Human glutathione S-transferase P1–1 (GST P1–1) is a homodimeric enzyme expressed in several organs as well as in the upper layers of epidermis, playing a role against carcinogenic and toxic compounds. A sophisticated mechanism of temperature adaptation has been developed by this enzyme. In fact, above 35 °C, glutathione (GSH) binding to GST P1–1 displays positive cooperativity, whereas negative cooperativity occurs below 25 °C. This binding mechanism minimizes changes of GSH affinity for GST P1–1 because of temperature fluctuation. This is a likely advantage for epithelial skin cells, which are naturally exposed to temperature variation and, incidentally, to carcinogenic compounds, always needing efficient detoxifying systems. As a whole, GST P1–1 represents the first enzyme which displays a temperature-dependent homotropic regulation of substrate (e.g. GSH) binding.

Although many enzymes are oligomeric proteins, substrate binding to one subunit does not alter generally the catalytic properties of the other one(s). Thus, the enzyme obeys to simple hyperbolic ligand binding behavior and standard Michaelis-Menten kinetics. However, in some oligomeric enzymes, substrate binding to one subunit may modify the catalytic properties of the functionally related one(s). This property, named “homotropic interaction,” yields nonhyperbolic ligand binding isotherms and nonstandard Michaelis-Menten kinetics. However, in some oligomeric enzymes, substrate binding to one subunit may modify the catalytic properties of the functionally related one(s). This property, named “homotropic interaction,” yields nonhyperbolic ligand binding isotherms and nonstandard Michaelis-Menten kinetics, representing a case of the general phenomenon named “cooperativity” (1, 2).

Some apparently noncooperative oligomeric enzymes display a latent cooperativity. Thus, the dimeric glutathione reductase from Escherichia coli shows homotropic catalytic behavior when Gly416, present at the subunit interface, is replaced by Trp (3). Also, tyrosyl-tRNA synthetase from Bacillus stearothermophilus displays a cooperative behavior upon mutation of Lys233, involved in the substrate binding, to Ala (4). Furthermore, trimeric aspartate transcarbamoylase from Bacillus subtilis and ornithine transcarbamoylase from Escherichia coli show a homotropic substrate binding behavior upon mutation of Arg99 and Arg106 present at the enzyme active site, to Ala and Gly, respectively (5, 6). Also, the homodimeric human glutathione S-transferase P1–1 (GST P1–1),1 by playing a central role in cellular detoxification processes against toxic and carcinogenic compounds (7, 8), displays latent cooperativity. In fact, the substitution of Gly41, Cys47, or Lys54 with Ala, Ser, and Ala, respectively, induces positive cooperativity for substrate binding at 25 °C (9–11).

In this study, we report direct evidence that positive and negative cooperativity occurs for substrate (e.g. glutathione; GSH) binding to wild type GST P1–1 above 35 °C and below 25 °C, respectively. This binding mechanism minimizes changes of GSH affinity because of temperature fluctuation. As a whole, GST P1–1 represents the first enzyme displaying a temperature-dependent homotropic regulation of the substrate (e.g. GSH) binding.

MATERIALS AND METHODS

Enzyme Preparation. The wild type GST P1–1 (EC 2.5.1.18) was obtained as previously reported (10). The Y49F mutant of GST P1–1 was prepared according to Landt et al. (12) in two steps of polymerase chain reaction. In the first step, the 5′ universal primer 5′-TCGAATTCGAAGGAACACAGCTATGAC and the 3′ universal primer 5′-AGCTTGCCGAATTAGGCAGG were used to generate a double-stranded mutated fragment with p18seq-1 plasmid (10) as a template and the high fidelity polymerase EXPAND (Roche Molecular Biochemicals, Italy). The amplified DNA fragment was purified from agarose gel and used as a primer in the second polymerase chain reaction in combination with the second 3′ universal primer 5′-TCGAATTCGT'TTTCCGTTTAGCCAGCACG. The amplified DNA was restricted with SphI and subsequently ligated in the expression plasmid pGST-1 to produce the mutant enzyme. Nucleotide sequence analysis was carried out by the chain termination method. The Y49F mutant of GST P1–1 was expressed and purified as previously reported (10).

