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Inhibition of human placenta glutathione transferase P1-1 by the antibiotic calvatic acid and its diazocyanide analogue

Evidence for multiple catalytic intermediates

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The inhibition mechanism of the dimeric human placenta glutathione transferase (GST) P1-1 by calvatic acid and the reaction intermediates, i.e. the diazocyanide analogue of calvatic acid, has been investigated at pH 7.0 and 30.0°C. Experiments performed at different molar ratios of inhibitor/GST P1-1 indicate that 1 mol calvatic acid inactivates 1 mol GST P1-1, containing two catalytically equivalent active sites. However, 2 mol of the diazocyanide analogue of calvatic acid inactivate 1 mol GST P1-1. Two disulfide bridges/dimer, probably between Cys47 and Cys101, have been formed during the reaction of GST P1-1 with calvatic acid and its diazocyanide analogue. The apparent second-order rate constants for GST P1-1 inactivation by calvatic acid and its diazocyanide analogue are $2.4 \pm 0.3\ M^{-1}\ s^{-1}$ and $(8.5 \pm 0.7) \times 10^3\ M^{-1}\ s^{-1}$, respectively. The reaction of calvatic acid with free L-cysteine can be described by a simple process with an apparent second-order rate constant of $(5.0 \pm 0.4) \times 10^1\ M^{-1}\ s^{-1}$. In contrast, a transient species occurs during the reaction of the diazocyanide analogue of calvatic acid with free L-cysteine. Kinetics may be described by a second-order process [the rate constant being $(8.0 \pm 0.5) \times 10^3\ M^{-1}\ s^{-1}$] followed by a first-order decay [the rate constant corresponding to $(1.2 \pm 0.1) \times 10^1\ s^{-1}$]. Calvatic acid represents an enzyme inhibitor acting much slower than its reaction intermediates (i.e. its diazocyanide analogue).

Keywords: human placenta glutathione transferase; enzyme inhibition; calvatic acid; cysteine oxidation; kinetics.

Glutathione transferase (GST) catalyzes the conjugation of glutathione with a variety of electrophilic compounds, representing one of the most efficient biological systems for the detoxification of alkylating agents (Mannervik, 1985; Mannervik and Danielson, 1988; Coles and Ketterer, 1990; Armstrong, 1991, 1994; Wilce and Parker, 1994). Moreover, this enzyme may be involved in cellular resistance to anticancer drugs, pesticides, herbicides, and specific inhibitors that may be useful for therapeutic applications (Waxman, 1990; O'Brien and Tew, 1996). Cytosolic GST, accounting for more than 80% of the total cellular enzyme activity, are non-covalent homodimers and heterodimers with molecular masses ranging between 23 kDa and 27 kDa (Mannervik, 1985). Each subunit contains one binding site for glutathione (G-subsite) and one cleft for the association of hydrophobic substrates (i.e., xenobiotics; H-subsite) (Mannervik, 1985). On the basis of their sequence similarities, substrate and inhibitor specificities and immunological properties, GST have been grouped into at least five distinct classes named α, μ, π, θ and σ (Mannervik et al., 1985, 1992; Meyer and Thomas, 1995; Meyer et al., 1991).

The antibiotic calvatic acid [2-(4-carboxyphenyl)diazenecarbonitrile 2-oxide; Fig. 1], isolated from the culture broth of *Calvatia lilacina* (Gasco et al., 1974), inhibits the growth of tumor cells and of microbial cultures (Umezawa et al., 1975; Calvino et al., 1986) and tubulin polymerization (Gadoni et al., 1989). Calvatic acid inactivates GST P1-1 by oxidizing the critical Cys47 residue, inducing the formation of two intrasubunit disulfide bridges, between Cys47 and Cys101 of each subunit (Ricci et al., 1991; Caccuri et al., 1994). The specific bond formation between Cys47 and Cys101 [at 1.8 nm in the reduced enzyme (Reinemer et al., 1992)] implies a remarkable conformational change of the protein (Caccuri et al., 1996; Ricci et al., 1996). Calvatic acid may therefore represent a model for the synthesis of more-specific GST–P1-1 inhibitors with therapeutic relevance, such as in drug resistance (O'Brien and Tew, 1996).

