Glutathione transferase P1-1: self-preservation of an anti-cancer enzyme

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Self-preservation is a typical property of living organisms, observed in the simplest prokaryotic cell as well as in the more complex pluricellular organisms. Surprisingly we found a self-preservation mechanism operating at the level of a single enzyme. Human glutathione transferase P1-1 operates in such a way towards either killer compounds (competitive and irreversible inhibitors) or physical factors (temperature and UV-rays), which could suppress its detoxicating and anti-cancer activity in the cell. This property, here termed 'co-operative self-preservation', is based on a structural intersubunit communication, by which one subunit, as a consequence of an inactivating modification, triggers

a defence arrangement in the other subunit. Paradoxically this ability, developed during evolution for the survival of the cell, may not always be advantageous for us. In fact, glutathione transferase P1-1 is overexpressed in most tumour cells and pharmacological attempts to inhibit this enzyme *in vivo*, to prevent the drug resistance phenomenon during chemotherapy, may be thwarted by such self-preservation.

Key words: detoxification, glutathione transferase (GST), self-preservation.

INTRODUCTION

Multimeric structures, observed in many enzymes, have often evolved their activity accompanied by sophisticated regulatory mechanisms, based on intersubunit communications triggered by etherotropic or homotropic effectors (co-operativity). We propose here that co-operativity may be utilized by glutathione transferase P1-1 (GSTP1-1) to provide self-preservation against chemical or physical attacks, which threaten its catalytic efficiency. This enzyme belongs to a superfamily of detoxicating enzymes able to catalyse the conjugation of GSH to the electrophilic centre of a wide range of toxic compounds and carcinogens [1–3]. Furthermore, GSTP1-1 interacts with the C-terminal of Jun-kinase modulating the induction of apoptosis [4]. It is highly expressed in several organs including kidney, spleen, skin, erythrocytes and placenta, and its anti-cancer role is underlined by increased skin carcinogenesis observed in mice lacking GSTP1-1 [5]. Paradoxically its detoxicating activity is, in part, responsible for the deleterious development of cellular multi-drug resistance towards a number of chemotherapeutic agents [2,6]. In fact, GSTP1-1 is overexpressed in most tumour cells (reaching 1–2 % of the cytosolic proteins) [7] and promotes the conjugation of GSH to alkylating cytostatic drugs, leading to their inactivation and extrusion from the cell by means of specific efflux pumps [8]. To counteract this phenomenon specific inhibitors of this enzyme have been designed. Ethacrynic acid (EA), for example, has been found to sensitize tumour cells to the cytotoxic effects of alkylating agents and, *in vivo*, to enhance the effects of chlorambucil in patients with chronic lymphoblastic leukaemia [9]. However, a number of unwanted clinical side effects limit its clinical utility [9]. More recently a number of glutathione derivatives, e.g. TER199, were found to be potent and specific inhibitors of GSTP1-1 and have been developed for clinical treatment in tumours overexpressing the enzyme [9]. These drug-design advances take advantage of the atomic structures of the enzyme,

which have been elucidated during the last few years. The crystal structures also show a strict structural identity of the two subunits, as well as of the two GSH-binding sites (G-sites), both able to bind GSH by at least eight polar contacts and numerous hydrophobic interactions [10]. Crucial for catalysis is a hydrogen bond between Tyr7 and the thiol group of GSH, which forces the latter into an ionized state and into a productive conformation [1]. For many years GSTP1-1 has been thought to be a non-co-operative enzyme, as both kinetics and substrate binding follow a normal hyperbolic behaviour. However, selected point mutations reveal a latent co-operativity [11–13] and a few old observations also suggest intersubunit communication. In 1989 Ricci et al. [14] found that any chemical modification of a Cys residue (Cys47 in the human enzyme) caused strong inactivation but, curiously, the equivalent Cys in the other subunit displayed non-equivalent reactivity. At that time, the identity of the two subunits being unknown, we could not appeal to a co-operative mechanism, but this behaviour can be now explained in terms of induced asymmetry, i.e. the modification of one Cys causes a shielding of the second, as a consequence of a structural modification in the adjacent subunit. In this paper we suggest that this curious non-equivalent reactivity of a crucial residue is a particular case of a more general mechanism termed here 'co-operative self-preservation' which has likely evolved to preserve activity under different stress conditions and is active whenever strong inhibitors or physical factors threaten the functionality of this enzyme. A rational explanation of this phenomenon at molecular level is proposed by means of molecular dynamics (MD) simulations.