GST Association to GST P1–1. Kinetic and Equilibrium Experiments—GST P1–1 steady-state kinetics was carried out at pH 6.5 (0.1 M potassium phosphate buffer) and between 5 and 43 °C, by varying GSH concentration from 10.0 μM to 10.0 mM, in the presence of CDNB as the co-substrate. The CDNB concentration (≈ 1.0 mM), corresponding approximately to that for the enzyme half-saturation, could not be further increased, accounting for the low co-substrate solubility (13). Kinetic experiments were also carried out at pH 5.0 (0.1 M sodium acetate buffer) and between 5 and 35 °C, by varying GSH concentration from 2.0 μM to 1.0 mM, in the presence of NBD-Cl as the co-substrate. The NBD-Cl concentration (≈ 0.2 mM) can be considered as saturating for the enzyme (14). The GST P1–1 concentration ranged between 10 μM and 100 μM.

Equilibrium experiments for GSH binding to GST P1–1 were performed at pH 6.5 (0.1 M potassium phosphate buffer), between 5 and 43 °C, by following the quenching of the enzyme intrinsic fluorescence, as a function of GSH concentration (ranging between 10 μM and 10 mM).
in the absence of any co-substrate (9). The GST P1–1 concentration was 3 μM.

Under all the experimental conditions, no GST P1–1 inactivation was observed within the experimental time. Furthermore, the effect of temperature on the catalytic activity and the spectroscopic properties of GST P1–1 was completely reversible.

**Data Analysis**—Steady-state kinetics, obtained in the presence of a constant concentration of the co-substrate (e.g. CDNB or NBD-Cl) and by varying the GSH concentration, were analyzed in the framework of the minimum overall enzyme reaction scheme (Scheme 1) (15),

\[
K + E + X-R + GSH \rightleftharpoons X-R-E-GSH \rightleftharpoons E + GS-R + HX
\]

\text{Scheme 1}

according to the Hill equation (Equation 1):

\[
Y = \frac{v/V_{\text{max}}}{(1 + [S]/K_1^a [S]/K_2^a)}
\]

where \( E \) is GST P1–1, \( GSH \) is the substrate; \( X-R \) is the co-substrate (e.g. an electrophilic harmful compound, such as CDNB and NBD-Cl); \( X-R-E-GSH \) indicates the reaction intermediate(s); \( GS-R \) and \( HX \) are the reaction products; \( K \) (e.g. \( K_1 \), the Michaelis constant) is the overall dissociation equilibrium constant, indicating the substrate (e.g. GSH) concentration corresponding to the half enzyme saturation (e.g. to \( V_{\text{max}}/2 \)); \( V_{\text{max}} \) is the maximum velocity, observed at saturating substrate (e.g. GSH) concentrations; \( v \) is the initial velocity, observed at nonsaturating substrate (e.g. GSH) concentrations; [S] is the GSH concentration; and \( n \) is the apparent Hill coefficient at the GSH concentration corresponding to the half enzyme saturation (e.g. at K). According to Equation 1, values of \( n \) may vary from 1, indicating the absence of cooperativity, to a maximum value, corresponding to the number of interacting subunits (e.g. 2 for the homodimeric GST P1–1) and indicating negative and positive cooperativity. Then, \( n \) values lower than 1 indicate negative cooperativity.

Steady-state kinetics were also analyzed in the framework of the minimum two sites Adair model for ligand binding to a homodimeric macromolecule (Scheme 2) (15, 16),

\[
E + X-R + GSH \rightleftharpoons X-R-E-GSH -\cdots -\cdots E + GS-R + HX
\]

\text{Scheme 2}

according to Equation 2,

\[
Y = \frac{v/V_{\text{max}}}{(1 + 2[S]/K_1^a + [S]/K_2^a)}
\]

where \( K_1 \) is the dissociation equilibrium constant for GSH binding to the substrate-free enzyme, \( K_2 \) (\( K_2 = K_1 \)) represents the dissociation equilibrium constant for GSH binding to the mononuclear enzyme, and \( \alpha \) is the adimensional interaction parameter coupling the two functionally linked GSH binding clefts (e.g. G-sites). According to Equation 2, the value of \( \alpha \) may correspond to 1, indicating no functional coupling between identical ligand binding sites (e.g. noncooperativity, \( K_1 = K_2 \)). Moreover, values of \( \alpha \) may be higher and lower than 1, indicating negative and positive cooperativity, respectively (e.g. \( K_1 < K_2 \) and \( K_1 > K_2 \), respectively).

