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A Chemical Model for the Enzymatic Mono De-Alkylation of (methyl and ethyl) Parathion by Glutathione-S-Transferase.

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Abstract: The NMR study of the reaction of methylparathion with thiol (glutathione) and a tertiary amine shows unambiguously a sequential transfer of the methyl group firstly to the amine and secondly to the thiol. The reaction stops after a single demethylation. This observation may account for regiospecificity of some glutathione-S-transferases. © 1997 Elsevier Science Ltd.

INTRODUCTION

There is considerable evidence to suggest that glutathione-S-transferases (GST : EC 2.5.1.18) in insects are very important for insecticide resistance. GSTs catalize the condensation of glutathione (GSH) with insecticidal molecules as a detoxification process.[1] High levels of GST activity have been detected in the hemolymph of resistant insects when exposed to insecticides.[2] GSH dependent insecticide metabolism is correlated with insecticide resistance.[3] Among insecticides, parathion (diethylparanitrophenylthiophosphate, DEPT) and methyl-parathion (Dimethylparanitrophenylthiophosphate, DMTP) are known to be good substrates for the enzymatic conjugation of alkyl or aryl groups with GSH. For example rat hepatic GSTs undergo both dealkylation and dearylation[4] of DMPT and DEPT. The reaction ratio varies markedly depending upon the alkyl group[5] of DNMPT/DEPT. The reaction can be attributed to distinct isoforms of GST, for example, GSTs from Diamondback Moth catalyse the conjugation of GSH only with the aryloxy group of DMPT or DEPT while with GSTs from human foetal liver, only the alkyloxy substituent of methylparathion is transfered to the thiol group of GSH.[6] In both cases, reactions lead to inactivation of the insecticide.

Structure/function analysis of GSTs has suggested that the catalytic mechanisms depends on the ability of the proteins to lower the pKa of the thiol group of GSH. This function has been assigned either to a tyrosine or to a serine depending on the GST.[7] The formation of the glutathione thiolate anion allows for the nucleophilic attack of the substrate. For DMPT of DEPT, the assumption of a SN_2P substitution between the GSH as nucleophile and the substrate seems the most probable. In that case, the pentacoordinated phosphorus intermediate would eject the better leaving group, i.e. the aromatic and not the alkyl one. In this study we examined the mechanism for the dealkylation of DMPT and DEPT by some of the GST isoenzymes.

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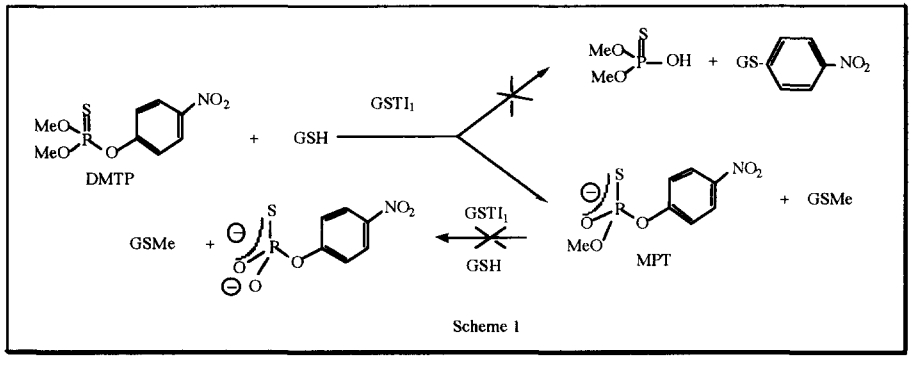
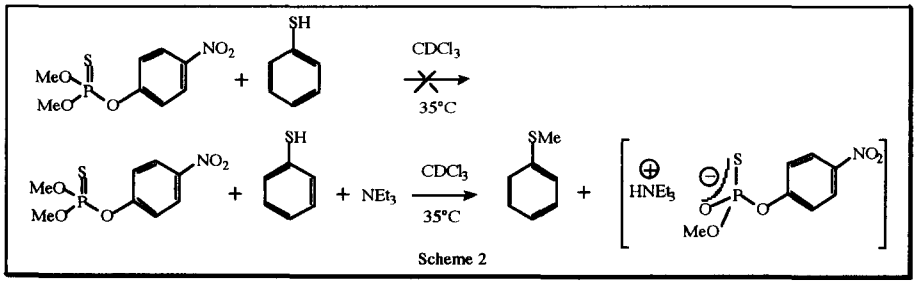
RESULTS

