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Glutathione *S*-Transferase Catalysed Dehalogenation of Haloaromatic Compounds which Lack Nitro Groups Near the Reaction Centre

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Abstract: A comparative kinetic study of the rat-liver glutathione *S*-transferase catalysed dehalogenation of fluoro- and chloroaromatic compounds showed no significant decrease of the F/Cl mobility ratio on going from substrates that carry an *ortho*-nitro group to those which lack nitro groups near the reaction centre. This suggests a so far unrecognized activity of *S*-transferases in providing "solvation" for the leaving halide anion at the hydrophobic reaction centre. © 1998 Elsevier Science Ltd. All rights reserved.

Glutathione *S*-transferases are a family of enzymes occurring as dimeric proteins that play two vital roles: detoxification of organisms and cellular translocation of material. Thus, they freed plants from herbicides[1] and animals from xenobiotics, like drugs and carcinogens,[2] and sequester anthocyanins for transport into vacuoles of angiosperms.[3] A strategy in common underlies these processes: in all cases a glutathione *S*-conjugate is formed between the thiol moiety of the tripeptide glutathione as a nucleophile and the electrophilic substrate, be that a herbicide,[3] an antocyanin,[3] or a xenobiotic,[2] while the mechanism of translocation of anthocyanins resembles that of herbicides.[3] These conclusions were based on a series of experimental studies (comprising protein encoding by dedicated genes,[3] Michaelis-Menten kinetics,[2a] and X-ray diffraction analysis for glutathione-substrate conjugates within glutathione *S*-transferases[2b]) as well as on theoretical calculations for model reactions.[2b]

Important mechanistic aspects of the detoxification processes induced by glutathione *S*-transferases are still unclear, however, which limits our possibilities of intervention in maintaining or restoring their full potential in the liver, which, at present high level of environmental pollution, represents our primary guard. A case in point is the glutathione *S*-transferase catalysed dehalogenation of haloaromatics, where kinetic studies with *ortho*-nitro substituted substrates showed the relative leaving-group mobility order $F > Cl \approx Br \approx I$.[2a] This is the order always observed for *ortho*-nitro-activated nucleophilic aromatic substitution occurring by the addition-elimination mechanism (S_NAr),[4] which was therefore assumed for the *S*-transferase processes too. These observations contrast with the reverse order of halogen mobility observed for other important detoxification processes by soil bacteria, exemplified by the dehalogenation of 4-halobenzoate by 4-chlorobenzoyl-CoA dehalogenases,[5] which was interpreted in the framework of the S_NAr mechanisms, generically assuming the leaving-group release in a slow step,[5b] or suggesting, by a faulty reasoning, that this may simply be due to a rapid formation of the intermediate.[5a] Radical mechanisms have been alternatively taken into consideration.[5a] What has never been questioned is why

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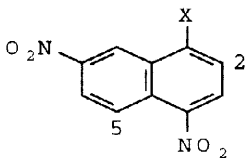
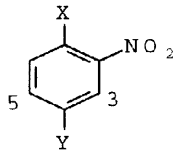
the release of fluoride may be fast or slow in the context of the active centre of the enzyme, which is of central relevance as to the mechanism of these detoxification processes. We reasoned that all aromatic substrates so far examined in processes catalysed by glutathione *S*-transferases possess an *ortho*-nitro group, which may represent an intramolecular catalytic centre.[4] By examining aromatic substrates which lack nitro groups in the vicinity of the reaction centre we obtained results that offer new clues as to the function of the active centre of the enzyme.

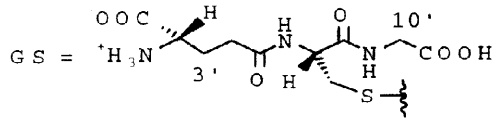
As substrates we chose 1-fluoro- **1** and 1-chloro-4,7-dinitronaphthalene **2**,[4,6] which, although lacking an



1 X=F
2 X=Cl
3 X=GS



4 X=F, Y=NO_2
5 X=Cl, Y=NO_2
6 X=Br, Y=NO_2
7 X=GS, Y=NO_2
8 X=F, Y=CF_3
9 X=Cl, Y=CF_3
10 X=GS, Y=CF_3



ortho-nitro group, are highly activated, thus allowing determination of the kinetics of halogen replacement under mild conditions. For comparison, the reactions of the *ortho*-nitro substituted substrates **4-6** and **8-9** were selected.