Equilibrium fluorescence data for GSH binding to GST P1–1, obtained in the absence of the co-substrate (e.g. CDNB and NBD-Cl), were also analyzed in the framework of the minimum overall reaction scheme (Scheme 3) (15),

\[
E + GSH \rightleftharpoons E-GSH
\]

\text{Scheme 3}

according to the Hill equation (Equation 3),

\[
Y = \frac{\Delta F_{H} / \Delta F_{\text{max}}}{([S]/K^E + [S]^a)}
\]

where \( \Delta F_{H} \) indicates the fluorescence quenching change observed at nonsaturating substrate (e.g. GSH) concentrations, and \( \Delta F_{\text{max}} \) is the maximum fluorescence quenching variation detected at saturating substrate (e.g. GSH) concentrations.

Equilibrium data were also analyzed in the framework of the minimum two sites Adair model for ligand binding to a homodimeric macromolecule (Scheme 4) (15, 16),

\[
K_1^E + E + GSH \rightleftharpoons E-GSH + GSH \rightleftharpoons E-GSH_2
\]

\text{Scheme 4}

according to Equation 4,

\[
Y = \frac{\Delta F_{H} / \Delta F_{\text{max}}}{([S]/K_1^E + [S]/\alpha K_1^E + (1 + 2[S]/K_1^E + [S]/\alpha K_1^E)^a)}
\]

In the absence of the co-substrate, Scheme 1 reduces to Scheme 3, and Scheme 2 reduces to Scheme 4.

**RESULTS**

**Effect of Temperature on GSH Association to Wild Type GST P1–1, in the Presence of the Co-substrate—**GST P1–1 displays standard Michaelis-Menten kinetics, between 25 and 35 °C, in the presence of the co-substrate CDNB (9–11). Accordingly, values of the Hill coefficient \( n \) and of the interaction parameter \( \alpha \) for GSH binding to GST P1–1 are close to 1 (see Fig. 1, and Tables I and II). However, positive cooperativity for substrate binding to GST P1–1 is observed above 35 °C. Thus, values of the Hill coefficient \( n \) and of the interaction parameter \( \alpha \) are 1.4 and 0.14, respectively, at 43 °C (see Fig. 1, and Tables I and II). Conversely, negative cooperativity occurs below 25 °C, values of the apparent Hill coefficient \( n \) and of the interaction parameter \( \alpha \) for GSH binding to GST P1–1 being 0.63 and 5.3, respectively, at 5 °C (see Fig. 1, and Tables I and II). As a whole, data given in Fig. 1 indicate that, at 43 °C, the affinity of GSH for the substrate-free enzyme is lower than that for substrate association to the GST monomer-GSH-polymerized GST P1–1 (e.g. \( K_1 > \alpha K_1 \)), but on the other hand, \( K_1 < \alpha K_1 \), at 5 °C.

The unusual temperature-dependent GST P1–1 properties were observed also in the presence of the co-substrate NBD-Cl, the Hill coefficient \( n \) ranging from 0.64, at 5 °C, to 1.2, at 35 °C. Consistently, the value of the interaction parameter \( \alpha \) decreases from 5.5, at 5 °C, to 0.42, at 35 °C (see Tables I and II).

**Effect of Temperature on GSH Association to Wild Type GST P1–1**
Temperature Adaptation of GST P1–1

**Table I**

| GST P1–1  | Co-substrate | Parameter<sup>a</sup> | 5 °C | 25 °C | 35 °C | 43 °C |
|-----------|--------------|------------------------|------|-------|-------|-------|
| Wild type | CDNB         | $K_1$ ($\mu$M)         | $1.5 \times 10^2$ | $2.2 \times 10^2$ | $3.9 \times 10^2$ | $7.2 \times 10^2$ |
| Wild type |             | $K_1$ ($\mu$M)         | 3.5  | 8.3   | 2.6 $\times 10^1$ | 1.1   |
| Wild type |             | $n$                    | 0.63 | 0.92  | 1.74  | 1.4   |
| Wild type |             | $K_1$ ($\mu$M)         | $7.9 \times 10^1$ | $1.3 \times 10^2$ | $2.3 \times 10^2$ | $5.1 \times 10^2$ |
| Wild type |             | $n$                    | 0.64 | 0.98  | 1.1   | 0.2   |
| C47S Mutant | CDNB         | $K_1$ ($\mu$M)         | $3.0 \times 10^2$ | $5.2 \times 10^2$ | $9.2 \times 10^2$ | $1.1 \times 10^3$ |
| C47S Mutant |             | $n$                    | 0.64 | 1.06  | 1.0   | 1.0   |