EXPERIMENTAL PROCEDURES

Enzyme

Human GSTP1-1 was expressed in *Escherichia coli* and purified as described previously [12]. Enzyme activity was measured

Abbreviations used: GSTP1-1, glutathione transferase P1-1; GST, glutathione transferase; EA, ethacrynic acid; G-site, GSH-binding site; NBD-Cl, 7-chloro-4-nitrobenzofurazan; MD, molecular dynamics; CDNB, 1-chloro-2,4-dinitrobenzene; DNDGIC, dinitrosyl-diglutathionyl-iron complex.

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by following the enzymic conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) (1 mM) to GSH (1 or 10 mM) in 1 ml (final volume) of 0.1 M potassium phosphate buffer, pH 6.5 at 25 °C. The reaction was monitored spectrophotometrically at 340 nm where the product absorbs (ϵ at 340 nm = $9.6 \text{ M}^{-1} \text{ cm}^{-1}$).

Inactivation by alkylating compounds

GSTP1-1 (2 μM) was reacted with 35 μM NBD-Cl (7-chloro-4-nitrobenzofurazan) in 0.1 M sodium acetate buffer, pH 5.0 at 25 °C. Alternatively, GSTP1-1 (9 μM) was reacted with EA (0.2 mM and 1 mM) in 0.1 M potassium phosphate buffer, pH 7.0 at 25 °C. At various times, aliquots of the solution were assayed for activity. In the case of EA, the same experiments were also performed on a crude homogenate of human placenta containing 4 μM GSTP1-1. Briefly, 1 g of fresh tissue was homogenized in 6 ml of 0.01 M potassium phosphate buffer, pH 7.0 and centrifuged at 12 000 *g* for 20 min. A 0.9 ml aliquot of the clear supernatant was brought to 1 ml final volume with 0.1 M potassium phosphate buffer, pH 7.0 and incubated with 0.2 mM EA.

Inhibition by DNDGIC (dinitrosyl-diglutathionyl-iron complex)

GSTP1-1 (4.4 μM) in 0.1 M potassium phosphate buffer, pH 7.0, was incubated with variable amounts of DNDGIC (from 0.5 μM to 9 μM) prepared as described previously [15]. After a 2 min incubation, 12 μl aliquots were assayed for residual activity at pH 6.5 (in 1 ml final volume) in the presence of 10 mM GSH and 1 mM CDNB. To reach equilibrium conditions in the activity test, the inhibited enzyme and 10 mM GSH were incubated for 5 min before addition of CDNB. This procedure differs from that reported previously [15]. K_i values were calculated by assuming that DNDGIC is a competitive inhibitor for GSH [15]. The same experiments were performed on a crude homogenate of human placenta containing 4 μM GSTP1-1 in the final incubation mixture.

Inactivation by UV irradiation

GSTP1-1 (4.4 μM) in 0.1 M potassium phosphate buffer, pH 7.0 was irradiated in a quartz cuvette (thermostated at 5 °C) with a deuterium lamp [wavelength (λ) max. = 254 nm] placed at a distance of 10 cm from the sample. At various times, aliquots of the solution were assayed for activity. The same experiments were performed also in the presence of 1 mM GSH.

Inactivation by dilution

GSTP1-1 ($5 \times 10^{-8} \text{ M}$) was incubated at 25 °C in 0.1 M potassium phosphate buffer, pH 6.5, in the absence or in the presence of 10 mM GSH. At various times aliquots were assayed for activity.

MD simulations

The MD simulation was performed as described previously [16], except for the following details. Initial coordinates were taken from the crystal structure of the complex with GSH (Protein Database entry 6GSS). The holo protein was centered in a truncated octahedron box, edge size 8.4 nm, resulting in a final fully hydrated system containing 8074 water molecules. The simulation was performed using the GROMOS 96 software and the 43A1 force field [17], and MOLMOL [18] was used for molecular graphics and to calculate solvent accessible surfaces. Equili-

bration of the system was performed during the first 50 ps, after which protein stability was demonstrated by stationary values of energies, total accessible surface, radius of gyration, intramolecular H-bonds and secondary structure.