Products **3, 7** and **10** were prepared by non-enzymatic reactions[7,8] and were fully characterized.[9] Rat-liver glutathione *S*-transferase (EC 2.5.1.18) was purchased from SIGMA as a lyophilized, purified powder;[10,11] Ellman-standardized[12] glutathione solutions in 0.1M potassium phosphate buffer at pH 6.5 were prepared under N_2 just before use. Pseudo-first-order kinetics were run by adding to 2 ml of these glutathione solutions containing enzyme 10^{-6}M (assayed spectrophotometrically[2a] at λ = 280 nm) 20-50 x 10^{-3} ml aliquots of either the benzene substrate in CH_3CN solution[2a] or the naphthalene substrate, the latter in DMSO for solubility reasons[7] (thus starting with initial concentrations 10^{-3} M glutathione and 10^{-5} M substrate) in a thermostatted cuvette at 25 ^{0}C under magnetic stirring. The reaction progress was monitored by the UV absorption increase at 380, 340 and 365 nm resulting from formation of the products **3**,[13] **7** or **10**, respectively. The electrical signal corresponding to the UV absorption was sampled every 0.1 s (f_c = 10 Hz) during 300 s.[14] The kinetic data were elaborated[15] by the Lineweaver-Burk linear version of the Michaelis-Menten equation. That the nature of the products under kinetic condition was the same (**3**, **7**, and **10**) as under preparative conditions was confirmed by HPLC analysis[7,16] for reactions carried out in flasks;[17] this also allowed to grossly confirm the kinetic data obtained by UV analysis.

The kinetic data for the benzene derivatives **5**, **6**, **8** and **9** (Table) conform to published values for a variety of glutathione *S*-transferases;[2] comparing these data with those for the naphthalene compounds (**1** and **2**) it is seen that there is no significant decrease of the F/Cl mobility ratio on going from substrates which carry an *ortho*-nitro to those which lack nitro groups near the reaction centre. This is shown both by the k_c^F/k_c^{Cl} ratio (3.7 with *ortho*-nitro group and 3.4 without) and the $(k_c/K_m)^F/(k_c/K_m)^{Cl}$ ratio (4.8 with *ortho*-nitro group and 0.8 without).

These observations can be interpreted in the light of the recognized role of glutathione *S*-transferases to provide a hydrophobic environment where a molecule of water is allowed to enter as a reactant:[2,5] our view is that an *ortho*-nitro group may exert catalytic assistance to the removal of a proton from the nucleophile[4] - a thiol proton in the glutathione *S*-transferase reactions[2,18] - thus masking the intrinsic mobility order F << Cl ≈ Br ≈ I in S_NAr.[4,19] Since, as seen above, in the glutathione *S*-transferase processes fluorine is a good leaving group even in absence

Table. Kinetic data for glutathione *S*-transferase catalysed dehalogenation of nitro- (and trifluoromethyl) activated haloaromatic compounds

	k_c (s^{-1})	K_m (μM)	k_c/K_m ($M^{-1} s^{-1}$)	k_c^X/k_c^{Cl}		$\frac{(k_c/K_m)^X}{(k_c/K_m)^{Cl}}$		
				DNN[a]	DNB[b]	DNN[a]	DNB[b]	TNB[c]
1-fluoro-4,7-dinitronaphthalene[d] (**1**)	15±1	350±10	(4.2±0.2)10^4	3.4		0.8		
1-chloro-4,7-dinitronaphthalene[d] (**2**)	4.4±0.4	81±3	(5.2±0.3)10^4	1.0		1.0		
1-fluoro-2,4-dinitrobenzene[d] (**4**)	19±2	92±4	(2.0±0.1)10^5		3.7		4.8	
1-chloro-2,4-dinitrobenzene[d] (**5**)	5.1±0.5	120±5	(4.2±0.2)10^4		1.0		1.0	
1-bromo-2,4-dinitrobenzene[d] (**6**)	7.6±0.6	150±6	(5.1±0.2)10^4		1.5		1.2	
1-fluoro-4-trifluoromethyl-2-nitro benzene[e] (**8**)			(1.01±0.06)10^3					9.0
1-chloro-4-trifluoromethyl-2-nitro benzene[e] (**9**)			(1.12±0.06)10^2					1.0

[a]Dinitronaphthalene series. [b]Dinitrobenzene series. [c]Trifluoromethylnitrobenzene series. [d]Data from the best linear fitting with r = 0.999. [e]Data at a single concentration, $4x10^{-3}$ M, which only allows the value of k_c/K_m to be determined.

of the potential assistance of an nitro group placed near the reaction centre, we suggest that these enzymes, while assisting deprotonation of glutathione SH or stabilisation of glutathione S⁻,[18] are able to provide "solvation" for the leaving group at the hydrophobic reaction centre. This should hold for all enzymes in this family and not only for the SIGMA preparation of glutathione *S*-transferase[10] since the kinetic properties of the subunits are additive, i.e. they act independently, as if the units were joined from the side opposite to the glutathione and substrate binding sites.[20]

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8. Glutathione was added to the respective fluoro-aromatic compound (**1**, **4**, and **8**) in methanol containing 1 M aqueous $NaHCO_3$ under stirring at r.t. The products were isolated by acidic work up and purified by reversed phase FC. No products of reduction of the nitroaromatic compounds were detected.

