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Kinetic characteristics of a thioredoxin-activated rat hepatic and renal low-K_m iodothyronine 5′-deiodinase

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The properties and kinetic characteristics of a non-GSH NADPH-dependent cofactor system activating rat hepatic and renal 5′-deiodinase (5′-DI), which we have previously demonstrated with partially purified cytosol Fractions A and B [Sawada, Hummel & Walfish (1986) Biochem. J. **234**, 391–398], were examined further. Although microsomal fractions prepared from either rat liver or kidneys could be activated by crude cytosol Fractions A and B from those tissues as well as from rat brain and heart, a homologous hepatic or renal system was the most potent in producing 5′-deiodination of reverse tri-iodothyronine (rT_3). At nanomolar concentrations both rT_3 and thyroxine (T_4) were deiodinated but with a much greater substrate preference for rT_3 than for T_4. However, at micromolar concentrations of these substrates no activation of 5′-DI could be detected. In this deiodinative system, T_4 and tri-iodothyronine (T_3) competitively inhibited 5′-deiodination of rT_3. Dicoumarol, iopanoate, arsenite and diamide were also inhibitory to the activation of hepatic or renal 5′-deiodination by this cofactor system. Purification of cofactor components in hepatic crude cytosolic Fractions A and B to near homogeneity, as assessed by their enzymic and physical properties, indicated that these co-purified with and were therefore identical with thioredoxin reductase and thioredoxin respectively, and accounted almost entirely for the observed activation of rT_3 5′-DI. When highly purified liver cytosolic thioredoxin reductase and thioredoxin were utilized to determine the kinetic characteristics of the reaction, evidence for a sequential mechanism operative at nanomolar but not micromolar concentrations of rT_3 and T_4 was obtained. The K_m for rT_3 was 1.4 nM. Inhibition by 6-n-propyl-2-thiouracil (K_i 6.7 μM) was competitive with respect to thioredoxin and non-competitive with respect to rT_3, whereas inhibition by T_4 (K_i 1.3 μM) was competitive. Since rT_3 is a potent inhibitor of T_4 5′-deiodination, this thioredoxin system activating deiodination of rT_3 may play an important role in regulating the rate of intracellular production of T_3 from T_4.

INTRODUCTION

The demonstration of iodothyronine 5- and 5′-deiodinations *in vitro* requires the presence of thiol compounds such as GSH or DTT to sustain 5′-DI activity by reducing the oxidized (inactive) enzyme to its original reduced (active) state [1–3].

Although their identities remain to be determined, other endogenous thiol compounds are also thought to be components of the 5′-DI activating system of cytosol *in vivo* [4]. Previous investigations of the reaction mechanism of iodothyronine deiodinations in various tissues have been carried out in the presence of DTT, which does not exist *in vivo* but is a potent artificial cofactor. Although the observed kinetic characteristics of these reactions have been observed to depend markedly on the concentration of added iodothyronines and DTT [5,6], there is some doubt as to the physiological significance of all such studies employing exogenous thiol activators.

We have recently reported that liver cytosol from fed normal rats contains a non-GSH NADPH-dependent cofactor system (CCS) that activated hepatic microsomal 5′-DI in the presence of 0.2 nM-rT_3 as substrate [7]. Evidence has been presented suggesting that this system contains a cytosolic dithiol component of M_r approx. 13000 (DF_B) that is reduced by another cytosolic component of M_r > 60000 (DF_A) (a putative reductase) in the presence of NADPH [7,8]. These studies utilized relatively crude cytosolic Fractions A and B containing DF_A and DF_B respectively and showed that this endogenous cofactor system, which supported the activity of a type I low-K_m 5′-DI, had properties similar to those of the thioredoxin system [7–9].

