Intermediate M_r cytosolic components potentiate hepatic 5'-deiodinase activation by thiols

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The role in the activation of microsomal 5'-deiodinase (5'-DI) of rat hepatic cytosolic components of M_r approx. 13000 (Fraction B) was studied in the presence of various concentrations of thiol compounds such as dithiothreitol (DTT), dihydrolipoamide (DHLA), GSH, and 2-mercaptoethanol (2-ME). Although Fraction B (which was prepared by gel filtration to exclude GSH and GSSG) had no intrinsic 5'-DI activity, could not stimulate microsomal 5'-DI activity in the absence of added thiol and did not contain GSH as a mixed disulphide, it could produce a 3-fold increase in the maximal deiodinase activity achievable with DTT as well as other thiols, with the order being the same as the activation potency of these thiols in the absence of Fraction B (i.e. DHLA > DTT > 2-ME > GSH). These observations suggest that: (1) a component of cytosolic Fraction B, designated 'deiodination factor B' (DF_B), operates as an efficient intermediary to enhance activation of microsomal 5'-DI by thiols through a mechanism independent of GSH; (2) thiols may participate in a non-specific thiol-disulphide exchange with inactive (oxidized) DF_B to convert it into an active form that contains one or more thiol groups and is more effective than GSH or other thiols in facilitating the re-activation of inactive (oxidized) microsomal 5'-DI thiol (ESI) to its active state (ESH).

INTRODUCTION

Since Visser *et al.* [1] reported that a soluble factor in rat liver cytosol required for the stimulation of microsomal 5'-deiodinase (5'-DI) activity could be replaced by exogenous thiols such as DTT, the dependence of the enzyme on soluble thiols has been well established [2–4]. It has also been proposed that thiols such as DTT and GSH may function as direct reductants of the enzyme, maintaining deiodinase in an active form [2–7]. Attempts to correlate hepatic intracellular thiols (particularly GSH) with 5'-DI activities in various pathophysiological conditions, however, have not been successful [8–10]. Although thiol compounds may act directly, it is also possible that an endogenous factor may function as an intermediary between the 5'-DI enzyme and thiol compounds.

Using subcellular fractions of rat liver, we have recently obtained evidence that cytosol contains a heat-stable non-dialysable factor of M_r approx. 13000 (herein designated 'deiodination factor B' or DF_B) which, although inactive by itself, is able to stimulate the 5'-DI (ESH) activity of washed microsomes when NADPH and a heat-labile cytosolic factor of $M_r > 67000$ (herein designated 'deiodination factor A' or DF_A) are added to the deiodination assay system [11,12]. This activating effect, achieved in the absence of free GSH or exogenous thiol, was attributed to the reduction of DF_B (catalysed by the

putative reductase, DF_A) in an NADPH-dependent reaction [12], as follows:

Thus it appeared that the reduced cofactor (DF_BH_2) could substitute for thiols of low M_r , such as DTT, in the reduction and re-activation of the inactive thienyl iodide intermediate (ESI) postulated by others [2–4], according to the following sequential reactions for the overall reaction of 5'-DI:

In view of the known requirement of 5'-DI for a thiol activator to maximize activity [1–4], we have proposed [11,12] that DF_BH_2 might contain thiol groups generated from an inactive disulphide form of the factor in a manner analogous to the reduction of thioredoxin by NADPH and thioredoxin reductase [13], as follows:

If eqn. (4) is correct and the inactive form of DF_B is

Abbreviations used: DTT, dithiothreitol; DHLA, dihydrolipoamide; BSA, bovine serum albumin; 2-ME, 2-mercaptoethanol; rT_3 , 3,3',5'-tri-iodothyronine (reverse tri-iodothyronine); T_2 , 3,3'-di-iodothyronine; T_3 , 3,5,3'-tri-iodothyronine; T_4 , 3,5,3',5'-tetraiodothyronine (thyroxine); 5'-DI, 5'-iodothyronine 5'-deiodinase; PB-EDTA, 0.125 M-potassium phosphate/1 mM-EDTA, pH 7.4; DF_A , deiodination reductase in Fraction A; DF_B , deiodination cofactor in Fraction B; ESI, inactive (oxidized) microsomal 5'-deiodinase thiol; ESH, its active state; SPF, soluble protein factor.

