Paradoxical inhibition of rat glutathione transferase 4–4 by indomethacin explained by substrate–inhibitor–enzyme complexes in a random-order sequential mechanism

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Under standard assay conditions, with 1-chloro-2,4-dinitrobenzene (CDNB) as electrophilic substrate, rat glutathione transferase 4–4 is strongly inhibited ($I_{50} = 1 \mu\text{M}$) by indomethacin. No other glutathione transferase investigated is significantly inhibited by micromolar concentrations of indomethacin. Paradoxically, the strong inhibition of glutathione transferase 4–4 was dependent on high (millimolar) concentrations of CDNB; at low concentrations of this substrate or with other substrates the effect of indomethacin on the enzyme was similar to the moderate inhibition noted for other glutathione transferases. In general, the inhibition of glutathione transferases can be explained by a random-order sequential mechanism, in which indomethacin acts as a competitive inhibitor with respect to the electrophilic substrate. In the specific case of glutathione transferase 4–4 with CDNB as substrate, indomethacin binds to enzyme–CDNB and enzyme–CDNB–GSH complexes with an even greater affinity than to the corresponding complexes lacking CDNB. Under presumed physiological conditions with low concentrations of electrophilic substrates, indomethacin is not specific for glutathione transferase 4–4 and may inhibit all forms of glutathione transferase.

INTRODUCTION

Indomethacin is an anti-inflammatory drug that inhibits the cyclo-oxygenase (Vane, 1971) and lipoxygenase (Siegel *et al.*, 1979; Wu & Mathews, 1983) pathways of arachidonate metabolism. It thereby prevents the production of leukotrienes and related compounds directly responsible for inflammatory effects. Clinical treatment with indomethacin is known to give rise to certain side effects that indicate that the action of the drug is not limited to anti-inflammatory action. Indomethacin has also been shown to inhibit O_2 uptake by gastric mucosal mitochondria (Priess & Sewing, 1985) and to have antitumoral activity (Rubio, 1984).

Glutathione transferase is among the enzymes that have been found to be inhibited by indomethacin (Wu & Mathews, 1983). Cytosolic glutathione transferases are dimeric proteins consisting of identical or non-identical subunits. Multiple forms of glutathione transferase have been isolated, characterized and grouped into one of three classes (Mannervik *et al.*, 1985). Within the same class the subunits are highly homologous and can hybridize with each other, even though there may be large differences in their substrate and inhibitor specificities. For example, rat subunits 3 and 4 display 77% sequence identity (Ålin *et al.*, 1986; Ding *et al.*, 1986; Lai *et al.*, 1986) and form a hybrid (transferase 3–4), but have distinct catalytic properties (Ålin *et al.*, 1985b). The functional properties of different forms of glutathione transferase appear to reflect the structure of their respective active-site regions (Danielson *et al.*, 1987). A variety of substrates and inhibitors may be used in the identification of different enzyme forms (Mannervik *et al.*, 1985). The inhibition by indomethacin has been

especially useful for identification of rat glutathione transferase 4–4, which is the only form strongly inhibited by this compound in the standard assay system (Nicholls & Ahokas, 1984; Ålin *et al.*, 1985b; Tahir *et al.*, 1985; Warholm *et al.*, 1986). The comparatively weaker inhibition by indomethacin of, for example, the homologous form transferase 3–3 may be due to a different overall reaction mechanism, a different mode of inhibition or a combination of these factors. The kinetics of rat transferases 3–3 (Jakobson *et al.*, 1977) and 1–1 (Schramm *et al.*, 1984) previously studied are consistent with a sequential mechanism with random-order addition of GSH and the electrophilic substrate. Neither the reaction mechanism of transferase 4–4 nor the mode of inhibition by indomethacin, with any enzyme form, has been reported previously.

Glutathione transferase is known as an important detoxication enzyme acting on endogenous and exogenous compounds (for a review see Mannervik, 1985). The physiological effect of indomethacin inhibition of glutathione transferase may involve impaired detoxication. Another potential effect may be inhibition of the biosynthesis of leukotriene C_4 , since it is also recognized as an enzyme capable of catalysing the conversion of leukotriene A_4 into the GSH conjugate leukotriene C_4 (Mannervik *et al.*, 1984). Glutathione transferase 4–4 is the form most sensitive to indomethacin inhibition and also the cytosolic transferase most active with leukotriene A_4 as substrate (Mannervik *et al.*, 1984).