RESULTS AND DISCUSSION

Self-preservation against killer compounds

The non-equivalence of the two Cys47 residues of the dimeric GSTP1-1 in their reaction with thiol reagents, is a first indication of a self-preservation mechanism operating in this enzyme. Two representative experiments are reported here, showing the biphasic inactivation (and thus the different reactivity of the two Cys47 residues) with classical thiol reagents, NBD-Cl (Figure 1a), and EA (Figure 1b), a well known inhibitor of GSTP1-1 used during chemotherapeutic treatments to minimize the multi-drug resistance [9]. Self-preservation against EA, which causes an almost half-site inactivation at moderate inhibitor concentrations, is equally recovered in a crude homogenate from human placenta, where GSTP1-1 is the prevalent isoenzyme (>90% of all GSTs, Figure 1b). Thus, the presence of many different proteins and endogenous compounds does not alter this co-operative behaviour.

A different case of self-preservation is found against DNDGIC, a natural NO carrier [19], which does not interact with Cys 47 but binds with extraordinary affinity to the G-site ($K_{i1} = 1 \times 10^{-9} \text{ M}$) such that it could suppress completely the usual conjugating activity in the cell (even at saturating GSH concentration) when present in amounts nearly stoichiometric to GSTP1-1. Binding of DNDGIC to one subunit triggers negative co-operativity in the apo subunit (Figure 1c), which loses its affinity for DNDGIC by about two orders of magnitude ($K_{i2} = 8 \times 10^{-8} \text{ M}$). The self-preservation against DNDGIC is still recovered in a crude homogenate of human placenta, suggesting that it could have physiological relevance (Figure 1c). Similar experiments performed under non-equilibrium conditions gave even higher affinity values [15].

Self-preservation against killer physical factors

Self-preservation in GSTP1-1 also happens when physical factors threaten the functionality of this enzyme. We observed that, when GSTP1-1 is exposed to high or low temperatures, self-preservation balances the negative effects of these stress conditions utilizing co-operativity [20]. Above 38 °C the affinity for GSH decreases remarkably, but the binding of GSH to one subunit increases the affinity of the adjacent one. Conversely, at low temperatures, a negative homotropic behaviour caused by GSH binding is observed (Figure 1d). Thus, at normal physiological GSH concentrations, the enzyme can work under proper substrate saturation irrespective of the temperature. This thermal self-preservation represents a likely advantage, since GSTP1-1 is the prevalent GST isoenzyme present in the upper layer of the epidermis [21], the only tissue exposed directly to thermal stress.

UV irradiation represents another noxious physical factor for an enzyme localized in the upper layer of epidermis. GSTP1-1 undergoes photoinactivation upon UV irradiation: Trp38 and Tyr7, both involved in GSH binding, appear to be modified based on analysis of spectral changes (results not shown). Again, after one subunit inactivation, the other one becomes more resistant to damage (Figure 1e).

Another case of self-preservation has been found under conditions of very low enzyme concentrations. Below $5 \times 10^{-7} \text{ M}$, GSTP1-1 inactivates in a time- and concentration-dependent