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indeed a disulphide, then thiols (RSH) added to DF_B could be expected to participate in a thiol-disulphide exchange with the production of DF_B(SH)₂, its active (reduced) form, as follows:

[Eqns. (4) and (5) imply that DF_B contains an intramolecular disulphide, whereas it may in fact be a mixed (DF_B-S-S-R') or symmetrical disulphide (DF_B-S-S-DF_B).]

The validity of the reaction shown by eqn. (5) is suggested by our recent finding that the activation of microsomal 5'-DI can be potentiated by the addition of a rat liver cytosolic fraction of *M_r* approx. 13000 that had been prepared by Sephacryl S-200 chromatography and showed properties very similar to those of Fraction B, i.e. it potentiated the activating effect of DHLA on microsomal 5'-DI [14]. It was therefore of interest to compare the potentiating effect of inactive Fraction B on the activation of 5'-DI by a variety of thiols over a range of concentrations to determine whether the potentiation of activation by DHLA was specific for that thiol or was a common characteristic of thiols in general. A second objective was to obtain further data relating to the mechanism of activation of 5'-deiodination by Fraction B.

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MATERIALS AND METHODS

The reagents used in the present study were of reagent grade or of the highest available purity. Outer-ring (3' or 5')-¹²⁵I-labelled reverse tri-iodothyronine ([¹²⁵I]rT3), 100 μCi/ml, with a specific radioactivity of > 1200 μCi/μg, was purchased from Amersham, Oakville, Ont., Canada. NADPH, lipoamide, GSH, DTT and BSA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-50 (fine grade), ribonuclease A and Blue Dextran were from Pharmacia Fine Chemicals (Uppsala, Sweden). 2-ME was from Eastman Kodak Co. (Rochester, NY, U.S.A.). DHLA was prepared by reduction of lipoamide with NaBH₄ as previously described [14].

Preparation of cytosol and microsomes

Livers of young male Sprague-Dawley rats aged 7-11 weeks were used to prepare subcellular fractions, as described previously [12].

Preparation of cytosol fractions

Pooled cytosolic Fractions A and B were prepared by chromatography of cytosol (5 ml containing 102 mg of protein) on Sephadex G-50 gel as described elsewhere [12]. A useful guide to the division between Fraction A and Fraction B was found to be the point at which the *A*₄₁₅ (presumed to be due to haemoglobin) has decreased to a value of < 0.1. Thus Fraction B contains components of *M_r* < 65000, consistent with our previous study using Sephacryl S-200 [14]. Individual effluent fractions having 5'-DI-stimulating activity when assayed with pooled Fraction A, NADPH and washed microsomes as described below were combined and designated 'Fraction B'. Within this preparation the principal 5'-DI

activator activity was of *M_r* approx. 13000 and was separated from GSH (*M_r* 307) and GSSG (*M_r* 612), which emerged later [14]. In some experiments Fraction B was further concentrated after column chromatography by high-pressure ultrafiltration through a type YM5 Diaflo membrane (Amicon Corp., Danvers, MA, U.S.A.), which retains solutes of *M_r* > 5000.

Determination of 5'-DI activity

The assay method used to determine 5'-DI activity was based on measurement of ¹²⁵I⁻ released from outer-ring (3' or 5')-¹²⁵I-labelled rT3 essentially as described by others [15] and utilized in our laboratory with minor modifications [12]. [¹²⁵I]rT3 was purified by paper electrophoresis before use by methods previously described [16]. Deiodination rates are expressed as the percentage of ¹²⁵I⁻ released/15 min, with 1% release equivalent to 4.8% total deiodination (including unlabelled I⁻) or 3 fmol of rT3 deiodinated [12].