The present investigation was undertaken in order to determine the reaction mechanism of rat glutathione transferase 4–4 and the mode of action of indomethacin *in vitro*, as well as to find out if, under physiological conditions, this enzyme form has a unique sensitivity to

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; tPBO, *trans*-4-phenylbut-3-en-2-one.

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indomethacin as compared with other glutathione transferases.

MATERIALS AND METHODS

Glutathione transferases were isolated from rat liver by the procedure described by Ålin *et al.* (1985b). The chemicals used were standard commercial compounds of highest purity.

The inhibition of glutathione transferase activity was determined at several different concentrations of substrate. The concentration of inhibitor giving 50% inhibition (I_{50}) was used as a measure of the inhibitory effect at a given substrate concentration. The I_{50} value for the classical linear inhibition patterns of the Michaelis-Menten equation (Cleland, 1963) can be expressed as a function of the substrate concentration:

Competitive inhibition:

$$I_{50} = K_i + \frac{K_i}{K_m}[S]$$

Uncompetitive inhibition:

$$I_{50} = K_i + \frac{K_i \cdot K_m}{[S]}$$

Non-competitive inhibition:

$$I_{50} = \frac{(K_m + [S])K_{ii} \cdot K_{is}}{K_m \cdot K_{is} + K_{ii} \cdot [S]}$$

Competitive and uncompetitive inhibitions are identified by linear plots of I_{50} versus $[S]$ and $1/[S]$ respectively, whereas non-competitive inhibition results in curvature. In cases of more complex inhibition kinetics, such as that of a steady-state random-order mechanism, corresponding linear functions are approached asymptotically at high substrate concentrations for competitive inhibition and at low substrate concentrations for uncompetitive inhibition. The examination of I_{50} values has previously been used for distinction of heterodimeric and homodimeric enzymes (Tahir & Mannervik, 1986); in the present investigation it was found to be a valuable complement to conventional kinetic analysis, especially for defining the inhibition type.

A range of 0–100 μM -indomethacin was sufficient to determine the I_{50} value for transferase 4–4 at substrate concentrations of 3 μM -, 10 μM -, 30 μM -, 100 μM -, 300 μM - and 1000 μM -1-chloro-2,4-dinitrobenzene (CDNB) and of 4 μM -, 10 μM -, 40 μM - and 100 μM -*trans*-4-phenylbut-3-en-2-one (tPBO). The concentrations cover the maximal ranges possible for accurate measurements with these substrates. The enzyme concentration was 4–40 nM. A standard assay system containing 0.2 M-sodium phosphate buffer, pH 6.5, 5% (v/v) ethanol and 1 mM-GSH (for CDNB) or 0.25 mM-GSH (for tPBO) was used at 30 °C. The initial velocity was determined in each point as the average of at least five replicates, and the standard deviation was used as a measure of the experimental error. The I_{50} value was also determined with 4-hydroxyalk-2-enals as substrate; the activity was measured as described by Ålin *et al.* (1985a), except that 2.5 mM-GSH and different concentrations of 4-hydroxyalkenal were used.

Non-linear-regression analysis was performed essentially as described previously (Mannervik, 1982; Mannervik *et al.*, 1986). The experimental error in the kinetic data was not constant, as judged from the replicate

Table 1. Inhibition of glutathione transferases 4–4 and 3–3 by indomethacin

I_{50} values are given for different concentrations of the substrates. For each substrate the catalytic efficiency is presented as $k_{\text{cat.}}/K_m$. Abbreviation: N.D., not determined.

Enzyme	Substrate	$k_{\text{cat.}}/K_m$ ($\mu\text{M}^{-1} \cdot \text{s}^{-1}$)	Concn. (μM)	I_{50} (μM)
4–4	CDNB	0.19*	3	37
			10	37
			100	22
			1000	0.9
	tPBO	0.065†	4	37
			10	45
			100	53
	4-Hydroxydec-2-enal	0.28‡	10	28
3–3	4-Hydroxyhex-2-enal	0.026‡	100	> 100
	CDNB	2.5*	5	12
			1000	100
	tPBO	< 0.002†	5	N.D.
	4-Hydroxydec-2-enal	0.16‡	10	≥ 25
	4-Hydroxyhex-2-enal	0.025‡	100	> 100

* From Ålin *et al.* (1985b).

† From Danielson & Mannervik (1985).