Figure 1 Self-preservation of GSTP1-1 under different stress conditions

(a) Inactivation by NBD-Cl. NBD-Cl (35 μ M) was incubated with GSTP1-1 (2 μ M) in 0.1 M sodium acetate buffer, pH 5.0. The modification of Cys 47 (results not shown) parallels the loss of activity. (b) Inactivation by EA. EA ($\blacktriangle$) 1 mM and ($\circ$) 0.2 mM were reacted with GSTP1-1 (9 μ M) in 0.1 M potassium phosphate buffer, pH 7.0. The modification of Cys 47 (results not shown) parallels the loss of activity. EA ($\bullet$) 0.2 mM incubated in a crude homogenate of human placenta containing approx. 4 μ M GSTP1-1. (c) Inhibition by DNDGIC. Purified GSTP1-1 (∇) and a crude homogenate of human placenta ($\blacktriangle$). By fitting the experimental data to a two-site inhibition model, inhibition constants are $K_{i1} = 10^{-9}$ M and $K_{i2} = 8 \times 10^{-8}$ M. (d) Temperature adaptation of GSTP1-1. The Hill plot has been reported in [20]. 5 $^{\circ}$ C ($\bullet$), 25 $^{\circ}$ C ($\circ$), 37 $^{\circ}$ C ($\blacksquare$) and, 43 $^{\circ}$ C ($\square$). At 43 $^{\circ}$ C, and at low GSH concentrations, K_D for GSH is 1.3 mM (low affinity state) while at 3 mM GSH K_D decreases to 0.2 mM (high affinity state); an opposite effect is observed at 5 $^{\circ}$ C. At physiological GSH concentrations, the enzyme displays a similar affinity for GSH between 5 $^{\circ}$ C and 43 $^{\circ}$ C. (e) Inactivation by UV irradiation in the presence ($\square$) or absence ($\blacksquare$) of 1 mM GSH. (f) Inactivation by dilution. GSTP1-1 (5×10^{-8} M) was incubated in 0.1 M potassium phosphate buffer, pH 6.5 in the presence ($\bullet$) or absence ($\circ$) of 10 mM GSH. Experimental details of each experiment are reported in the Experimental procedures. In panels (a, b, e) and (f), k_1 and k_2 are the pseudo-first-order kinetic constants for observed biphasic inactivations obtained by fitting the data to a bi-exponential decay equation. In all cases the extent of the first phase is within 45–55% of the initial activity. Each experimental point is the mean of three independent experiments, and S.E. for each point does not exceed 6%.

process [22]. While the apo-enzyme gives a relevant biphasic inactivation pattern, in the holo-enzyme the damage is restrained to an half-site inactivation (Figure 1f). These results complete previously reported observations [22].

MD simulation

We have used MD simulations to gain further understanding of this self-preservation phenomenon at the molecular level. Preliminary MD simulations of the isolated monomer showed that the active site 'breathes', interconverting between at least two families of conformers with a different exposure to solvent [23]. This result has been confirmed recently by dynamic fluorescence data [24] and by NMR experiments [25], indicating that this breathing, mainly due to the high flexibility of helix α_2 , is evident in the

apo-enzyme but is still present in the holo-enzyme, albeit to a lesser extent [24]. We have now run simulations of the dimer, focusing on intersubunit communications. Fluctuations of the two active sites do not appear either random or synchronous, but rather are largely anti-correlated (covariance = -0.4), so that when a closed structure is present in one subunit, the corresponding open conformer is favoured in the adjacent monomer (Figure 2, upper panel). This suggests a mechanistic interpretation of the observed self-preservation; any physical or chemical factor, which 'freezes' the open conformation in one subunit may favour the corresponding 'closed' conformation in the adjacent subunit. Interestingly, the hydrogen bonding interaction between GSH and Tyr7 is not permanent but only present for a fraction of the time (about 24%) as a consequence of an on-off switching. Up to 400 ps, our MD simulation shows a fully active dimer with

Figure 2 Solvent-accessible surface of the GSH S_{γ} atom in the two subunits during MD simulation, when the enzyme displays fully active conformation (50–400 ps)

A clear anticorrelation in the 'breathing' of the two active sites is observed (top panel) where the continuous line represents subunit A and the broken line is subunit B (covariance = -0.4). The middle and bottom panels show the comparison between the C_{α} root mean square fluctuations calculated from crystallographic temperature factors (broken line) and those derived from the MD simulation (continuous line), after the GSH molecule of monomer A has lost its proper binding (600–1000 ps). This 'half inactivation' is followed by a higher flexibility in the segment around helix $\alpha 2$ (residues 35–50) of the A subunit (middle panel), which is accompanied by a decrease in the fluctuations of the same region in the B subunit (bottom panel).

