monitor *Adh* position effects, we assayed the relative enzyme activities in a number of T(Y : 2) translocation stocks. In these the heterochromatic Y-chromosome was attached to chromosome 2 at various distances to the left and the right of *Adh.* These stocks were homozygous for the Adh^F (fast electrophoretic mobility) allele.

ADH activity assays of 40 mg of flies from 7 different T(Y : 2) stocks[5] are shown in the figure, with translocation breakpoints given by Lindsley et al.[5]. It is clear from this figure that there are repeatable differences in activity from stock to stock. Thus, since the stocks are believed to be genetically identical, except for the Y and chromosome 2 breakpoints, it appears that ADH activity can be influenced by either the amount or proximity of heterochromatin.

The results of detailed assays of four translocation stocks are shown in the table. In these assays, we have corrected for fly size, since ADH activity is apparently not directly correlated with adult b.w[6]. We have also eliminated the possibility that additional Y-chromosomes are present. As in the figure, it can be seen that the activity of stock P58, in which the Y-heterochromatin is inserted proximal to *Adh,* is consistently lower than the activity of strains with heterochromatin inserted distal to *Adh.* Although the amount of Y-heterochromatin differs among stocks G74, A80 and P58, the makeup of stocks P58 and B210 appears to be about the same. Yet the ADH activity is higher in B210 than in P58, in which the breakpoint and proximity of



ADH activities in 7 T(Y:2) translocation stocks of *Drosophila melanogaster.* The activity scale is a temporal one, based upon the time (in sec) required to change the OD a standard amount in a reaction catalyzed by ADH. Thus, lowest activities require the longest time (50) and highest activities the shortest time (less than 10 sec).