In the present study we investigated whether the cytosolic components supporting 5′-DI activity in the absence of DTT co-purified with the thioredoxin components and thus whether or not any endogenous cofactor activity other than that of the thioredoxin system could be detected under our assay conditions. In addition, the homogeneous components of this endogenous cofactor system have been utilized to characterize further their interaction with 5′-DI without the addition of exogenous thiol and to compare the

Abbreviations used: T_3, 3,5,3′-tri-iodothyronine; T_4, thryoxine (3,5,3′,5′-tetraiodothyronine); rT_3, reverse tri-iodothyronine (3,3′,5′-tri-iodothyronine); 5′-DI, iodothyronine 5′-deiodinase; DTT, dithiothreitol; PTU, 6-n-propyl-2-thiouracil; CCS, non-GSH NADPH-dependent cytosolic cofactor system; ESI, deiodinase, oxidized by substrate; ESH, deiodinase, reduced active form; DF_A, 5′-DI cytosolic cofactor in Fraction A; DF_B, 5′-DI cytosolic cofactor in Fraction B; PB-EDTA, 0.125 M-potassium phosphate/1 mM-EDTA buffer, pH 7.5.

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activator systems in hepatic and renal cytosol with a wide range of iodothyronine substrate concentrations. From such studies, the kinetic characteristics, substrate preferences and the effects of a variety of known inhibitors on the endogenous NADPH-dependent cofactor-supported 5′-DI activity could be assessed. The data thus obtained were expected to contribute to a more complete understanding of the deiodinative mechanism *in vivo* than has been reached on the basis of previous studies that used exogenous thiol activators such as DTT [3,4].

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MATERIALS AND METHODS

Materials

Outer-ring- (3′- or 5′-)labelled [^{125}I]rT_3 (100 μCi/ml with a specific radioactivity of 1200 μCi/μg) and L-[^{125}I]T_4 (300 μCi/ml with a specific radioactivity of 1200 μCi/μg) were purchased from Amersham (Don Mills, Ont., Canada) and New England Nuclear (Boston, MA, U.S.A.) respectively. L-T_4, L-T_3, PTU, NADPH, DTT and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and rT_3 was from Henning (Berlin, West Germany). Sephadex G-50, DEAE-Sephacel and CM-Sepharose were from Pharmacia Fine Chemicals (Uppsala, Sweden). 2′,5′-Bisphosphoadenosine–hexyl-agarose was from P-L Biochemicals (Milwaukee, WI, U.S.A.). All other reagents were of the highest purity commercially available.

Preparation of microsomes and cytosolic Fractions A and B

Male Sprague–Dawley rats (7–11 weeks of age when killed) maintained on Purina chow *ad libitum* were used. Rats were killed under light diethyl ether anaesthesia by exsanguination. Liver and kidney were homogenized in 0.125 M-potassium phosphate/1 mM-EDTA buffer, pH 7.5 (PB-EDTA), with the use of 3 ml/g of tissue. The microsomal fractions (referred to below simply as microsomes) were obtained by standard differential centrifugation as described previously [9]. Cytosol was chromatographed on a Sephadex G-50 column to obtain cytosolic fractions of $M_r > 60000$ (designated Fraction A) containing a putative reductase (DF_B) and to exclude GSH and GSSG, which emerged later [7]. The subcellular fractions were stored at −70 °C in PB-EDTA until assayed. To avoid the activating effects of added thiol compounds and thus simplify interpretation of reaction kinetics of the 5′-DI activation by endogenous cofactor system, no thiol compounds such as DTT were added during either the preparation of microsomes or their storage at −70 °C.

Purification of DF_A and DF_B

Pooled frozen livers (120 g per fractionation) from male Sprague–Dawley rats were obtained from Charles River (St. Constant, Que., Canada) and used after storage for several weeks at −70 °C. The fractionation procedures used were those of Engström *et al.* [10] and Luthman & Holmgren [11] with the following modifications: (*a*) the precipitate obtained with 85%-saturated $(NH_4)_2SO_4$ was dissolved in equilibration buffer, adjusted to 5 mM-DTT and chromatographed on a 5 cm × 75 cm column of Sephadex G-50 pre-equilibrated with PB-EDTA; (*b*) pooled fractions containing DF_A thus obtained were concentrated and equilibrated with 10 mM-Tris/HCl/1 mM-EDTA buffer, pH 7.4, by repeated pressure ultrafiltration under N_2 at 4 °C on a YM-5 membrane (Amicon), and DTT was added to reach 5 mM before gradient elution chromatography on a DEAE-Sephacel (instead of DEAE-cellulose [11]) column equilibrated with the same buffer; (*c*) final purification of DF_A was by affinity chromatography on 2′,5′-bisphosphoadenosine–hexyl-agarose (instead of 2′,5′-bisphosphoadenosine–Sepharose 4B followed by 6-aminohexyl-agarose [11]) pre-equilibrated with 50 mM-Tris/HCl/1 mM-EDTA buffer, pH 7.5; after the column had been washed free of unadsorbed components with the same buffer, elution was accomplished with 0.2 M-potassium phosphate/1 mM-EDTA buffer, pH 7.5; (*d*) pooled fractions containing DF_B obtained from the Sephadex G-50 column were made 15 mM in sodium acetate at pH 5.4 and 5 mM in DTT and then applied to a 1 cm × 35 cm column of CM-Sepharose (instead of CM-cellulose [11]) pre-equilibrated with 15 mM-sodium acetate buffer, pH 5.4. Elution was with a linear gradient of sodium acetate from 15 to 125 mM, followed by 1 M-NaCl.