In the present study, a detailed analysis of the reaction of GST P1-1, a class-π isoenzyme (Mannervik et al., 1992), with calvatic acid and its diazocyanide analogue [2-(4-carboxyphenyl)diazenecarbonitrile; Fig. 1] is reported. Present data indicate that 1 mol calvatic acid reacts with 4 mol cysteine to form two cystines, while its diazocyanide analogue induces the formation

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Abbreviations. GST, glutathione transferase; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid).

Enzyme. Glutathione transferase (EC 2.5.1.18).

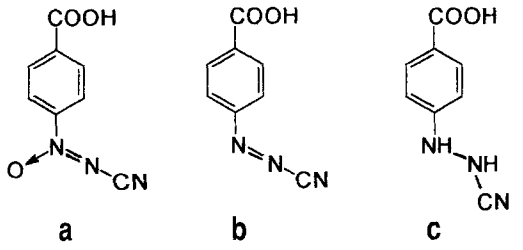

Fig. 1. Chemical structures of calvatic acid (a), and of its diazocyanide (b) and hydrazocyanide (c) derivatives.

of one disulfide bridge. Moreover, the intermediate product(s) of the reaction of GST P1-1 with calvatic acid (i.e. its diazocyanide analogue) inactivates the enzyme much faster than the calvatic acid itself. A transient species has been observed during the reaction of the diazocyanide analogue of calvatic acid with free L-cysteine.

EXPERIMENTAL PROCEDURES

Materials. *GST P1-1.* GST P1-1 was expressed in *Escherichia coli* and purified as previously reported (Lo Bello et al., 1995). Fully active enzyme showed a specific activity of 80 U/mg, at pH 6.5 and 25.0°C. The isoenzyme concentration was determined according to Lowry et al. (1951). The molarity of dimeric GST P1-1 was calculated on the basis of the 46-kDa molecular mass (Mannervik, 1985). The isoenzyme activity was assayed spectrophotometrically at pH 6.5 (0.1 M potassium phosphate) and 25.0°C, according to Habig et al. (1974). Moreover, GST P1-1 sulfhydryl groups were titrated with an excess of 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2; 1 mM) at pH 8.0, the reaction being followed spectrophotometrically at 412 nm (ε = 13.6 mM^{-1} cm^{-1}) (Ellman, 1959). Under all the experimental conditions, the spontaneous inactivation of GST P1-1 was assayed and found to be negligible.

Calvatic acid. Calvatic acid [2-(4-carboxyphenyl)diazenecarbonitrile 2-oxide; Fig. 1] was synthetized as previously reported (Sorba et al., 1995). The concentration of calvatic acid was determined spectrophotometrically on the basis of ε = 11.1 mM^{-1} cm^{-1} at 310 nm, pH 7.0 (0.01 M potassium phosphate) and 30.0°C. Under all the experimental conditions, the spontaneous inactivation of calvatic acid was assayed and found to be negligible.