We used one isoenzyme which is over-expressed in an insecticide resistant house fly strain. This isoenzyme (GSTI1) has been cloned, sequenced, expressed in *E. coli* and purified by affinity chromatography.[8] The reaction products of DMPT and GSH in the presence og GSTI1 were assayed using light absorption spectrophotometry over time. The dearylation product can be detected as S-paranitrophenylglutathione which is described to show a typical λ_{max} at 342 nm.[6] No variation was observed at this wavelenght even after a 6h incubation showing that dearylation was not detectable. Then, TLC analysis was carried out to study the dealkylation reaction of DMPT and DEPT. Several potential degradation products were co-analysed as standard references : the paranitrophenol, the mono and bis-dealkylated parathion. The chromatography patterns obtained shows no metabolites with DEPT and exhibit the presence of a single metabolite with DMPT which was identified, without ambiguity, as methylparanitrophenylthiophosphate diester (MPT). As expected, no spontaneous hydrolysis occurs at pH 7, therefore GST and GSH are required for metabolisation of DMPT. Furthermore, no paranitrophenylthiophosphate monoester was observed showing that a two fold dealkylation does not occur.



Scheme 1

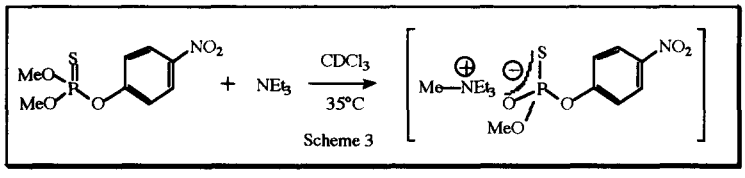
Taking into account this first results, we tried to chemically modelize the enzymatic reaction. We first use DMPT and benzenethiol as analog of glutathione and the progress of the reaction was followed by NMR spectroscopy. In $CDCl_3$, at 35°C, we did not observe any reaction between DMPT and benzenethiol. On the other hand, the presence of the organic base triethylamine allowed the reaction to proceed. A single methyl group was transfered to the benzenethiol and the mono-anion, $MPT^{(-)}$ and the cation triethylammonium$^{(+)}$ were produced (A and B conditions in the experimental part) (scheme 2).



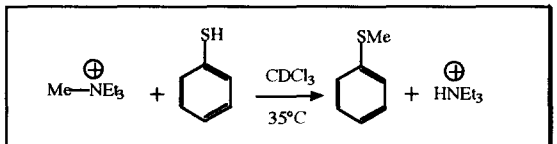
Scheme 2

The reaction was complete at this temperature after 100 hours. All the species were identified by ^{31}P and ^{1}H NMR without ambiguity. Only the mono demethylation reaction of DMPT was observed as for the enzymatically catalysed reaction between GSH and DMPT. The reaction was brought to completion in $CDCl_3$ at 35°C after

100 hours. The study of these chemical models unambigously showed the following sequential pathway : the first step, observed by NMR spectroscopy, is the the transfer of the methyl group from the thiophosphate to the nitrogen of the base (scheme 3). The second and last step, also oserved by the same spectroscopy, corresponds to the transfer of the methyl group from the methyltriethylammonium ion to the thiol with formation of methylphenylthioether and the triethylammonium ion (the three types of methyl were fairly distinguishable on the ^1H NMR spectra) (scheme 4). When the experiment was performed in the same conditions but in absence of benzenethiol, a mono demethylation reaction occured between triethylamine and DMPT.[9]