‡ From Danielson *et al.* (1987).

measurements. Consequently, empirical error functions were determined by fitting the estimated standard deviations (s.d.) to alternative models by the procedures previously described (Mannervik *et al.*, 1986). The best error models were based on substrate and inhibitor concentrations and were subsequently used to define weighting factors in the regression analysis of the kinetic data sets. For data (31 experimental points with at least five replicates) in which tPBO served as substrate the best error function was:

$$\text{s.d.} = \frac{2.1[S] + 0.040[S][I] + 0.0015[S]^2[I]}{350 + [S] + 0.61[S][I]}$$

s.d. is given in units of s^{-1} and substrate and inhibitor concentrations in μM . For CDNB experiments (54 points with at least five replicates):

$$\text{s.d.} = \frac{1.9[S] + 8.9[I]}{258 + [S] + 110[I]}$$

It is noteworthy that the same error function could not be used to describe s.d. of the data sets obtained by measurements with the different substrates, even though the expressions are not significantly different in the absence of inhibitor ($[I] = 0$).

RESULTS

In order to characterize the effect of indomethacin on glutathione transferases, the inhibition was studied under different conditions.

Fig. 1. Inhibition of glutathione transferase 4-4 by indomethacin

Initial velocities are given per mol of enzyme (M_r 50000). In the insets activity for each substrate concentration is expressed as percentage of the activity in the absence of inhibitor. The curves represent the predicted values obtained by regression analysis (Tables 2 and 4). (a) Activity measured with 1 mM-GSH and the following concentrations of CDNB: 3, 10, 30, 100, 300 and 1000 μM, as indicated by the curves (from bottom to top). (b) Activity measured with 0.25 mM-GSH and the following concentrations of tPBO: 4, 10, 40 and 1000 μM, as indicated by the curves (from bottom to top).

The inhibition of transferases 4-4 and 3-3 was initially measured at high and at low concentrations of the substrates CDNB, tPBO, 4-hydroxdec-2-enal and 4-hydroxyhex-2-enal. These 4-hydroxyalkenals are homologous substrates giving high and low activity respectively with the transferases studied. All experiments were carried out with constant concentrations of GSH in the

millimolar range, which are physiologically relevant and which essentially saturate the enzyme. The results, expressed as I_{50} values, are presented in Table 1. The catalytic efficiencies (k_{cat}/K_m) with each substrate, estimated under first-order conditions (cf. Danielson & Mannervik, 1985), are also given.

The data show that indomethacin is a particularly

strong inhibitor of transferase 4-4 ($I_{50} = 1 \mu\text{M}$) only at high concentrations (1 mM) of CDNB. With no other enzyme or substrate tested was an equally low I_{50} value obtained with indomethacin. The inhibition of transferase 4-4 depended on the CDNB concentration in a unique manner and increased with increasing substrate concentration. In all other cases the inhibition decreased as the substrate concentration increased.

CDNB is one of the substrates giving the highest activity with the glutathione transferases, but the unusual effect of indomethacin could not be correlated to the efficiency of the catalysis, as shown by the values of $k_{\text{cat.}}/K_m$ for 4-hydroxyalkenals (Table 1).

Inhibition of transferase 4-4

The unique effect of indomethacin on transferase 4-4 was further investigated by studies of the inhibition at different concentrations of tPBO and CDNB. Fig. 1 shows the activity as a function of the indomethacin concentration at different concentrations of CDNB (Fig. 1a) and tPBO (Fig. 1b). The shape of the curves and the effect of substrate concentration are clearly different in the two cases.

Determination of rate equation and reaction mechanism

Initial-velocity data from the inhibition studies involving transferase 4-4 and indomethacin were analysed in order to determine a rate equation for each of the two substrates CDNB and tPBO (Fig. 1). Several rate equations were fitted to the initial-velocity data by using non-linear regression. The rate equation was found to require second-degree terms in substrate concentration, and the model must consequently contain two steps for the binding of the electrophilic substrate. For an analogous reason, the model must contain two steps for binding of inhibitor when CDNB served as substrate. Graphically, this rate behaviour was expressed by non-linear Lineweaver-Burk and Dixon plots. The dependence of I_{50} on substrate concentration indicated that indomethacin was a generalized competitive inhibitor (Mannervik, 1978) with tPBO and non-competitive with CDNB, in agreement with principles described in the Materials and methods section.

The second step in the analysis involved the construction of realistic reaction mechanisms, taking into account the essential features of the rate equation. The rate equations were derived by use of the procedure of Cornish-Bowden (1977) for each of the mechanisms considered.

tPBO reaction

The simplest rate equation corresponding to a realistic reaction mechanism and giving a good fit to the experimental data with tPBO as substrate is presented in Table 2. The structure of the equation and the values of the parameters obtained by regression analysis are shown. The choice of the rate equation and the parameter estimation were not critically dependent on the choice of the error function.