Diazocyanide analogue of calvatic acid. The diazocyanide analogue of calvatic acid [2-(4-carboxyphenyl)diazenecarbonitrile; Fig. 1] was synthesized as follows. To a stirred and ice-water-cooled solution of 2-(4-carboxyphenyl)diazenecarboxyamide (Umezawa et al., 1975) (2.00 g, 10 mmol) and dry pyridine (4.8 ml, 60 mmol) in dry tetrahydrofuran (40 ml) was added dropwise over 45 min trifluoracetic anhydride (4.2 ml, 30 mmol). The reaction mixture was stirred for 30 min at room temperature and then poured into ice water. The orange precipitate formed was collected by filtration, washed with water and dissolved in hot benzene (20 ml). The insoluble impurities were eliminated by filtration and the solution was allowed to cool; the orange precipitate was collected by filtration, washed with benzene and dried (0.89 g, 51%). Decomposition, 156–158°C. Analysis, ($C_8H_5N_3O_2$) C,H,N ^{1}H-NMR, δ 8.29–8.07 (m,4H). ^{13}C-NMR, δ 166.39 (COOH), 156.15 (C4), 137.97 (C1), 132.09 (C3, C5), 124.97 (C2, C6), 112.53 (CN). MS *m/z* 175. ^{1}H-NMR and ^{13}C-NMR spectra were recorded in [^{2}H]acetonitrile at 200 MHz and 50 MHz, respectively, with a Bruker AC-200 spectrometer. The elemental analysis was performed by means of REDOX (Cologno Monzese, Milan, Italy) and the results are within 0.4% of theoretical values. The concentration of the diazocyanide analogue of calvatic acid was determined spectrophotometrically on the basis of ε = 14.1 mM^{-1} cm^{-1} at 337 nm, pH 7.0 (0.01 M potassium phosphate) and 30.0°C. The spontaneous inactivation of the diazocyanide analogue of calvatic acid, at pH 7.0 (0.01 M potassium phosphate) and 30.0°C, occurs with an half-time of about 4 h. Under all the experimental conditions, the spontaneous inactivation of the diazocyanide analogue of calvatic acid was assayed and found to be negligible.

Chemicals. Nbs_2, dithiothreitol and L-cysteine were purchased from Sigma Chemical Co.

Methods. *Reaction of calvatic acid and of its diazocyanide analogue with GST P1-1.* The reaction of GST P1-1 with calvatic acid was monitored as previously described (Caccuri et al., 1994). The diazocyanide analogue of calvatic acid was allowed to react with GST P1-1 as follows. In a typical experiment, the fully active enzyme (0.60 μM) was incubated with inhibitor (0.15, 0.30, 0.60 and 1.2 μM) dissolved in Me_2SO, at pH 7.0 (0.01 M potassium phosphate) and 30.0°C. Small aliquots of the incubation mixture were assayed for GST P1-1 activity at different times (from 30 s to 2 h). The reactivation of GST P1-1 was performed by passing the isoenzyme solution throughout a G-25 Sephadex column (1 cm×40 cm) equilibrated at pH 7.0 (0.01 M potassium phosphate), to remove the inhibitor and its by-products. GST P1-1 was incubated with an excess of the reducing agent dithiothreitol. The activity and the free thiol groups of GST P1-1 were determined before and after incubation with dithiothreitol.

Reaction of calvatic acid and of its diazocyanide analogue with free L-cysteine. Calvatic acid and its diazocyanide analogue were allowed to react with L-cysteine as follows. In a typical experiment, the inhibitor (30 μM), dissolved in Me_2SO, was mixed with cysteine (from 15 μM to 30 mM), at pH 7.0 (0.01 M potassium phosphate) and 30.0°C. The reaction was followed spectrophotometrically between 220 nm and 400 nm. Rapid kinetic experiments were performed on an Applied Photophysics kinetic spectrometer stopped-flow instrument equipped with a temperature-regulated observation chamber with a 1-cm light path. Typically, 200 points were collected on a faster time base, then 200 points were collected on a slower time base. Time-dependent spectra were reconstructed from single-wavelength observations (between 220 nm and 400 nm) by repetitively changing the wavelength after reagent-mixing steps. The synthesis of cystine, after following the reaction of cysteine with calvatic acid and its diazocyanide analogue, was monitored by amino acid analysis.

Data analysis. Optical deconvolution of time-dependent-spectra sets was performed by means of the software MATLAB (MathWorks), running on an Intel-486-based computer by means of singular-value decomposition in combination with curve-fitting algorithms, according to Henry and Hofrichter (1992). The best fit of the experimental data to the desired kinetic scheme was performed by means of the program FACSIMILE (AEA). The matrix of time-dependent spectra (A) is decomposed by singular-value decomposition into the product of three matrices, $A = U \times S \times V^T$, where the U columns are the basis spectra, and their time dependence is represented by the V columns. The diagonal values of the S matrix (the singular values) yield the relative occupancies of the basis spectra in the data set. If a data set is contributed by more than one optical transition, deconvolution of the optical components (provided they have different time-courses) can be achieved by simultaneously fitting the chosen V-column subset to the desired kinetic scheme. The resulting amplitude matrix can be used to reconstruct the optical species from the chosen subset of spectra.

