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Relaxed thiol substrate specificity of glutathione transferase effected by a non-substrate glutathione derivative

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Rat glutathione transferase 4-4 catalyzed the conjugation of 2-mercaptoethanol with 1-chloro-2,4-dinitrobenzene in the presence of *S*-methyl-glutathione. The reaction was linearly dependent on enzyme concentration and saturation was seen with respect to both 2-mercaptoethanol and *S*-methyl-glutathione concentration. High concentrations of *S*-methyl-glutathione were inhibitory. The results suggest that the natural substrate glutathione has two distinct functions in the normal catalytic reaction, (i) induction of a catalytically competent conformation of the enzyme and (ii) provision of the substrate sulfhydryl group in the reaction catalyzed.

Conformational change; Thiol substrate specificity; Reaction mechanism; Glutathione transferase; Glutathione derivative

1. INTRODUCTION

The specificity of glutathione transferases for glutathione as a thiol substrate is remarkably high, especially in comparison with the broad specificity for the electrophilic second substrate [1]. The glutathione analogues γ-glutamylcysteine [2] and homoglutathione [3] are the only thiols known to serve as alternative substrates. Other thiols tested include L-cysteine, *N*-acetyl-L-cysteine, 2-mercaptoethanol [3–5], dithiothreitol [5] and 2-propyl-thiouracil [6,7]. Since the size of these molecules should be small enough in comparison with glutathione to fit into the active site, the lack of activity may be attributed to failure of binding of these alternative thiols or to formation of non-productive complexes with an incorrect positioning of the reactive sulfhydryl group. It is conceivable that a highly specific orientation of the thiol of glutathione is afforded by a conformational change induced in the enzyme by the binding of the tripeptide.

The work presented here was undertaken in order to test the hypothesis that other thiols, e.g. 2-mercaptoethanol, could function as substrates for glutathione transferases in the presence of a non-substrate glutathione analogue serving as an activator of the enzyme.

2. EXPERIMENTAL

Rat liver glutathione transferase 4-4 was purified by use of affinity chromatography and FPLC chromatofocusing [8]. The enzyme was judged to be homogeneous by dodecyl sulfate-polyacrylamide slab gel electrophoresis. The concentration of active enzyme was determined using the specific activity with 1-chloro-2,4-dinitrobenzene (CDNB) [8]. Enzyme activity was measured spectrophotometrically at 30°C in a standard assay system containing CDNB in 0.2 M sodium phosphate buffer, pH 6.5. Glutathione, 2-mercaptoethanol and *S*-methyl-glutathione were products from Sigma and were present in the concentrations indicated below. The reaction was started by addition of enzyme.

CNDB conjugates were analyzed by HPLC using a reverse-phase C_{18} column (Chrompack) eluted isocratically with methanol/H_2O/acetic acid (30:70:5, v/v). The CDNB conjugates of 2-mercaptoethanol and glutathione, detected photometrically at 254 nm, were well resolved with retention times of 14.9 and 11.9 min, respectively.

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Abbreviation: CDNB, 1-chloro-2,4-dinitrobenzene

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3. RESULTS

2-Mercaptoethanol was demonstrated to function as substrate for glutathione transferase 4-4 in the presence of 1-chloro-2,4-dinitrobenzene (CDNB), the acceptor substrate, and *S*-methyl-glutathione, the activator. The reaction resulted in an increase in absorbance at 340 nm attributable to formation of a thioether linkage.

The reaction product was identified by HPLC with the *S*-conjugate of CDNB and 2-mercaptoethanol, synthesized nonenzymatically. No trace of *S*-2,4-dinitrophenyl-glutathione was detected. Thus, the possibility was excluded that free glutathione was first generated enzymatically from *S*-methyl-glutathione and then conjugated with CDNB. The enzymatic process involving 2-mercaptoethanol was consequently considered analogous to the reaction with glutathione, which involves nucleophilic displacement of the chlorine atom of CDNB by the thiol group. There was no observable activity in the absence of either CDNB, 2-mercaptoethanol or *S*-methyl-glutathione. An effect due to glutathione contamination in the *S*-methyl-glutathione preparation was excluded, since no reaction was detectable in the absence of 2-mercaptoethanol. The extinction coefficient of the 2-mercaptoethanol conjugate was similar to that determined for the reaction between CDNB and glutathione (ϵ = 9.6 $mM^{-1} \cdot cm^{-1}$, [3]), as determined by allowing a limited amount of CDNB to react to completion in the presence of excess 2-mercaptoethanol. The reaction rate was linearly dependent on the enzyme concentration, as shown in fig.1. A nonenzymatic reaction was observed and corresponds to the intercept on the *y*-axis in fig.1.