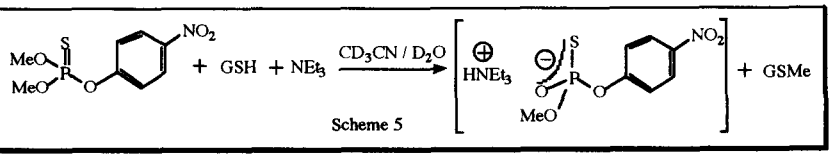


Scheme 3



Scheme 4

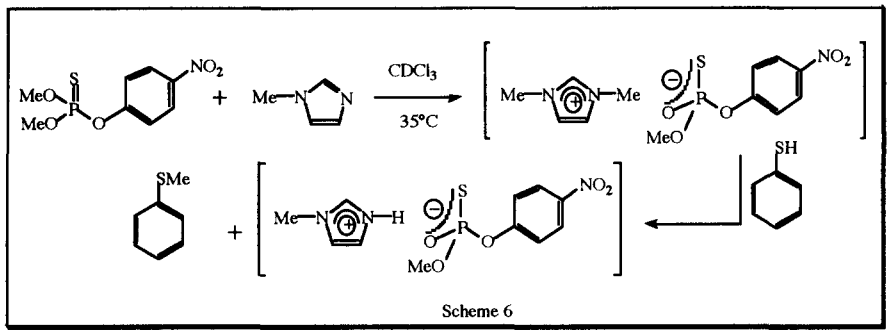
In these previous experimental conditions (A and B conditions, see experimental part), the reaction did not proceed further than the mono demethylation, even with an excess of thiol or base, because it is evidently difficult for a nucleophile to be added to an anionic species. Two reactions mechanisms were expected. The first one involves activation of the thiol by a base (general basic catalysis) and the relative nucleophilicity of the reactants. The nucleophilic constants[10] n_{CH3I} are 5.7, 6.66 and 9.92 for benzenethiol, triethylamine and benzenethiolate respectively and the pK (ethanol) of benzenethiol and triethylamine are 6.52 and 10.7. The probability of a general basic catalysis is high ; nevertheless in the point of view of the HSAB theory we can understand the sequence of the attack taking into account the hardness of the nitrogen of the base and the softness of thiol or thiolate. The second expected mechanism is SN_2P substitution, the reaction proceeding through a pentacoordianted intermediate. This reaction could be probable for a neutral ester and the product of the reaction would be nitrophenol. Experimental observations are in discordance with these two mechanisms, we consequently conclude that a sequential reaction takes place in which the initial step is the nucleophilic attack on the methyl group of methylparathion by the triethylamine. The transfer reaction between GSH and DMPT in the same experimental conditions ($CDCl_3$, 35°C, Et_3N) could not be studied because of the weak solubility of GSH in $CDCl_3$. But in a more polar solvent (water / acetonitrile (1:1)) mono demethylation of DMPT was observed with transfer of the methyl group to GSH. After 24 hours at 35°C the 50% of the products had followed this reaction (scheme 5).(For this last solvent mixture the methyl transfer between DMPT and benzenethiol in the presence of triethylamine is strongly accelerated compared with CDCl3 and complete after 1 hour at 35°C).



Scheme 5

N-methylimidazole was selected as a tertiary base which is structurally similar to the lateral chain of histidine in the active site of the enzyme (Scheme 6). N-methylimidazole and benzenethiol, in CDCl3 (B conditions, see

experimental part) were shown to produce signals corresponding to MPT and dimethylimidazolium after 24 hours using ^{31}P and ^{1}H NMR spectroscopy. After 100 hours, the progress of the reaction was 60%. The reaction was achieved after a week at 35°C. In these conditions the demethylation and the transfer towards the benzenethiol takes place in the presence of a clearly weaker base (pKa ≈ 7) than triethylamine.



Scheme 6

In order to better understanding of the results obtained with the enzyme, the de-alkylation reaction was also studied with three other organophosphorus substrates : trimethylthiophosphate, bis-(diisopropylamino)-methoxyphosphorothioamidate and DEPT which are involved in our study of the enzyme actvity. These results show trimethylthiophosphate undergoes mono demethylation (A conditions : 50% of progress after 24 hours ; B conditions : 70% after 24 hours). For the bis-(diisopropylamino)-methoxyphosphorothioamidate no reaction was detected whatever the conditions. For the DEPT, mono de-ethylation was observed in A conditions, but the reaction was clearly slower (15% of progress after 20 days) than mono demethylation of DMPT. The slowness of the reaction for DEPT can be attributed to the steric hindrance of the ethyl groups and resonance stabilization in the adjoining carbons ; the absence of reaction with the second substrate can be related to the strong back coordination of the two nitrogens towards phosphorus (the two nitogens are exactly planar).

CONCLUSION

The reaction model studied in a non-enzymatic medium can help to explain the regiospecificity of GSTs. When dearylation takes place, a general basic catalysis can be proposed in which the thiol of the GSH is activated by a nearby tyrosine or serine. When monodealkylation takes place, results obtained here seem to indicate the required presence of a basic group in the active site of the enzyme. This basic group functions as an intermediate acceptor of the alkyl group. The lateral chain of an histidine is a potential candidate to play this role in some of these enzymes.

EXPERIMENTAL

Reagents : benzenethiol, methylimidazole and glutathione were purchased from Aldrich ; DMPT and DEPT were purchased from C.I.L. (Cluzeau Info Labo France).

Trimethylthiophosphate was prepared by sulfuration of trimethylphosphite with S_8.

The synthesis of bis-(diisopropylamino)-methoxy thiophosphoramidate was carried out by reaction of dichloromethoxyphosphite with four equivalents of diisopropylamine in anhydrous ether at 0°C. The bis-(isopropylamino)-methoxyphosphite obtained was sulfurated by addition of one equivalent of S_8 at room temperature. The reaction was followed by ^{31}P NMR. The precipitate was filtered and recrystillized in ethanol.