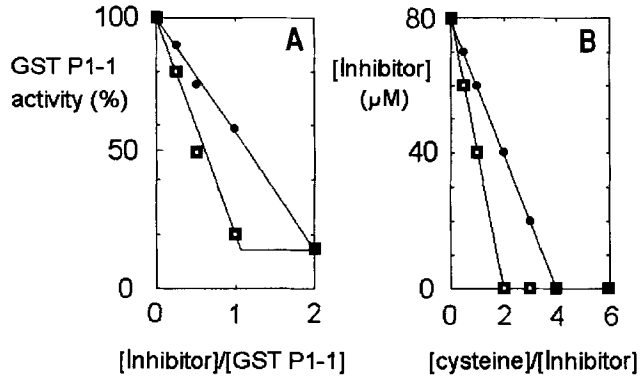

Fig. 2. Effect of inhibitor concentration on GST P1-1 inhibition and cystine formation. The dependence of the GST P1-1 activity on the inhibitor/GST P1-1 molar ratio is shown in (A). The dependence of the inhibitor concentration on the cysteine/inhibitor molar ratio is given in (B). The intercept of the straight lines indicates the stoichiometry of the reaction. □, calvatic acid; ●, diazocyanide analogue.

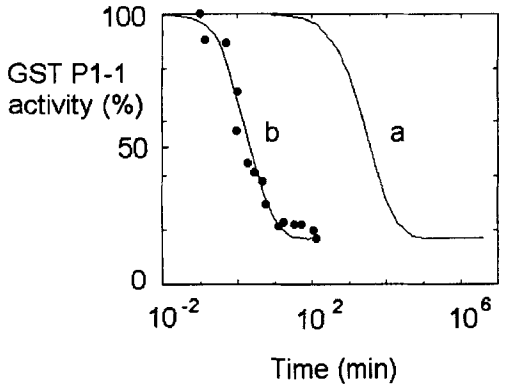

Fig. 3. Time-course of GST P1-1 inactivation by calvatic acid and by its diazocyanide analogue. Kinetics of GST P1-1 inactivation by calvatic acid (a) was computer simulated taking into account the same experimental conditions (0.6 μM enzyme, 1.2 μM inhibitor) for enzyme inactivation by the diazocyanide analogue (b; ●, experimental data). Values of rate constants for GST P1-1 inactivation, calculated from the best fit of the experimental data, according to Eqn 1, are given in Table 1.

RESULTS AND DISCUSSION

Data shown in Fig. 2 indicate that 1 mol calvatic acid reacts with 1 mol GST P1-1 (Caccuri et al., 1994), while 2 mol of its diazocyanide analogue inactivate 1 mol enzyme. 1 mol calvatic acid and 2 mol of its diazocyanide analogue react with both the catalytic sites, containing four cysteine residues, present in the dimeric isoenzyme.

The free thiol groups of GST P1-1 have been quantified before and after the reaction with the inhibitors. The isoenzyme contains four cysteine residues/monomer (i.e. eight/GST P1-1 dimer), but one thiol residue (i.e. two/dimer) is masked and does not react with specific reagents even in the presence of denaturants (Ricci et al., 1989). Therefore, the fully active dimeric isoenzyme shows six cysteine residues titratable with Nbs_2. After the complete reaction of GST P1-1 with calvatic acid and with its diazocyanide analogue (i.e., at inhibitor/GST P1-1 molar ratios of 1:1 and 2:1, respectively; Fig. 2A), only two cysteine side chains/dimer were titratable. Incubation of the inactive isoenzyme with excess dithiothreitol, a specific reducing agent of disulfide bridges (Cleland, 1964), restored the original isoenzyme catalytic activity, and six cysteine residues/dimer become titratable with Nbs_2.