Steps (*a*)–(*d*) above yielded preparations of DF_A and DF_B free of DTT. This was confirmed by 5,5′-dithiobis-(2-nitrobenzoic acid) assay [12].

Measurement of enzyme activities in chromatographic fractions

A portion of each chromatographic fraction was adjusted to 0.1 mM-DTT immediately after collection for determination of thioredoxin activity by the insulin reduction assay and of thioredoxin reductase activity by the 5,5′-dithiobis-(2-nitrobenzoic acid) assay method of Luthman & Holmgren [11]. No DTT was added to fractions before determination of DF_A or DF_B (i.e. 5′-DI-supporting) activity. Thiol transferase activity was assayed by the method of Larson *et al.* [13], and glutaredoxin and glutathione reductase activities were assayed as described by Luthman & Holmgren [11].

Determination of 5′-DI activity

The assay method used to determine 5′-DI activity was based on $^{125}I^-$ released from outer-ring- (3′- or 5′-)labelled [^{125}I]T_3 or [^{125}I]T_4 as described by others [14] and utilized in our laboratory with minor modifications [7]. [^{125}I]rT_3 and [^{125}I]T_4 were purified by paper electrophoresis before use by methods previously described [15].

For assays of rT_3 5′-DI activity, carried out in PB-EDTA, the reaction mixture contained (except as otherwise noted), in a total volume of 300 μl, 20 μg of microsomes, 1 μg of DF_A (or 150 μg of Fraction A), 10 μg of DF_B (or 35 μg of Fraction B), 0.2 mM-NADPH and [^{125}I]rT_3 (50000 c.p.m.) to which, in some cases, had been added various amounts of unlabelled rT_3 or T_4 as specified in the Figure legends and text. Assays of T_4 5′-DI activity were performed in reaction mixtures containing 150 μg of microsomes, 750 μg of Fraction A, 175 μg of Fraction B, 1.0 mM-NADPH, [^{125}I]T_4 (50000 c.p.m.) and the specified concentrations of unlabelled L-T_4 in a total volume of 300 μl. These assays of 5′-DI activity were carried out in the absence of DTT,

which was always removed during chromatographic purification of DF_A (thioredoxin reductase) and DF_B (thioredoxin). The reactions were started by the addition of appropriate amounts of substrates and continued for 15 min for the rT_3 5′-DI assay and for 60 min for the T_4 5′-DI assay at 37 °C. The remainder of the assay was carried out as described previously [7].

Polyacrylamide-gel electrophoresis

Gel electrophoresis under non-denaturing conditions was performed in 10% polyacrylamide slab gels (0.75 mm) with Tris/glycine buffer, pH 8.4, at 15–20 mA constant current for 5–6 h. SDS/polyacrylamide-gel electrophoresis was performed with 15% polyacrylamide gel as described by Laemmli [16]. The gels were stained with Coomassie Brilliant Blue G-250.

Determination of protein concentrations

Protein concentrations were determined by the method of Lowry *et al.* [17], with bovine serum albumin as a standard. Weights of microsomes, Fraction A or Fraction B refer to protein.

Methods of data analyses

Double-reciprocal (Lineweaver–Burk) plots of data were determined by unweighted linear-regression analysis with all data obtained in any one experiment wherein duplicate values for each concentration of substrate, inhibitor or activator were obtained. K_m and K_i values were also obtained from secondary plots of ordinate intercepts versus concentration [18] with the use of unweighted linear-regression analysis. Each experiment was carried out two or more times and data for representative experiments are shown in the Figures.