Synthesis of dealkylated parathion : MPT was performed as follow : 20 mg of DMPT were dissolved in 1 ml of 50% triethylamine in $CDCl_3$. The reaction is total after five days of incubation at room temperature. Identities of all products were confirmed by ^{31}P and ^{1}H NMR spectroscopy. (^{31}P NMR DMPT δ ppm 65.8, MPT δ ppm 53.8). The reaction mixture was then evaporated to a syrup. The salt obtained, MPT triethylammonium salt, was diluted in water and was protonated by traitement with DOWEX 8X50 anion exchange resin. This final solution was used as reference for TLC analysis of metabolites without further purification.

Insecticide metabolization by GST : Dearylation was tested by incubating the insecticide (DMPT, 0.3 mM) at 25°C with 0.15 mg of GSTI1 in 1 ml of 50 mM phosphate buffer pH 7, in presence or in absence of 0.1 mM GSH . The dearylation product of DMPT degradation, S-paranitrophenylglutathione, is described to show a typical λ_{max} at 342nm.[6] Spectral scans between 300 and 400 nm were done at various incubing time on the same reaction without being able to detect any absorbtion at this wave length. For the analysis of dealkylation products, 0.3 mM DMPT was incubated with 0.15 mg of GSTI1 in 500 µl of 50 mM phosphate buffer pH 7, in presence or in absence of 0.1 mM GSH. The reaction was carried out at 25°C and stopped after 1h by adding 50 µl of 1.2 M HCl. The metabolites were extracted twice by 200 µl of Et_2O. As control, each potential metabolites was incubed in the same conditions without enzyme. The products of metabolism were separated by TLC on Whatman silica F254 plates. The plates were developed first with chloroform/acetone 9:1 to a height of 14.5 cm. They were then dried at room temperature and run in the same direction for 5.8 cm with isopropanol/water/acetic acid 60:10:5. Compounds were located under UV light and identified by comparison with standard samples: the bis dealkylated parathion (paranitrophenylthiophosphate) $R_f = 0.17$, the single dealkylated parathion (MPT) $R_f = 0.32$, the paranitrophenol $R_f = 0.52$, the DMPT $R_f = 0.72$.

Experimental conditions for the study of demethylation : two sets of conditions were used.

A conditions : 20 mg of methylparathion (70 µmole) are put in a NMR tube (5mm) with 0.5 ml of deuterated solvant. 7 µmoles of the tertiary base and 70 µmole of benzenethiol (or GSH) are added. Temperature was regulated at 35°C.

B conditions : the same experimental conditions as above are used except for the quantity of base, which was higher : 70 µmole.

NMR Spectroscopy : Spectra were recorded on a BRUCKER AC 80 and AC 200 machine, working at 80 and 200 MHz for ^{1}H and 81 MHz for ^{31}P. Solvent : $CDCl_3$. δ ppm . Accuracy : ± 2% of the integrated signals.

DMPT : ^{1}H NMR 7.77 (m, 4H, phenyl) ; 3.86 (d, 6H, OCH3). ^{31}P NMR 65.8. Triethylamine : ^{1}H NMR 2.37 (q, 6H, -CH2-) ; 0.79 (t, 9H,CH3). Methylphenylthioether : ^{1}H NMR 7.2 (m, 6H, phenyl) ; 2.4 (s, 3H, CH3-). Triethylammonium cation : ^{1}H NMR 3.1 (q, 6H, -CH2-) ; 1.3 (t, 9H, CH3-). MPT : ^{1}H NMR 7.8 (m, 4H, phenyl) ; 3.7 (d, 3H, OCH3). ^{31}P NMR 53.8 . Methyltriethylammonium cation : ^{1}H NMR 3.55 (q, 6H, -CH2-) ; 3.1 (s, 3H, $CH3N^+$) ; 1.3 (t, 9H, CH3-). S-methylglutathione : the transfered methyl group on the sulfur atom of GSH shows a ^{1}H NMR signal at δ 2.05 ppm. Methylimidazole : ^{1}H NMR 6.9 (m, 3H, -CH-) ; 3.6 (s, 3H, CH3N). Dimethylimidazolium cation : ^{1}H NMR 6.8 (m, 3H, -CH-) ; 4 (s, 3H, $CH3N^+$) ; 3.5 (s, 3H, CH3N). DEPT: ^{31}P NMR 61.6. Ethylparanitrophenylthiophosphate : ^{31}P NMR 50.8.

ACKNOWLEDGMENTS

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