As already reported for calvatic acid (Caccuri et al., 1994), its diazocyanide analogue inactivates GST P1-1 via a redox process that induces the disappearance of four titratable thiol groups/dimeric isoenzyme, probably due to the formation of the Cys47-Cys101 disulfide bridge in each subunit. Oxidation of Cys47 and Cys101 to cystine switches the isoenzyme towards an inactive conformation, which prevents the binding of the substrate to the active center (Caccuri et al., 1992).

Fig. 3 reports the time-course of GST P1-1inactivation by calvatic acid and by its diazocyanide analogue. The best fit of the experimental data, according to Eqn (1)

$$\text{inhibitor} + \text{GST P1-1} \rightarrow \text{inactive enzyme}, \quad (1)$$

allowed us to determine values of the second-order rate constant for GST P1-1 inactivation by calvatic acid ($2.4 \pm 0.3\ M^{-1}\ s^{-1}$; Table 1; Caccuri et al., 1994) and its diazocyanide analogue [$(8.5 \pm 0.7) \times 10^3\ M^{-1}\ s^{-1}$; Table 1]. This is a case of an enzyme inhibitor (i.e. calvatic acid) acting much slower (about 4000-fold) than the intermediate reaction product(s) (i.e. its diazocyanide derivative).

Fig. 4 shows the time-course, at 305 nm, for the reaction of calvatic acid with cysteine at different concentrations. The best fit of the experimental data, according to Eqn (2)

Table 1. Values of kinetic parameters for the reaction of calvatic acid and of its diazocyanide analogue with GST P1-1 and cysteine.

Inhibitor	Reagent	Rate constant
		$M^{-1}\ s^{-1}$
Calvatic acid	GST P1-1	2.4 ± 0.3 [a]
Diazocyanide analogue	GST P1-1	$(8.5 \pm 0.7) \times 10^3$ [b]
Calvatic acid	cysteine	50 ± 4 [c]
Diazocyanide analogue	cysteine	$(8.0 \pm 0.5) \times 10^3$ [d]

[a] Data from Caccuri et al. (1994).
[b] Calculated from data given in Fig. 3, according to Eqn (1).
[c] Calculated from data given in Fig. 4, according to Eqn (2).
[d] Calculated from data given in Fig. 5, according to Eqn (3).

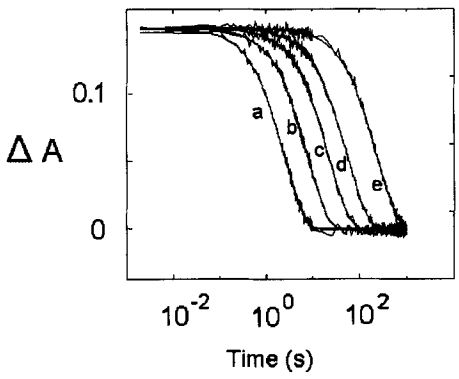

Fig. 4. Time-course of the absorbance change at 305 nm recorded during the reaction of calvatic acid with cysteine. Kinetics were obtained by mixing calvatic acid (30 μM) with cysteine (30, 10, 3, 1 and 0.3 mM; a–e, respectively). The value of the rate constant for cysteine oxidation, calculated from the best fit of the experimental data according to Eqn 2, is given in Table 1.

$$\text{calvatic acid} + \text{cysteine} \rightarrow \text{products}, \quad (2)$$

allowed us to determine the value of the second-order rate constant for cystine formation [$(5.0 \pm 0.4) \times 10^1\ M^{-1}\ s^{-1}$; Table 1].