RESULTS

Purification of DF_A and DF_B

All fractions from the Sephadex G-50, DEAE-Sephacel, 2′,5′-bisphosphoadenosine–hexyl-agarose and CM-Sepharose columns were assayed for 5′-DI-supportive activity, thioredoxin reductase, thioredoxin, thiol transferase and glutaredoxin activities. At each stage of purification the distributions of DF_A and thioredoxin reductase among the fractions closely paralleled one another, as did those of DF_B and thioredoxin (Tables 1 and 2).

Chromatography on Sephadex G-50 before that on DEAE-Sephacel, rather than the reverse [11], allowed complete separation of DF_A and thioredoxin reductase from DF_B and thioredoxin, and the use of DEAE-Sephacel rather than DEAE-cellulose [11] resulted in recovery of all the glutathione reductase activity in the flow-through fractions before application of the salt gradient.

DF_A or thioredoxin reductase from the 2′,5′-bisphosphoadenosine–hexyl-agarose step and DF_B or thioredoxin from the CM-Sepharose step were nearly

Table 1. Purification of DF_A or thioredoxin reductase from 120 g of rat liver

For full experimental details see the text. Units of DF_A activity are expressed as pmol of I^- released/h; units of thioredoxin reductase activity are expressed as μmol of NADPH oxidized/min. A final preparation of thioredoxin reductase with a specific activity of 35 units/mg and an apparent 4000-fold purification with 15% yield based on activity measurements of the crude extract was achieved.

		DF_A			Thioredoxin reductase		
Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Activity (units)	Specific activity (units/mg)	Purification (fold)
Sephadex G-50	1452	2499	1.72	1.0	46.9	0.032	1.0
DEAE-Sephacel (eluted at 140 mM-NaCl)	164	1107	6.75	3.9	19.9	0.121	3.8
2′-,5′-Bisphosphoadenosine–hexyl-agarose	0.47	848	1804	1049	17.2	36.6	1144

Table 2. Purification of DF_B or thioredoxin from 120 g of rat liver

For full experimental details see the text. Units of DF_B activity are expressed as pmol of I^- released/h; units of thioredoxin activity are expressed as μmol of NADPH oxidized/min. A final preparation of thioredoxin with a specific activity of 50 units/mg and an apparent 400-fold purification with 8% yield based on activity measurements of the crude extract was achieved.

		DF_B			Thioredoxin		
Fraction	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (fold)	Activity (units)	Specific activity (units/mg)	Purification (fold)
Sephadex G-50	54.7	332	60.9	1.0	164	3	1.0
CM-Sepharose (eluted at 70 mM-sodium acetate)	2.9	2684	925.5	15.2	142	49	16.3

homogeneous on polyacrylamide-gel electrophoresis under both denaturing and non-denaturing conditions (results not shown).

Characteristics of the CCS-activated 5′-DI system

(*a*) With partially purifed DF_A and DF_B (Fractions A and B). Chromatographic Fractions A (or B) from cytosol of kidney, heart, cerebrum and cerebellum as well as liver were examined for their DF_A (or DF_B) activity in reaction mixtures containing liver microsomal 5′-DI Fraction B (or A). Both DF_A and DF_B activities, required for activation of hepatic 5′-DI in the presence of NADPH, were found in these tissues. Cofactor activity in heart, cerebrum and cerebellum, though lower than those in liver or kidney, were significant, i.e. 48, 21 and 23% respectively of the DF_B activity (means; $n = 3$) found in liver. The cross-reactivity between liver and kidney tissue preparations was examined in reaction mixtures containing washed microsomes and cytosolic Fractions A and B from each tissue as well as NADPH with $[^{125}I]rT_3$ as substrate. The subcellular fractions of each tissue were found to be interchangeable with corresponding fractions of the others in supporting 5′-DI activity. However, both the liver and the kidney CCS activated the homologous microsomal enzyme more effectively than the non-homologous system (Fig. 1). Studies of 5′-deiodination of T_4 with use of the endogenous CCS also showed similar cross-reactivity between liver and kidney components (results not shown).