<sup>a</sup> Experiments have been performed in triplicate. The standard error for values of $K$ and $n$ does not exceed ±8%.

**Table II**

| GST P1–1  | Co-substrate | Parameter<sup>a</sup> | 5 °C | 25 °C | 35 °C | 43 °C |
|-----------|--------------|------------------------|------|-------|-------|-------|
| Wild type | CDNB         | $K_1$ ($\mu$M)         | $6.4 \times 10^1$ | $1.8 \times 10^2$ | $4.6 \times 10^2$ | $1.9 \times 10^3$ |
| Wild type |             | $aK_1 = K_a$ ($\mu$M)  | $3.4 \times 10^2$ | $2.6 \times 10^2$ | $3.4 \times 10^2$ | $2.7 \times 10^2$ |
| Wild type |             | $\alpha$               | 5.3  | 1.4   | 0.74  | 0.14  |
| Wild type |             | $K_1$ ($\mu$M)         | 1.5  | 8.0   | 4.0 $\times 10^1$ | 1.7 $\times 10^1$ |
| Wild type |             | $n$                    | 8.2  | 8.7   | 1.7 $\times 10^1$ | 0.42  |
| Wild type |             | $K_1$ ($\mu$M)         | $3.5 \times 10^2$ | $1.0 \times 10^2$ | $2.9 \times 10^2$ | $1.3 \times 10^3$ |
| Wild type |             | $\alpha$               | 5.5  | 1.1   | 1.0   | 0.15  |
| Wild type |             | $aK_1 = K_a$ ($\mu$M)  | $1.8 \times 10^2$ | $1.8 \times 10^2$ | $1.9 \times 10^2$ | $1.9 \times 10^2$ |
| Wild type |             | $\alpha$               | 5.1  | 1.8   | 0.66  | 0.15  |
| Wild type |             | $n$                    | 8.4 $\times 10^2$ | $8.9 \times 10^2$ | $1.1 \times 10^3$ | $1.1 \times 10^3$ |
| Wild type |             | $K_1$ ($\mu$M)         | 1.1 $\times 10^2$ | $3.1 \times 10^2$ | $7.7 \times 10^2$ | $1.2 \times 10^3$ |
| Wild type |             | $\alpha$               | 7.6  | 2.9   | 1.4   | 0.92  |
| Wild type |             | $aK_1 = K_a$ ($\mu$M)  | $4.4 \times 10^2$ | $4.1 \times 10^2$ | $2.9 \times 10^2$ | $2.9 \times 10^2$ |
| Wild type |             | $\alpha$               | 0.07 | 0.07  | 0.07  | 0.07  |
| C47S mutant | CDNB         | $K_1$ ($\mu$M)         | $1.0 \times 10^2$ | $2.4 \times 10^2$ | $2.7 \times 10^2$ | $2.7 \times 10^2$ |
| C47S mutant |             | $aK_1 = K_a$ ($\mu$M)  | $1.4 \times 10^3$ | $1.4 \times 10^3$ | $1.4 \times 10^3$ | $1.4 \times 10^3$ |
| C47S mutant |             | $\alpha$               | 0.11 | 0.11  | 0.11  | 0.11  |

<sup>a</sup> Experiments have been performed in triplicate. The standard error for values of $K$, $aK_1$ (= $K_a$) and $\alpha$ does not exceed ±8%.