The same absorbance dependence on the reaction time was observed at all the wavelengths explored in the 220–400-nm

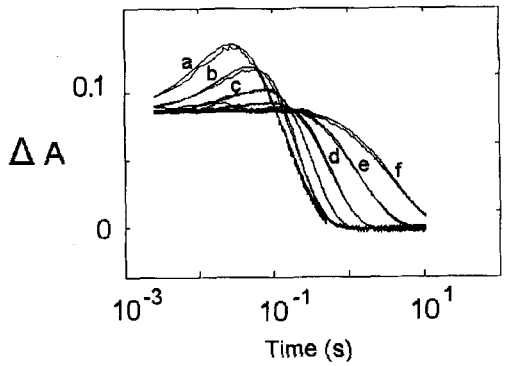

Fig. 5. Time-course of the absorbance change at 305 nm recorded during the reaction of the diazocyanide analogue of calvatic acid with cysteine. Kinetics were obtained by mixing the diazocyanide analogue of calvatic acid (30 μM) with different concentrations of cysteine (30, 10, 3, 1, 0.3 and 0.1 mM; a–f, respectively). Values of the rate constants for cysteine oxidation, calculated from the best fit of the experimental data according to Eqn 3, are given in Table 1.

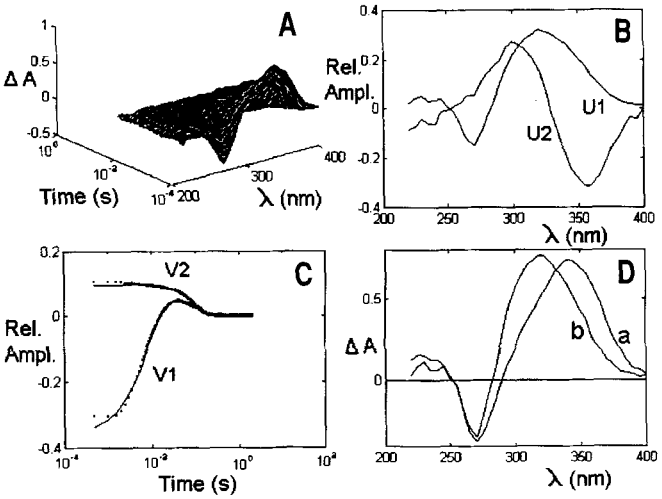

Fig. 6. Singular-value-decomposition analysis of the reaction of the diazocyanide analogue of calvatic acid with cysteine. Calvatic acid was used at 30 μM and cysteine at 30 mM. Time-courses (52, each containing 200 absorbance observations from 2 ms to 1 s) recorded at different wavelength from 220 nm to 400 nm (2.5-nm intervals) (A). The time-courses were arranged in a single matrix and plotted in a three-dimensional frame taking as reference the final spectrum of the reaction product(s) (i.e., the hydrazocyanide derivative of calvatic acid; Fig. 7). *U* columns 1 and 2 were obtained from singular-value-decomposition analysis of the wavelength/time/absorbance matrix given in (A) (B). The corresponding *S* values are 21.98 and 2.66. *V* columns 1 and 2 obtained from the singular-value-decomposition analysis (C). Dots represent the experimental points and the continuous line the best fit of data according to Eqn 3 with 7.9×10^3 M^{-1} s^{-1} (Table 1) and 1.1×10^1 s^{-1}. Calculated difference spectra of the diazocyanide analogue of calvatic acid minus the final product(s) (i.e. the hydrazocyanide derivative) (a), and of the transient species minus the hydrazocyanide derivative (b) (D).

spectral range. The singular-value-decomposition analysis performed on the spectral array revealed the presence of only one optical transition.

Fig. 5 shows the time-course, at 305 nm, for the reaction of the diazocyanide analogue of calvatic acid with different concentrations of cysteine. At the highest cysteine concentration, a biphasic time-course was observed, while a monoexponential process was recorded at the lowest cysteine concentration (Fig. 5), which was still in excess over that of the diazocyanide analogue of calvatic acid. The minimum reaction (Eqn 3) that accounts for the experimental data, involves a bimolecular process followed by a concentration-independent step:

$$\begin{aligned} &\text{diazocyanide analogue} + \text{cysteine} \rightarrow \text{transient} \\ &\text{transient} \rightarrow \text{products} . \end{aligned} \qquad (3)$$

Analysis of the data reported in Fig. 5 allowed us to determine values of the second-order rate constant [$(8.0\pm0.5)\times10^3$ M^{-1} s^{-1}; Table 1] and of the first-order rate constant (12 ± 1 s^{-1}) for cystine formation. According to Eqn (3), the transient species becomes populated only under conditions where its formation is faster than its (cysteine-concentration independent) decay (i.e. at the highest cysteine concentrations).