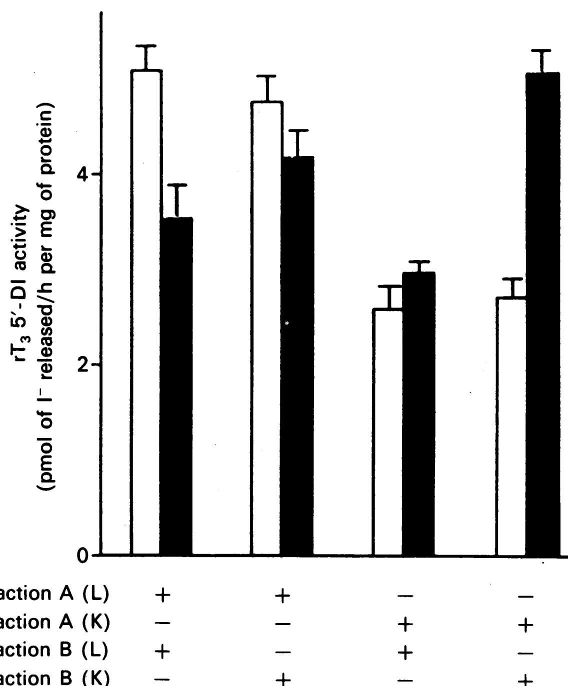

Fig. 1. Effect of interchanging hepatic and renal components of the partially purified CCS activating 5′-deiodination

The 5′-DI activity was assayed in reaction mixtures containing Fraction A and Fraction B. Each component from liver or kidney was substituted for the corresponding component from the other tissue. Data points represent the means ± S.D. for two experiments, each done in duplicate. □, Liver microsomes; ■, kidney microsomes. Fractions A and B: (L), from liver; (K), from kidney.

(*b*) Kinetic studies with highly purified DF_A and DF_B. Three components of CCS, DF_A, DF_B and NADPH, regulate the activation of 5′-DI by this CCS [7], and previous studies [7,8] are consistent with the proposal that the degree of activation by this system depends on the concentration of reduced DF_B available for the reduction of the oxidized enzyme, ESI [19,20]. In the present study, the experimental conditions in which the concentration of reduced DF_B depends on the amount of oxidized DF_B were manipulated by varying the concentration of DF_B at fixed concentrations of DF_A and a constant excess of NADPH [8]. Since preliminary studies with nanomolar concentrations of rT_3 as substrate showed the optimal pH to be 7.5 and the optimal temperature to be 37 °C for the reaction with both liver and kidney preparations, all experiments, whether performed with partially or highly purified CCS components, used these assay conditions.

Kinetic analysis of rT_3 5′-DI activation by hepatic CCS at low (nanomolar) substrate concentrations was performed in reaction mixtures in which the concentrations of DF_B and rT_3 were varied systematically while the other experimental conditions were as described in the Materials and methods section. Lineweaver–Burk plots of reaction velocities versus rT_3 concentrations yielded a series of lines intersecting in the second quadrant above the abscissa (Fig. 2*a*). A similar plot of reaction velocities versus DF_B concentrations yielded a series of lines intersecting on the abscissa (Fig. 2*b*). Secondary plots of the ordinate intercepts against $1/[DF_B]$ or against $1/[rT_3]$ yielded straight lines (Fig. 2*a* inset and Fig. 2*b* inset respectively). These observations are compatible with a sequential mechanism [18,21] for activation that is proportional to the concentration of added DF_B. As shown in Table 3, the calculated K_m and $V_{max.}$ for rT_3 were 1.4 nM and 182 pmol/h per mg of microsomal protein for liver respectively. From Fig. 2(*a*) inset the K_m for DF_B was found to be 6.6 µg/300 µl, or 1.8 µM assuming M_r 12000, with the use of purified DF_A and DF_B.

Parallel kinetic studies with Fraction A in place of DF_A and Fraction B in place of DF_B yielded results that were qualitatively similar to those shown in Fig. 2 (results not shown) but gave values for K_m and $V_{max.}$ that differed from those obtained with the highly purifed cofactor system (Table 3).

When high (micromolar) concentrations of rT_3 or T_4 were used with either the crude or the highly purified cofactor systems, however, activation of 5′-DI by the CCS could not be detected even when higher concentrations of microsomal enzyme, cytosolic components and NADPH were used.