Protocol for study of activation of 5'-DI by thiols

The reaction mixtures consisted of the various combinations of washed microsomes containing deiodinase (50 μl, containing 4 μg of protein), Fraction B (100 μl, containing 22 μg of protein), [¹²⁵I]rT3 in PB-EDTA (50 μl containing 10⁵ c.p.m., final concn. 0.21 nM) to which was added either DHLA, DTT, GSH, 2-ME in PB-EDTA, or buffer (50 μl) in a final volume of 300 μl. Control tubes containing microsomes denatured by heat at 90 °C for 5 min or containing buffer instead of denatured microsomes were incubated under the same conditions. The ¹²⁵I content of the supernatant in the presence of denatured microsomes was the same as that when microsomes were replaced by buffer (i.e., less than 5% of the total). At the same time, the effects of DTT, 2-ME and GSH in the presence of buffer alone were studied, but no influence by these materials on the free ¹²⁵I content of the trichloroacetic acid supernatant was observed. Therefore, tubes containing buffer instead of microsomes were used as controls.

Analysis of GSH-containing mixed disulphides in Fraction B

Release of free glutathione from combined form in Fraction B was achieved by incubating Fraction B (50 μl) with 10 mM-NaBH₄ at pH approx. 12 and 30 °C for 2 h, followed by removal of excess BH₄⁻ by acidification with HCl, re-adjustment of the pH with NaOH to approx. 7.4 and dilution to 300 μl. An appropriate control was prepared by acidification and neutralizing the same quantity of NaBH₄ before addition of Fraction B (150 μl) and adjustment of the final volume to 300 μl. Treated Fraction B (150 μl) and untreated Fraction B (50 μl) were subsequently assayed for GSH by the method of Tietze [24].

Determination of protein concentrations

The method used was that of Lowry *et al.* [17], with BSA as a standard.

Statistics

Statistical analyses of data were performed by a non-paired Student's *t* test to obtain two-tailed *P* values with a value of *P* < 0.05 considered to be significant.

Fig. 1. Effect of chromatographic Fraction B of cytosol and DTT on microsomal deiodinase activity

Five concentrations of Fraction B with various concentrations of DTT were tested by using the standard protocol (see the text for experimental details). * $P < 0.001$ for 5'-DI activities in the presence of Fraction B versus those in the absence of Fraction B at various concentrations of DTT ($n = 4$). Amounts of Fraction B (volume) per tube: ●, none (buffer only); ▲, 25 μ l; △, 50 μ l; ○, 75 μ l; ■, 100 μ l.

RESULTS

Fig. 1 shows the effect of adding various volumes of Fraction B on microsomal 5'-DI activity in the presence of DTT. When the concentration of DTT was raised from 0 mM to 1.0 mM in the absence of Fraction B, microsomal 5'-DI activity was increased from 0.05 ± 0.11 (S.D.) to 1.73 ± 0.15 ($P < 0.001$). The effect of DTT on the microsomal 5'-DI was significant in the absence of Fraction B at concentrations as low as 4 μ M ($P < 0.001$) and was maximal at 0.125 mM.

In the absence of DTT, 5'-DI activity of microsomes was not significantly enhanced by the addition of various volumes of Fraction B up to 100 μ l ($P > 0.05$), but the stimulatory effect of Fraction B at concentrations as low as 25 μ l/300 μ l of reaction mixture in the presence of DTT could be demonstrated and reached a maximum at approx. 0.125 mM-DTT. In the presence of Fraction B, 5'-DI activity was significantly greater than in its absence at all DTT concentrations between 0.015 and 1.0 mM (Fig. 1). In further studies, 150 μ l of Fraction B (concentrated 2.3-fold by high-pressure ultrafiltration), when tested without microsomes, showed no intrinsic 5'-DI activity in the presence of 1 mM-DTT, but produced a 7-fold increase in deiodination rate by microsomes (22 μ g of protein) when incubated in the presence of 1 mM-DTT in a total volume of 300 μ l (results not shown).