both G-sites bonding GSH in a conformation very similar to that seen in the crystal structure. However, between 400 and 600 ps of our 1 ns simulation, the GSH molecule of one subunit (monomer A) spontaneously leaves its hydrogen-bond interaction with Tyr7 in the active site and assumes a stable but unproductive geometry with its sulphur atom pointing towards the oxygen atom of Ile103 (Figure 3), and this non-productive binding is maintained for the rest of the simulation. This event leaves the average overall structure of both monomers largely unmodified, being accompanied only by a slight unfolding of the helix $\alpha 2$. This is reminiscent of the finding in crystals of the enzyme grown at low pH where the GSH sulphur atom points away from Tyr7 in a non-productive orientation [10]. The structural asymmetry found in our simulations could correspond to the experimentally observed half-inactivation at high dilutions, since in MD simulations the enzyme has no interactions with other protein molecules. How-

Figure 3 Representative structures of the active site in the two subunits, after the GSH of the A monomer has lost its native interactions (in particular the H-bond with Tyr7, represented in magenta) and has assumed a new stable unproductive binding geometry with its sulphur atom interacting with the oxygen atom of Ile103

Spheres denote the key atoms. S_{γ} of GSH (yellow sphere) and the O atoms of Tyr7 and of Ile103 (red sphere). The GSH molecule is cyan and the protein is blue.

ever, a cause due to simulation conditions (e.g. temperature, force field, approximations) cannot be discounted. Whatever the cause, this event gives us the opportunity to examine what might happen to one subunit when the other one is inactivated. It turns out that productive GSH binding in the monomer B is stabilized; the permanence of the crucial hydrogen bond almost doubles when GSH loses its interaction with Tyr7 in monomer A. The effect of the observed 'half-inactivation' on protein dynamics has also been analysed. In the fully active enzyme, the fluctuations calculated from the simulation are close to those derived from the crystallographic temperature factors, but they are slightly increased as expected on the basis of the high temperature and of the solution state of the enzyme. However, in monomer A, after inactivation, the fluctuations of helix $\alpha 2$ are greatly increased when compared with other flexible portions of the enzyme (Figure 2, middle panel). Conversely, the corresponding helix $\alpha 2$ in the adjacent monomer B (which displays properly bound GSH) acquires a relatively increased rigidity (Figure 2, lower panel). Thus, when an active site is forced towards an abnormally increased flexibility, the other one becomes less flexible. Tyr49, which contacts helix $\alpha 2$ of the adjacent subunit (via helix $\alpha 4$), is a likely player in intersubunit communication, acting as a hinge which forces the G-site into the proper (and more rigid) conformation when the

Scheme 1 Model for the co-operative self-preservation in GSTP1-1

The model assumes that the reagent R, which causes irreversible inactivation, may interact only with the open conformation (●), freezing it. Alternatively, R may be a perturbing physical factor that stabilizes the open conformation. Closed conformation (■).

opposite G-site undergoes some structural perturbation (and increased flexibility). Clearly, we cannot be sure that the asymmetric structure as reported in Figure 3 represents exactly the snapshot of the half-inactivated enzyme obtained experimentally at high dilution, but the computational data account well for all the experimental results. Scheme 1 summarizes our model for self-preservation in GSTP1-1.

CONCLUDING REMARKS

The observed self-preservation phenomenon observed in GSTP1-1 towards chemical and physical killer factors enlarges our knowledge about the potential inherent in multimeric proteins, and is a fascinating example of Le Chatelier principle: 'If there is a change in the condition of a system in equilibrium, the system will adjust itself in such a way as to counteract, as far as possible, the effect of that change' [30]. Structural communication between subunits in enzymes is often utilized for a fine-tuning regulation of activity, whilst in GSTP1-1 it appears aimed at the fundamental survival of its detoxicating activity. Co-operative self-preservation could be present in a number of oligomeric enzymes. The non-equivalent reactivity of identical subunits towards killer compounds, like that observed in creatine kinase [26], deoxycytidylate hydroxymethylase [27], transketolase [28], succinyl CoA synthetase [29], and in other enzymes could now be revised in this new light.

The killer-resistance of GSTP1-1 may be an important contributing cause to the failure of pharmacologists in suppressing multi-drug resistance. It is well known that this phenomenon is partially due to GSTP1-1 and, even now, pharmacologists are trying to design efficient inhibitors for this enzyme. The self-preservation of GSTP1-1 towards EA, even observed in a crude homogenate (Figure 1), is convincing evidence that this mechanism may defeat, in part, pharmacological approaches to inhibit this enzyme. Results presented here may help pharmacologists to rationalize failures and overcome them with new approaches.

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