Inhibition by PTU of 5′-deiodination activated by highly purified DF_A and DF_B

The mechanism of inhibition by PTU of the hepatic CCS activating 5′-deiodination was studied with use of the same experimental conditions employed in the study of reaction mixtures as described above. PTU inhibited 5′-deiodination at concentrations greater than 0.1 µM, with 90% inhibition by 1 mM-PTU when 35 µg of Fraction B protein or 10 µg of purified DF_B was used for activation (results not shown). Lineweaver–Burk plots of reaction velocities versus rT_3 concentrations at various fixed non-saturating PTU concentrations showed non-competitive kinetics over a range of rT_3 concentations

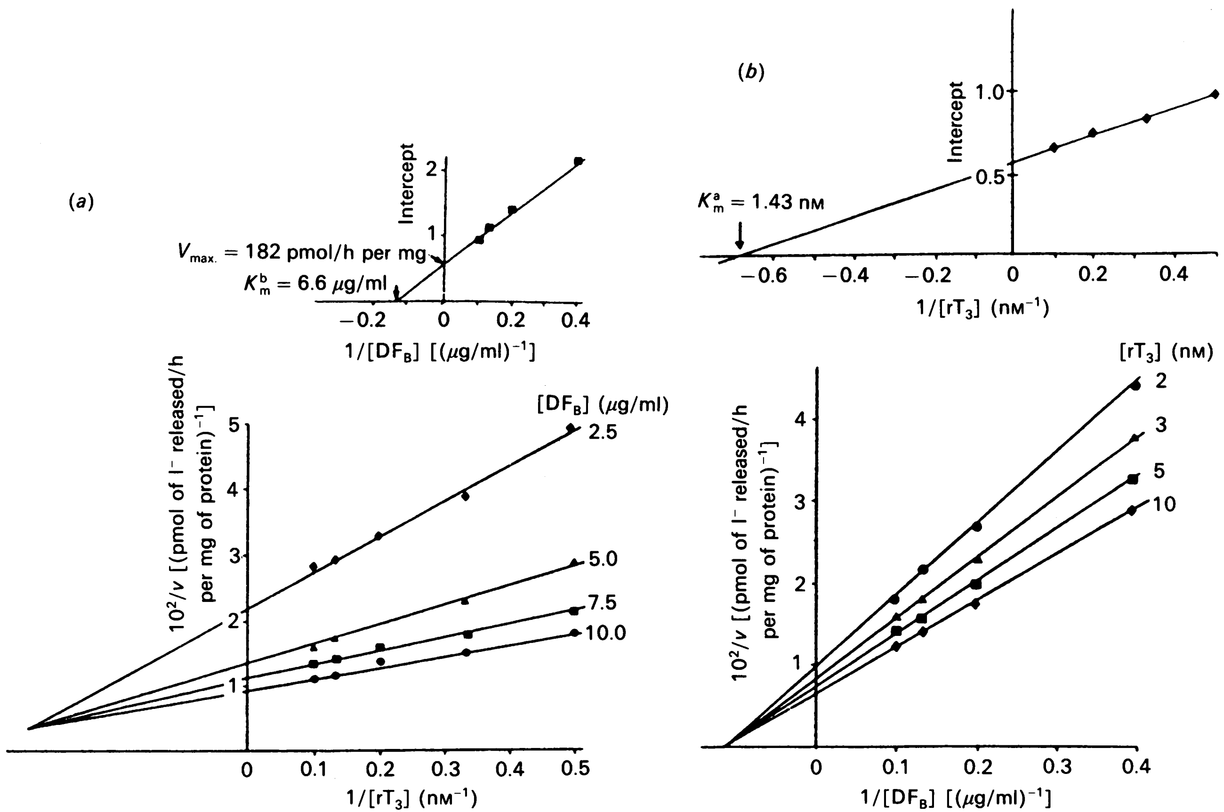

Fig. 2. Stimulation of hepatic 5′-deiodination of rT_3 at nanomolar substrate concentration by the CCS

The 5′-DI activities were measured with (*a*) DF_B at several fixed concentrations over a range of substrate concentrations and (*b*) rT_3 at several fixed concentrations over a range of DF_B concentrations. Assay conditions were as described in the text. In (*a*) an intercept replot (inset) was linear, giving a $V_{max.}$ of 182 pmol/h per mg of protein. In (*b*) an intercept replot (inset) was linear giving a K_m of 1.43 nM. The data are from one of three closely agreeing experiments. The concentration of DF_A was 0.1 mg of protein/ml.