The effect of incubation time on release of $^{125}\text{I}^-$ by microsomal deiodinase in the presence of 1 mM-DTT and various concentrations of Fraction B is illustrated in Fig. 2. The deiodination rates were linear up to 20 min in the presence of various concentrations of Fraction B and were significantly higher than those without Fraction B.

The stimulatory effect of Fraction B on microsomal

5'-DI was then studied by substituting the thiol compounds DHLA, 2-ME and GSH for DTT. The results are summarized in Table 1. The 5'-DI activity induced by the added thiols was consistently increased by the addition of Fraction B. In either the presence or absence of Fraction B, both 5 and 10 mM-2-ME produced a stimulating effect similar in magnitude to that of 0.125 mM-DTT, whereas GSH at these concentrations was only one-fifth to one-third as potent as 0.125 mM-DTT. The relative potencies of the thiols (i.e. DHLA > DTT > 2-ME > GSH) were found to be the same whether in the presence or absence of Fraction B.

Reduction of Fraction B by borohydride resulted in no detectable increase in GSH content compared with the amount present before reduction (results not shown). Measured rates of increase of A_{412} did not differ from the very low blank value attributable to a slow, direct, reduction of dithionitrobenzoic acid by NADPH and glutathione reductase in the absence of added GSH [24].

DISCUSSION

The present data demonstrate that the potentiating effect of Fraction B on the activation of 5'-deiodination by DHLA, as previously reported [14], is also obtainable to various extents with DTT, GSH and 2-ME (Table 1) and thereby indicates that the effect of thiols is non-specific. However, pronounced differences in the 5'-DI activator potency of these thiols are evident, in agreement with observations by others [1]. In the presence of the cytosolic Fraction B, all of these thiols showed apparently enhanced effectiveness as activators of 5'-DI, but maintained their original relative order of potency. The non-specific character of this effect suggests that each

Fig. 2. Effect of incubation time and concentration of Fraction B on the microsomal 5'-deiodination in the presence of 1 mM-DTT

See the text for experimental details. * $P < 0.001$ for 5'-DI activities in the presence of Fraction B versus those in the absence of Fraction B ($n = 4$). Volumes of Fraction B per tube: ●, none (buffer only); ○, 50 μ l; ▲, 100 μ l.

thiol participates in a thiol-disulphide interaction with a component of Fraction B, DF_B (eqn. 5) with a characteristic equilibrium constant determined by the reduction potential of the thiol employed. The relatively low potential of DTT, a potent activator of DF_B , compared with that of GSH, a weak activator (Table 1) ($E'_0 = -0.33$ V and -0.25 V respectively [18]), is consistent with this interpretation. DTT at concentrations of 1–100 mM has been used extensively to maximize deiodinase activity (at a fixed iodothyronine concentration) and to measure apparent absolute enzyme concentration in subcellular fractions and homogenates of liver [7], kidney [2], placenta [26], pituitary [27] and cerebral cortex [28]. Our observations suggest that, when 5'-DI activity is being determined in the presence of excess DTT, the possible presence of variable concentrations of DF_B in subcellular fractions or homogenates should be considered.

Since Fraction B contains cytosolic components of M_r approx. 13000, it would be expected to include such possible activators of 5'-DI as glutaredoxin (M_r approx. 11000) [20], thioredoxin (M_r approx. 12000) [13], thiol transferase (M_r approx. 12000) [21], and a thiol-transferase-like soluble protein factor (SPF) (M_r approx. 11000) [22]. The ability of Fraction B to activate 5'-DI in the complete absence of GSSG and GSH, whether free or in mixed disulphide (the presence of a mixed disulphide involving thiols other than GSH was not excluded) form (shown by failure to detect GSH after reduction by borohydride), as well as the absolute requirement of Fraction B for NADPH in the absence of thiols [12] appears to distinguish DF_B from glutaredoxin, thiol transferase and SPF, all of which function efficiently without NADPH if sufficient GSH is available. On the other hand, DF_B is similar to thioredoxin in that both require NADPH and are potentiated by thiols in the absence of NADPH [19, the present study]. However, it appears that our Fraction B/Fraction A/NADPH system may differ from the thioredoxin/thioredoxin reductase/NADPH system, since the latter was reported not to stimulate 5'-DI by rat kidney [22] or liver [23] microsomes using a higher iodothyronine concentration.