P1–1, in the Absence of the Co-substrate—GST P1–1, as observed by following the quenching of the enzyme intrinsic fluorescence, displays temperature-dependent cooperativity also in the absence of CDNB and NBD-Cl (see Tables I and II). In fact, the value of the Hill coefficient $n$ increases from 0.65, at 5 °C, to 1.3, at 43 °C. In agreement, the value of the interaction parameter $\alpha$ decreases from 0.92, at 43 °C, to 0.14, at 5 °C (see Table II). As shown in Fig. 1 and in Tables I and II, the apparent $\Delta H_{aK1} (= \Delta H_{K_a})$ approaching zero. As expected, the value of the interaction parameter $\alpha$ decreases from 0.92, at 43 °C, to 0.14, at 5 °C, from 0.92, at 43 °C, to 0.14, at 5 °C, in the presence of CDNB, as shown for the wild type enzyme below 25 °C (see Fig. 1, and Tables I and II). In fact, values of the Hill coefficient $n$ decrease from 1.0, at 43 °C, to 0.64, at 5 °C (see Table I). In parallel, values of the interaction parameter $\alpha$ increase from 0.92, at 43 °C, to 7.6, at 5 °C (see Table II). As a whole, the affinity of GST for the substrate-free Y49F mutant is higher than that of for the GST-monomoligated species below 43 °C (e.g. $K_1 < aK_1$ (see Table II)).

Effect of the Y49F Mutation on GST Association to the GST P1–1 in the Presence of the Co-substrate—Tyr<sup>49</sup> appears to be a crucial residue for the intersubunit communication in GST P1–1, contacting helices 4 and 5 of the opposite chain (17, 18) (see Fig. 2). Upon mutation of Tyr<sup>49</sup> to Phe, negative cooperativity for GST binding to the Y49F mutant of GST P1–1 occurs below 43 °C, in the presence of CDNB, as shown for the wild type enzyme below 25 °C (see Fig. 1, and Tables I and II). In fact, values of the Hill coefficient $n$ decrease from 1.0, at 43 °C, to 0.64, at 5 °C (see Table I). In parallel, values of the interaction parameter $\alpha$ increase from 0.92, at 43 °C, to 7.6, at 5 °C (see Table II). As a whole, the affinity of GST for the substrate-free Y49F enzyme is higher than that for the GST-monomoligated species below 43 °C (e.g. $K_1 < aK_1$ (see Table II)).

Effect of the C47S Point Mutation on GST Association to the GST P1–1 in the Presence and Absence of the Co-substrate—Point mutation of residues Gly<sup>41</sup>, Cys<sup>47</sup> and Lys<sup>54</sup>, belonging to helix 2 and forming the G-site (17, 18) (see Fig. 2), induces positive cooperativity for GST binding to GST P1–1, at 25 °C (9–11).
replacement of Cys47 with Ser triggers homotropic behavior, at 25 °C \((e.g. \, n \sim 1.4 \text{ and } \alpha \sim 0.09)\), as observed in the native enzyme at 43 °C \((e.g. \, n \sim 1.3 \text{ and } \alpha \sim 0.14, \text{ at } 43 ^\circ C)\) (see Tables I and II). Therefore, the affinity of GSH for the substrate-free C47S enzyme is lower than that for the GSH-monoligated species at 25 °C \((e.g. \, K_1 > \alpha K_1)\) (see Table II).

**DISCUSSION**

Here, reported data indicate that GST P1–1 represents the first enzyme displaying a temperature-dependent homotropic regulation of substrate \((e.g. \, GSH)\) binding to the two G-sites present in the homodimer. In fact, negative cooperativity for GSH binding to GST P1–1 occurs below 25 °C. On the other hand, positive cooperativity is observed above 35 °C (see Fig. 1, and Tables I and II).

Temperature has been previously reported to reverse allostERIC phenomena in rat liver fructose-1,6-diphosphatase, in phosphofructokinase from Bacillus stearothermophilus and in carbamoylphosphate synthetase from Escherichia coli. In fact, when varying the temperature, the AMP, ADP, or IMP action changes from inhibition to activation. Therefore, these enzymes display a temperature-dependent etherotropic regulation for substrate and allosteric ligand binding \((19, 20)\).