9. **3**: UV (EtOH), λ_{max} (ϵ, M^{-1} cm^{-1}) 378(5300), 294(5800), 254 nm(11000). ^1H NMR in CD_3OD (here and in the following taken at 299.94 MHz, δ_H in ppm with respect to the solvent at δ_H 3.31, *J* in Hz, assignments confirmed by selective decoupling irradiations, $^1H,^1H$- and $^1H,^{13}C$-COSY) δ_H 8.44 (d, $J_{2,3}$ 8.1, 2-H); 7.95 (d, $J_{3,2}$ 8.1, 3-H); 8.73 (d, $J_{5,6}$ 9.6, 5-H); 8.46 (dd, $J_{6,5}$ 9.6, $J_{6,8}$ 2.1, 6-H); 9.26 (d, $J_{8,6}$ 2.1, 8-H); 3.80 and 3.54 ((dd, *J* 14.1, 5.1) and (dd, *J* 14.1, 9.0), resp., H_2-CS); 4.01 (m, 2'-H); 2.19 (m, 3'-H_2); 2.59 (t, $J_{4',3'}$ 6.9, 4'-H_2); 4.75 (dd, *J*9.0; 5,1, 7'-H); 3.92 (s, 10'-H_2). ^{13}C NMR in CD_3OD (here and in the following taken at 75.43 MHz, δ_C in ppm with respect to the solvent at δ_C 49.00, C-multiplicities by DEPT) δ_C 174.51, 172.68, 172.18 and 171.68 (all s, C-1', C-5', C-8', C-11'), 147.38, 146.67, 145.51, 132.56 and 129.08 (all s, C-1, C-4, C-7, C-9, C-10), 128.30(d, C-2), 127.27 d, C-3), 127.20 (d, C-5), 123.59 (d, C-6), 121.81 (d, C-8), 53.60 (d, C-2'), 53.42 (d, C-7'), 41.86 (t, C-10'), 35.62 (t, CH_2S), 32.39 (t, C-4'), 26.96 (t, C-3'). FAB-MS (glycerol, H^+) m/z 524 (MH^+, 3%).

7: UV (MeOH), λ_{max} (ϵ) 340 (10500), 262 nm (6500). ^1H NMR (CD_3OD) δ_H 8.99 (d, $J_{3,5}$ 2.5, 3-H); 8.49 (dd, $J_{5,6}$ 9.0, $J_{5,3}$ 2.5, 5-H); 8.02 (d, $J_{6,5}$ 9.0,6-H); 3.77 and 3.43 ((dd, *J* 13.9, 5.2) and (dd, *J* 13.9, 9.0), resp., H_2-CS); 4.04 (m, 2'-H); 2.20 (m, 3'-H_2); 2.63 (t, $J_{4',3'}$ 7.2, 4'-H_2); 4.78 (dd, *J* 9.0; 5,1, 7'-H); 3.91 (s, 10'-H_2). ^{13}C NMR (CD_3OD) δ_C 174.53, 172.75, 172.08, 171.66 (all s, C-1', C-5', C-8', C-11'), 129.39 and 128.43 (two d, C-5 and C-6), 122.28 (d, C-3), 146.86 (three s, C-1, C-2, C-4), 145.67, 146.09, 53.84 (d, C-7'), 53.60 (d, C-2'), 41.80 (t, C-10'), 34.99 (t, CH_2S), 32.39 (t, C-4'), 26.96 (t, C-3'),. FAB-MS (glycerol,H^+) m/z 474 (MH^+, 2%).

10: UV (MeOH), λ_{max} (ϵ) 365 (2500), 267 (6700), 250 (10200). ^1H NMR (CD_3OD) δ_H 8.45 (s, 3-H); 7.97 (s, 5-H and 6-H); 3.69 and 3.37 ((dd, *J* 13.9, 5.1) and (dd, *J* 13.9, 9.0), resp., H_2-CS); 4.05 (t, $J_{2',3'}$ 6.9, 2'-H); 2.19 (m, 3'-H_2); 2.59 (t, $J_{4',3'}$ 6.9, 4'-H_2); 4.74 (dd, *J* 9.0; 5,1, 7'-H); 3.94 (s, 10'-H_2). ^{13}C NMR (CD_3OD) δ_C 174.47, 172.28, 171.56, 171.41 (all s, C-1', C-5', C-8', C-11'), 147.50, 142.50 (two s, C-1 and C-2), 130.93 (d , J_{C-F} 4.0, C-5), 129.64 (d, C-6), 128.20 (s, J_{C-F} 34, C-4), 126.50 (s, J_{C-F} 272, CF_3), 124.04 (d, J_{C-F} 4.0, C-3), 53.48 (d, C-2'), 52.95 (d, C-7'), 41.95 (t, C-10'), 34.83 (t, CH_2S), 32.33 (t, C-4'), 26.97 (t, C-3'),. FAB-MS (glycerol,H^+) m/z 497 (MH^+, 2%).

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13. For compounds **1** and **2**, which absorb light of the wavelength selected for our measurements (380nm, ϵ 1900 and 4000, respectively), the kinetics were followed from $\Delta\epsilon$ between $\epsilon_{product\ 3}$ and $\epsilon_{appropriate\ substrate\ (1\ or\ 2)}$.
14. Sampling was accomplished with a home-computerized spectrophotometer (Perkin-Elmer Lambda 3) equipped with a data acquisition board (AT-MIO-16X National Instruments).
15. In order to satisfy the initial rate condition, only data points in the range 0-40 s were used for the linear regression analysis, which was carried out by a home designed program based on the National Instruments LabView software.
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