Table 1. Effect of Fraction B on 5'-DI activity of washed microsomes in the presence of various thiols

Results are presented as means $\pm$ S.D. significances for microsomes with versus without Fraction B: * $P < 0.001$; ** $P < 0.01$. Values of 5'-DI activity in parentheses are relative and expressed as a percentage of activity in the presence of 0.125 mM-DTT without added Fraction B.

Thiol activator and concentration (mM)	5'-DI activity (% of ^{125}I -released/15 min) ($n = 3$)	
	Microsomes alone	Microsomes + Fraction B
(a) Control (no thiol)	0.3 $\pm$ 0.15 (18)	0.3 $\pm$ 0.10 (18)
(b) 2-ME		
(1.0)	0.6 $\pm$ 0.34 (38)	2.4 $\pm$ 0.54** (160)
(5.0)	1.8 $\pm$ 0.13 (120)	4.1 $\pm$ 0.24 * (273)
(10.0)	1.7 $\pm$ 0.13 (113)	3.9 $\pm$ 0.05 * (260)
(c) GSH		
(1.0)	0.1 $\pm$ 0.10 (7)	0.2 $\pm$ 0.13 (15)
(5.0)	0.3 $\pm$ 0.02 (20)	0.7 $\pm$ 0.02** (47)
(10.0)	0.6 $\pm$ 0.05 (42)	1.5 $\pm$ 0.21** (102)
(d) DTT (0.125)	1.5 $\pm$ 0.10 (100)	4.4 $\pm$ 0.26 (293)
(e) DHLA (0.016)	1.4 $\pm$ 0.20 (93)	4.0 $\pm$ 0.20 (267)

Scheme 1. Proposed mechanism of potentiation of iodothyronine 5'-deiodination of [125 I]rT $_3$ by Fraction B in the presence of DTT

A reduced component of Fraction B [DF $_B$ (SH $_2$)] is the direct reductant of the oxidized enzyme (ESI), whereas thiols such as DTT function as indirect activators to the extent that they are able to convert an oxidized (inactive) component of Fraction B (DF $_B$ S $_2$) into DF $_B$ (SH $_2$). GSH and exogenous thiols also act upon ESI directly, but less rapidly than does DF $_B$ (SH $_2$).

From previous evidence that the activator activity of Fraction B is dependent upon both cytosolic components of high M_r (Fraction A) and NADPH [11,12], we suggested that a component of Fraction B (now designated DF $_B$) is reduced to either a dithiol as in activated thioredoxin from an intramolecular disulphide or to a monothiol from a mixed disulphide such as DF $_B$ -S-S-G. On the basis of the analogous activation of oxidized (inactive) thioredoxin or glutaredoxin by DTT and DHLA [19], a postulated mechanism for the activation of Fraction B by thiols is illustrated in Scheme 1 and constitutes a further extension of our previously proposed mechanism of 5'-DI activation [12]. Thus, in the present case, reduction of DF $_B$ to its activated form is achieved by exogenous thiols rather than by cytosolic DF $_A$ and NADPH [11,12]. The data presented herein support the conclusion that the reduced Fraction B contains a thiol component that is a more efficient reductant of ESI than is GSH or any of the other thiols tested.

The apparently higher efficiency of DF $_B$ compared with DTT or DHLA in accelerating the deiodination of rT $_3$ may be due to the binding of reduced DF $_B$ to the deiodinase at, or near, the enzyme catalytic site before the approach of the substrate, thus making it immediately available to react with ESI. However, thiols of low M_r , such as DTT or GSH, may have relatively low affinity for the enzyme and therefore react with ESI at rates limited by diffusion. The relative rates of reaction of various thiols with ESI and with DF $_B$ remain to be determined.

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