The inspection of the three-dimensional model of GST P1–1 \((17, 18)\) (see Fig. 2) fulfills a structural interpretation of the homotropic binding behavior of GSH. Structural perturbation occurring at the G-site by GSH binding would be transmitted to the G-site present in the adjacent subunit via helix 4 (residues 90–109), which represents the monomer-monomer interface and contains residues that interact with the active site. A key residue involved in the intersubunit communication may be Tyr49, which contacts, with its aromatic ring, the hydrophobic pocket located between helices 4 and 5 of the adjacent subunit. In particular, in the presence of GSH, the Tyr49 residue forms 14 van der Waals contacts with the adjacent monomer. Next, the hydroxyl group of Tyr49 is hydrogen bonded with the carboxyl oxygen atom of the Met91 residue of the opposite subunit. However, in the absence of the substrate, Tyr49 forms only 8 van der Waals contacts and loses some intersubunit interac-

![Ribbon representation of the GSH binding domain (G-site) of the homodimeric human GST P1–1. Subunits are represented in yellow and red. The S-hexyl-GSH is shown in ball and sticks. Residues involved in the cooperative intersubunit communication are shown. The picture was based on the crystal structure of the GST P1–1-S-hexyl-GSH complex (17).](Image 275x454 to 555x729)

Tyr49 interacts with the hydrophobic pocket located between helices 4 and 5 of the adjacent subunit. In particular, in the presence of GSH, the Tyr49 residue forms 14 van der Waals contacts with the adjacent monomer. Next, the hydroxyl group of Tyr49 is hydrogen bonded with the carboxyl oxygen atom of the Met91 residue of the opposite subunit. However, in the absence of the substrate, Tyr49 forms only 8 van der Waals contacts and loses some intersubunit interac-

![Ribbon representation of the GSH binding domain (G-site) of the homodimeric human GST P1–1. Subunits are represented in yellow and red. The S-hexyl-GSH is shown in ball and sticks. Residues involved in the cooperative intersubunit communication are shown. The picture was based on the crystal structure of the GST P1–1-S-hexyl-GSH complex (17).](Image 275x454 to 555x729)

Conversely, G41A, C47S, and K54A point mutations at 25 °C revert negative cooperativity induced by Tyr49 to Phe substitution \(\text{(see Fig. 1, and Tables I and II). The positive cooperativity resulting from Gly41, Cys47, and Lys54 point mutations could be explained by the structural perturbation of helix 2 and by the resulting effects being transmitted through residue Tyr49 to the opposite subunit (9–11, 17, 18). In this respect, Cys47 acts as a hinge which limits the extent of frequency of conformational transitions involving helix 2. In its absence, helix 2 would become more flexible (21). A similar effect may be obtained by a higher temperature-enhancing motion of this flexible enzyme region.}\)

As a whole, GST P1–1 somewhat behaves as a thermodynamic system which obeys the Le Chatelier principle. In fact, whenever a physical or chemical factor forces the G-site to assume a low-affinity conformation for substrate binding, GST P1–1 opposes this perturbation by developing positive cooperativity, thus increasing the GSH affinity for the substrate-monoligated enzyme \((e.g. \, K_1 > \alpha K_1)\). This scenario is observed in the wild type GST P1–1 above 35 °C as well as in G41A, C47S, and K54A enzyme mutants at 25 °C \((9–11)\). On the other hand, negative cooperativity occurs when the G-site is forced toward a high-affinity conformation, \(e.g. \, \text{in the wild type enzyme at below } 25 \text{ °C and in the Y49F GST P1–1 mutant below } 43 \text{ °C.} \)

The physiological advantage of the GSH binding homotropic modulation mechanism becomes evident when considering that GST P1–1 is present in the human skin \((e.g. \, \text{in the upper layers of epidermis})\) (22) and may suffer very low and high tempera-
tures. Thus, at the human skin GSH concentration (about 1–3 mM) (23), GST P1-1 displays an almost unchanged affinity for GSH in a wide range of temperatures (e.g. between 5 and 43 °C; see Fig. 1). This aspect is particularly relevant considering the increased skin cancerogenesis observed in mice lacking Pi class GST, and indicates the crucial role of these enzyme(s) in cancer prevention (24).

Finally, the maintenance of the enzyme-substrate affinity is a well known phenomenon for evolutionary biochemists and, in organisms living at different temperatures, is pursued by the expression of homologous enzymes able to keep unchanged substrate affinity at the average body temperature of each species (25, 26). In this respect, GST is of particular interest as the conservation of the substrate (e.g. GSH) affinity is recovered in the same enzyme by triggering positive and negative cooperativity, representing a case of molecular adaptation and evolution.

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