The enzymatic rate of 2-mercaptoethanol conjugation was low in comparison to the uncatalyzed reaction, when measured under standard conditions for the 1-chloro-2,4-dinitrobenzene assay (1 mM for each of the thiol and electrophilic substrates, at pH 6.5). The optimal conditions of the reaction were therefore determined and used in further activity measurements. The ratio between the enzymatic and the nonenzymatic rate was dependent on the CDNB concentration and the pH of the assay. Table 1 shows that relatively low concentrations (100 μM) of the electrophilic substrate were required to reduce the contribution of the nonenzymatic activity. At higher concentrations (400 μM) the ratio between the enzymatic and the uncatalyzed rates was significantly lower, whereas at low concentration (10 μM) the accuracy of the measurements was lower. The rate of the enzymatic reaction increased with the pH, shown in

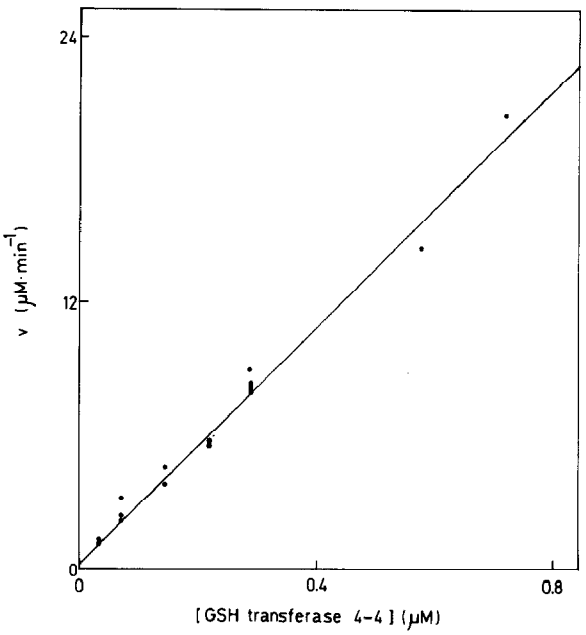

Fig.1. Reaction between 2-mercaptoethanol and 1-chloro-2,4-dinitrobenzene as a function of the concentration of rat glutathione transferase 4-4. The reaction was measured spectrophotometrically at 340 nm in 0.2 M sodium phosphate buffer, pH 6.5, containing 2 mM *S*-methyl-glutathione, 30 mM 2-mercaptoethanol and 100 μM 1-chloro-2,4-dinitrobenzene at 30°C.

Table 1

Activity of rat glutathione transferase 4-4 with 2-mercaptoethanol as thiol substrate at different concentrations of 1-chloro-2,4-dinitrobenzene

CDNB concentration (μM)	Enzymatic activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Enzymatic/ nonenzymatic activity
10	0.16 ± 0.02	8.7 ± 2.1
100	0.60 ± 0.11	11.2 ± 1.5
400	1.01 ± 0.06	4.3 ± 0.6

The assays were performed at 30°C in 0.2 M sodium phosphate buffer, pH 6.5, containing 2 mM *S*-methyl-glutathione, 25 mM 2-mercaptoethanol, and 1-chloro-2,4-dinitrobenzene at the concentrations indicated

Table 2

Activity of rat glutathione transferase 4-4 with 2-mercaptoethanol and 1-chloro-2,4-dinitrobenzene at different pH values

pH	Enzymatic activity (μmol · min^{-1} · mg^{-1})	Enzymatic/ nonenzymatic activity
6.0	0.16 ± 0.03	4.0 ± 1.0
6.5	0.60 ± 0.11	11.2 ± 1.5
7.0	0.76 ± 0.14	5.3 ± 1.2
7.5	1.22 ± 0.04	3.7 ± 0.2

The assays were performed at 30°C in 0.2 M sodium phosphate buffer, containing 100 μM 1-chloro-2,4-dinitrobenzene, 2 mM *S*-methyl-glutathione and 25 mM 2-mercaptoethanol

table 2. However, a greater increase was observed for the nonenzymatic reaction and the optimal ratio between the two reactions was obtained at pH 6.5 (table 2).

The activating effect of *S*-methyl-glutathione was found to be concentration dependent, having a maximum in a narrow concentration interval. Fig.2 shows a marked increase with activator concentrations up to 2 mM. Although saturation is reached, the reaction is inhibited by higher (>4 mM) concentrations of the activator.

The reaction was dependent on the 2-mercaptoethanol concentration, in a hyperbolic relationship between substrate and initial rate (fig.3). The corresponding double-reciprocal plot

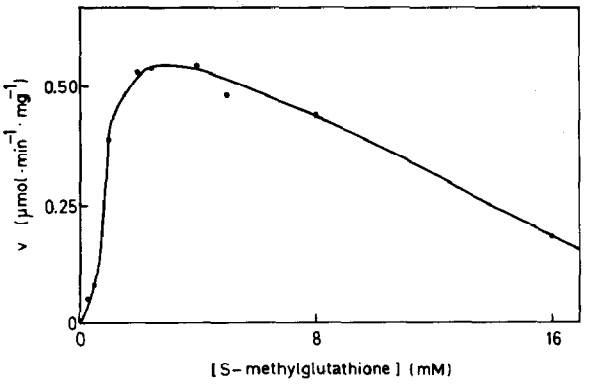

Fig.2. Activity of rat glutathione transferase 4-4 with 2-mercaptoethanol and 1-chloro-2,4-dinitrobenzene as a function of *S*-methyl-glutathione concentration. The reaction was measured at 30°C with 1-chloro-2,4-dinitrobenzene (100 μM) and 2-mercaptoethanol (30 mM) in 0.2 M sodium phosphate buffer, pH 6.5.