The reaction mechanism corresponding to the rate equation chosen is a steady-state random-order mechanism, in which indomethacin acts as a competitive inhibitor versus tPBO and a non-competitive inhibitor versus GSH (Scheme 1). Indomethacin may bind to free enzyme (E) or the enzyme-GSH complex (EG).

The model predicts a value for $k_{\text{cat.}}/K_m = P_1/P_3$, and

Table 2. Rate equation for glutathione transferase 4-4 tPBO-conjugating activity in the presence of indomethacin

The model presented was the best of a number of alternatives tested and corresponds to Scheme 1. The parameter values have large standard errors, mainly because of the limited number of data points in comparison with the number of parameters. Proper units of the parameters can be deduced on the basis of units for substrate and inhibitor concentrations (μM) and $v/[E]_0$ (s^{-1}).

$$v/[E]_0 = \frac{P_1[S] + P_2[S]^2}{P_3 + P_4[S] + P_5[S]^2 + P_6[S][I] + P_7[I]}$$

$$\begin{array}{ll} P_1 = 1.85 \pm 2.06 & P_5 = 0.0008 \pm 0.0037 \\ P_2 = 0.036 \pm 0.024 & P_6 = 0.019 \pm 0.007 \\ P_3 = 22.1 \pm 28.2 & P_7 = 0.53 \pm 0.55 \\ P_4 = 1.0 & \end{array}$$

Scheme 1. Reaction and inhibition mechanism of transferase 4-4 tPBO-conjugating activity in the presence of indomethacin

Key: E, enzyme; B, tPBO; G, GSH; I, indomethacin; P, conjugate of GSH and tPBO.

by using the parameter values obtained by regression analysis $k_{\text{cat.}}/K_m$ was calculated to be $0.084 \mu\text{M}^{-1} \cdot \text{s}^{-1}$, which is slightly higher than, but not significantly different from, the published value of $0.065 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (Danielson & Mannervik, 1985). The predicted value represents the maximal rate of the reaction under first-order conditions with respect to tPBO concentration.

The I_{50} value can be expressed as a function of tPBO concentration [S] and parameters of the underlying rate equation (cf. Table 2):

$$I_{50} = \frac{P_3 + P_4[S] + P_5[S]^2}{P_7 + P_6[S]} \quad (1)$$

When the parameter values presented in Table 2 are used this equation gives I_{50} values close to those found experimentally, as shown in Table 3.

Maximal inhibition is obtained at infinitely low [S], where the I_{50} value is equal to P_3/P_7 . In the case of the reaction of transferase 4-4 with tPBO the calculated I_{50} value for maximal inhibition is $42 \mu\text{M}$. The corresponding I_{50} value experimentally obtained was $37 \mu\text{M}$ at $4 \mu\text{M}$ -tPBO, which is in reasonable agreement with the fitted parameters.

CDNB reaction

The initial velocities of the reaction with CDNB as substrate could not be described by the rate equation

Table 3. Predicted and experimental I_{50} values for indomethacin inhibition of glutathione transferase 4-4 tPBO-conjugating activity

I_{50} values were predicted from eqn. (1) and parameter values in Table 2.

[S] (μM)	I_{50} (μM)	
	Experimental	Predicted
4	37	43
10	45	45
40	48	49
100	53	53

Scheme 2. Reaction and inhibition mechanism of transferase 4-4 CDNB-conjugating activity in the presence of indomethacin

Key: E, enzyme; C, CDNB; G, GSH; I, indomethacin; P, S-(2, 4-dinitrophenyl)glutathione and Cl⁻.

that was adequate for the data obtained with tPBO as substrate (Table 2).

The simplest mechanism accurately describing the inhibition when CDNB was used as substrate is presented in Scheme 2. The essential features are the same as for the tPBO reaction (Scheme 1), with the addition of ECI and EGCI complexes. Table 4 presents the rate equation corresponding to this model and the parameter values obtained by weighted regression analysis. This rate

Table 5. Predicted and experimental I₅₀ values for indomethacin inhibition of glutathione transferase 4-4 CDNB-conjugating activity

I_{50} values were predicted from the rate equation and parameter values presented in Table 4.