As expected from the values of kinetic constants reported in Table 1, no accumulation of intermediate(s) (such as the diazocyanide analogue of calvatic acid) was observed during the reaction of calvatic acid with cysteine and, as already reported, with GST P1-1 (Caccuri et al., 1994).

To reconstruct the absorption spectrum of the transient species (Eqn 3), kinetics (in the 220–400-nm spectral region) was recorded after mixing the diazocyanide analogue of calvatic acid with the highest cysteine concentration (30 mM; Fig. 6A). Singular-value-decomposition analysis of the kinetics revealed the presence of two optical components (*U* columns 1 and 2; Fig. 6B), whose time-courses were fitted to the minimum reaction Eqn 3 (*V* columns 1 and 2; Fig. 6C). The successive *U* and *V* columns did not contain spectral information but noise. The analysis of data allowed us to obtain the same values for the rate constants resulted from the fit performed at a single wavelength and different concentrations of cysteine (Figs 5 and 6). The difference spectrum of the transient species was reconstructed (Fig. 6D) from the fitted amplitudes (for details see Experimental Procedures).

Fig. 7 shows the optical spectra of calvatic acid, of its diazocyanide analogue, of the transient species occurring during

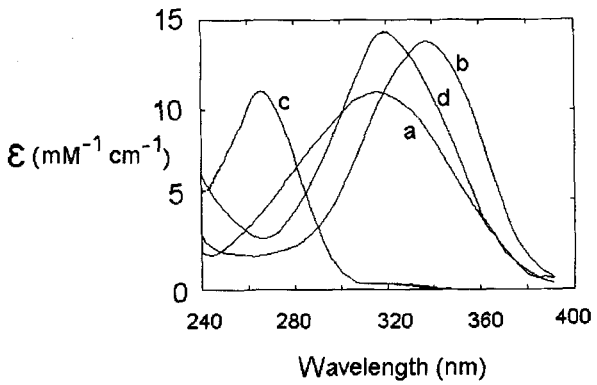

Fig. 7. Absorption spectra of calvatic acid (a), of its diazocyanide (b) and hydrazocyanide (c) analogues, and of the transient species occurring during the reaction of the diazocyanide derivative of calvatic acid with cysteine (d).

the reaction of the diazocyanide analogue of calvatic acid with cysteine, and of the final reaction product(s), such as the idrazocyanide analogue of calvatic acid [2-(4-carboxyphenyl)-hydrazinecarbonitrile; Fig. 1]. Small differences were observed in the spectra of the final reaction product(s) (ε = 11.1 ± 0.2 mM^{-1} cm^{-1}, λ_{max} = 263 ± 1 nm). The conversion of calvatic acid and of its diazocyanide analogue under the action of thiols may involve a number of less-populated pathways, leading to different minor product(s) (Haverkate et al., 1971).

The results reported here indicate that GST P1-1 inactivation by calvatic acid involves the formation of the diazocyanide ana-

logue. This chemical may interact with either the cysteine residues belonging to a native enzyme dimer or with the cysteine residues present in the non-reacted subunit of a half-inhibited enzyme dimer. However, it cannot be excluded that, given its very high reactivity, the diazocyanide analogue of calvatic acid might react preferentially with the proximal cysteine residues within the same half-inhibited enzyme. GST P1-1 inhibition by calvatic acid might represent a case for chronosteric effects, indicating transient enzyme and inhibitor properties preceding the formation of the stable inactive product(s) (Antonini et al., 1983).

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