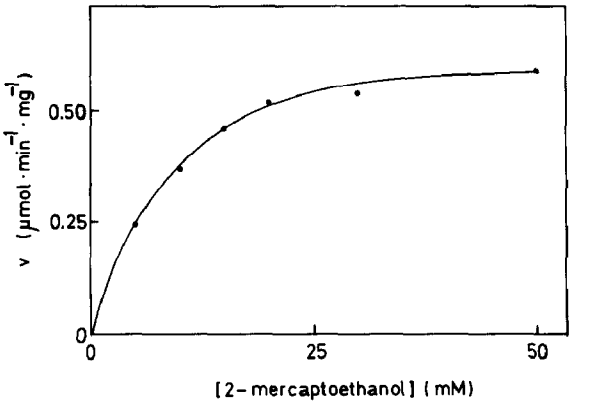

Fig.3. Activity of rat glutathione transferase 4-4 with 1-chloro-2,4-dinitrobenzene as a function of 2-mercaptoethanol concentration. The reaction was measured at 30°C with 1-chloro-2,4-dinitrobenzene (100 μM) and *S*-methyl-glutathione (2 mM) in 0.2 M sodium phosphate buffer, pH 6.5.

(not shown) is linear and predicts a K_m value ≈10 mM.

4. DISCUSSION

The results of this investigation show that 2-mercaptoethanol can serve as an alternative thiol substrate for rat glutathione transferase 4-4, provided that *S*-methyl-glutathione is present. The reaction is a true enzyme-catalyzed process, as evidenced by its linear dependence on enzyme concentration (fig.1). Further evidence for an enzymatic process was provided by the finding that 1 mM *S*-*p*-bromobenzyl-glutathione abolished the effect of the enzyme (not shown). This glutathione analog is a competitive inhibitor against glutathione in the normal enzymatic reaction. In the absence of 2-mercaptoethanol or *S*-methyl-glutathione, no enzyme activity was detectable.

The rate of the reaction was low in comparison with that obtained in the presence of glutathione, the natural thiol substrate, but was still significant. A major difference in the kinetic parameters is obvious from the apparent K_m value, which is approximately three orders of magnitude higher for 2-mercaptoethanol than for glutathione (0.05 mM [8]). In contrast to the results obtained with glutathione [1], the dependence of the initial velocity on 2-mercaptoethanol concentration appeared essentially hyperbolic in the range investigated (fig.2).

The most plausible interpretation of the results, is that the peptide part of glutathione induces a conformation change in the protein which is necessary for catalysis to occur. Once the tripeptide part has afforded the active conformation of the enzyme any sterically acceptable thiol compound could serve as substrate in the reaction catalyzed.

Indirect evidence from several sources suggest that glutathione transferases do undergo conformational transitions. For example, the solubility and crystallizability of rat glutathione transferase 4-4 change upon addition of *S*-substituted glutathione derivatives (Tibbelin, G. and Mannervik, B., unpublished). Similar results have been obtained with transferase 3-3 [9]. Binding of other substances such as bilirubin or even other proteins also appear to change the structure of glutathione transferases in a manner affecting their catalytic functions [10].

Finally, the non-hyperbolic steady-state kinetics observed for most of the glutathione transferases [1] would have a reasonable explanation by the suggested conformational transitions induced by glutathione.

Consequently, we propose that the natural substrate glutathione has two distinct functions in the normal catalytic reaction. Firstly, it induces the formation of a catalytically competent conformation of the protein. Secondly, it provides the thiol group which serves as one of the reactants in the reaction catalyzed. The first function does not require the thiol group of glutathione, as evidenced by the activating effect of *S*-methyl-glutathione. The second function can be exerted by an alternative thiol, such as 2-mercaptoethanol, once the conformational transition has occurred.

Experiments are in progress which show that also other combinations of thiols and non-substrate glutathione derivatives may serve as alternatives to glutathione in the novel enzymatic reaction described in this report. Studies of such reactions may be used to probe the various steps in the catalytic mechanism. Other glutathione transferases as well as glyoxalase I have also been shown to be active with alternative thiol substrates in the presence of a glutathione analog. It appears that several glutathione dependent enzymes may have the dual requirements of glutathione involving a peptide-induced conformational change in addition to the function as the thiol substrate.

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