	$I_{50} (\mu M)$	
$[S] (\mu M)$	Experimental	Predicted
3	37	25
10	37	29
30	30	33
100	22	25
300	5.2	6.1
1000	0.9	0.89

equation was the best of a number of alternative models used to describe the experimental data, as judged by the residual sum of squares and other goodness-of-fit criteria (Mannervik, 1982). Additional pathways in the reaction scheme, e.g. $EI \rightleftharpoons ECI$ and $EGI \rightleftharpoons EGCI$, are also possible, but the data analysis did not call for corresponding third- or fourth-degree terms in the rate equation. On the other hand, under the experimental conditions (i.e. high CDNB concentration) that favour the reaction loop involving ECI and EGCI, the concentrations of E and EG are low, disfavouring pathways including these enzyme forms. Consequently, the simplest scheme involving the essential features of the kinetics has been adopted.

The value for $k_{\text{cat.}}/K_m$ predicted by this mechanism is $P_1/P_5 = 0.16 \mu\text{M}^{-1} \cdot \text{s}^{-1}$, which is close to the published value of $0.19 \mu\text{M}^{-1} \cdot \text{s}^{-1}$ (Ålin *et al.*, 1985b).

The rate equation and parameters presented in Table 4 were used to calculate the inhibitor concentration giving 50 % of the uninhibited reaction rate. These I_{50} values, predicted by the model, are presented with the experimental values in Table 5.

The model presented in Scheme 2 explains the non-competitive behaviour versus CDNB as well as GSH. Alternative models considered, but rejected, involved the production of enzyme-product-inhibitor complexes, and consequently predicted increased inhibition by addition of the products Cl^- and S -(2,4-dinitrophenyl)-

Table 4. Rate equation for glutathione transferase 4-4 CDNB-conjugating activity in the presence of indomethacin

The model presented was the best of a number of alternatives tested and corresponds to Scheme 2. The model is mathematically overdetermined, but elimination of any of the apparently redundant parameters (e.g. P_7) gives an inferior fit of the equation to the experimental data. Proper units of the parameters can be deduced on the basis of units for substrate and inhibitor concentrations (μM) and $v/[E]_0$ (s^{-1}).

$$v/[E]_0 = \text{NL} \frac{P_1[S] + P_2[S]^2 + P_3[S][I] + P_4[S]^2[I]}{P_5 + P_6[S] + P_7[S]^2 + P_8[S][I] + P_9[S]^2[I] + P_{10}[S][I]^2 + P_{11}[S]^2[I]^2 + P_{12}[I]}$$

$$\begin{array}{ll} P_1 = 6.7 \pm 0.82 & P_7 = < 10^{-10} \\ P_2 = 0.030 \pm 0.0018 & P_8 = 0.088 \pm 0.052 \\ P_3 = 0.24 \pm 0.20 & P_9 = 1.6 \times 10^{-3} \pm 0.4 \times 10^{-3} \\ P_4 = 9.3 \times 10^{-3} \pm 4.3 \times 10^{-3} & P_{10} = 2.7 \times 10^{-3} \pm 1.4 \times 10^{-3} \\ P_5 = 42 \pm 6.4 & P_{11} = 1.4 \times 10^{-5} \pm 1.8 \times 10^{-5} \\ P_6 = 1.0 & P_{12} = 4.9 \pm 2.4 \end{array}$$

glutathione. However, these products even at high concentration were not found to affect the strength of the inhibition (results not shown).

DISCUSSION

The kinetic reaction mechanism proposed for glutathione transferase 4-4 was found to be consistent with a steady-state random-order mechanism for both CDNB and tPBO as second substrate (Schemes 1 and 2). GSH was present at near saturating concentrations in the experiments and was not explicitly accounted for in the rate equation. However, the data require a model including two forms of substrate-free (E and EG) and two forms of substrate-containing (ES and EGS) enzyme, representing the species with and without bound GSH.

The inhibition mechanism involves the binding of inhibitor to more than one enzyme form. In the experiments with tPBO as substrate, the inhibition was competitive versus tPBO, since indomethacin binds only to forms not including tPBO or product.

The tPBO activity of transferase 4-4 appears to be well characterized by the model, as judged by close agreement of experimental data for $k_{\text{cat.}}/K_m$ and I_{50} values with those predicted by the model. Alternative rate equations did not represent the data with similar accuracy.

The model presented in Scheme 1 describes the binding of indomethacin to the enzyme forms lacking the second substrate, resulting in EI and EGI complexes. This reaction scheme appears to apply generally for the action of indomethacin on transferase 4-4 as well as the other transferases tested. The exception, however, is transferase 4-4 with CDNB as second substrate, which in addition to the general inhibition pattern also exhibits a kinetically most significant binding of indomethacin to the enzyme-substrate complexes, forming ECI and EGCI. In this case the inhibition increases with the substrate concentration and results in a stronger inhibition than in the simpler case. Maximal inhibition is reached at infinitely high substrate concentrations. This paradoxical inhibition has so far not been observed with any other substrate nor with any other transferase.

In the search for an explanation of the paradoxical inhibition pattern of indomethacin observed when CDNB served as a substrate for glutathione transferase 4-4, several alternatives were excluded. CDNB is a particularly good substrate in comparison with most of the conventional substrates for the glutathione transferases (Ålin *et al.*, 1985b). However, comparison with results obtained with the even better substrate 4-hydroxydec-2-enal shows that the unusual inhibition is not dependent on the rate of the catalytic step, as measured by values of $k_{\text{cat.}}/K_m$ (Table 1). Further, the differences in the chemical reaction type were not found to be of significance for the inhibition. In the tPBO reaction GSH conjugation takes place by an addition reaction, whereas in the CDNB reaction the conjugate is formed via a substitution reaction, yielding Cl^- as a second product. However, addition of the products of the CDNB reaction did not enhance the inhibition with indomethacin. Further, the effect of indomethacin on transferase 3-3 is essentially the same with CDNB as with 4-hydroalkenals (cf. Table 1), which argues against mechanism type as a determinant for the strength of inhibition. Other models considered included mnemonic mechanisms (Ricard *et al.*, 1974), but these were not

found to be superior to the model proposed here and depended on assumptions about kinetically distinct conformational states of the enzyme. No experimental support exists for kinetically significant conformational changes under steady-state conditions.

Among the rat (Ålin *et al.*, 1985b), mouse (Warholm *et al.*, 1986) and human (Tahir *et al.*, 1985) transferases investigated, none but rat glutathione transferase 4-4 is strongly inhibited by indomethacin. It is particularly remarkable that rat glutathione transferase 3-3, which is structurally most closely related to transferase 4-4, does not show the inhibition properties of the latter isoenzyme. The inhibition of transferase 3-3 with CDNB as substrate is consistent with the general action of indomethacin, as described for the tPBO mechanism for transferase 4-4 (Scheme 1). This is also the behaviour of transferase 3-3 observed with other substrates.

Substrate specificity data suggest that the active site of transferase 4-4 permits the binding of bulky molecules (Danielson *et al.*, 1987). The binding of indomethacin might therefore be bound in the active-site cavity simultaneously with CDNB. The kinetic data (Table 1 and Fig. 1a) show that CDNB actually promotes the binding of indomethacin. In contrast, tPBO appears to restrict the binding of indomethacin, as judged by the kinetic data obtained with this substrate.

In contrast with earlier results, based on measurements with high CDNB concentrations, the present investigation demonstrates that the inhibition of glutathione transferases by indomethacin is not limited to transferase 4-4 under physiological conditions, where the electrophilic substrates are assumed to be present in low concentrations. The natural substrates are sought among products of oxidative metabolism such as epoxides, organic hydroperoxides and activated alkenes (Mannervik, 1986). The maximal inhibition is dependent on the degree of enzyme saturation with a particular substrate. Indomethacin can bind to the free enzyme, and for all substrates studied, except CDNB, a low substrate concentration will give the strongest inhibition. The intracellular concentrations of indomethacin resulting from clinical treatment may inhibit the activity of the enzyme significantly.

The previous findings that rat glutathione transferase 4-4 was both the most active cytosolic enzyme in the formation of leukotriene C_4 (Mannervik *et al.*, 1984) and the form most sensitive to indomethacin inhibition (Nicholls & Ahokas, 1984; Ålin *et al.*, 1985b) appears to be of less importance for the synthesis of leukotriene C_4 *in vivo* than was initially supposed. Firstly, it has been shown that indomethacin is not a strong inhibitor when leukotriene A_4 serves as a substrate for any of the enzyme forms tested (L. Örning, M. Söderström, S. Hammarström & B. Mannervik, unpublished work), and, secondly, the major part of the leukotriene C_4 synthesis *in vivo* is carried out by a membrane-bound protein, leukotriene C synthase (Bach *et al.*, 1984; Jakschik *et al.*, 1982; Yoshimoto *et al.*, 1985). This enzyme is not significantly inhibited by indomethacin (Söderström *et al.*, 1988). We therefore conclude that the effect of indomethacin on glutathione transferase *in vivo* probably would affect primarily its detoxication function.

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