Table 3. Kinetic constants for 5′-DI activated by DF_B (thioredoxin)

	rT_3 substrate						T_4 substrate	
Microsome source	K_m (DF_B) (μM)	K_m (rT_3) (nM)	$V_{max.}$ (pmol of I^- released/h per mg of microsomal protein)	K_i (T_4) (μM)	K_i (T_3) (μM)	K_i (PTU) (μM)	K_m (T_4) (nM)	$V_{max.}$ (pmol of I^- released/h per mg of microsomal protein)
Liver:								
Fraction A/ Fraction B		2.3	96	2.3	3.0	6.7*	5.5	0.50
DF_A/DF_B	1.8‡	1.4	182	1.3		3.6†		
Kidney:								
Fraction A/ Fraction B		3.2	96				1.4	0.4

* With 35 μg of Fraction B protein.
† With 10 μg of DF_B/300 μl = 3 μM.
‡ See Fig. 2(*a*) inset.

(0.1–5 nM), and a replot of the intercepts on the ordinate against the PTU concentrations was linear (results not shown) and revealed a K_i of 3.6 μM with the use of 10 μg of DF_B (Table 3). Another Lineweaver–Burk plot of reaction velocities versus DF_B concentrations at four fixed PTU concentrations showed competitive inhibition kinetics (results not shown). These characteristics of PTU inhibition of 5′-deiodination were also observed

Table 4. Effect of various compounds on the CCS-activated rT_3 5′-deiodination

The 5′-DI activity was assayed in reaction mixtures containing microsomes (20 μg), Fraction A (150 μg), Fraction B (35 μg), 200 μM-NADPH and various concentrations of compounds. [^{125}I]rT_3 (0.2 nM) was used as a substrate. Each value was obtained from two experiments, each done in duplicate.

Compound	Concentration producing 50% inhibition of rT_3 5′-deiodination (I_{50}) (μM) Liver	Kidney
Iopanoic acid	0.09	0.07
Sodium arsenite	2.5	1.4
L-T_4	3.3	0.99
Dicoumarol	4.6	2.1
L-T_3	6.0	0.56
Diamide	250	120

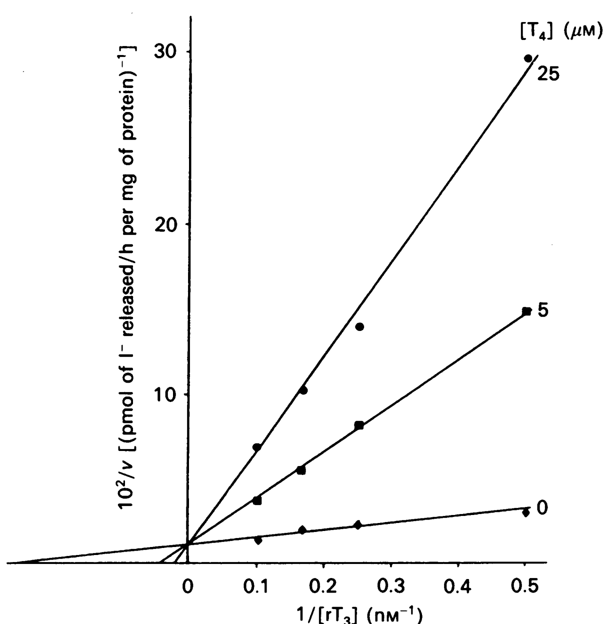

Fig. 3. Competitive inhibition of the CCS-activated hepatic rT_3 5′-DI activity by L-T_4 at nanomolar substrate concentrations

The 5′-DI activity was assayed in reaction mixtures containing DF_A, DF_B and 2–10 nM-rT_3 as substrate with L-T_4 at three different concentrations (0–25 μM). The data are from one of three similar experiments in close agreement. A K_i value of 1.3 μM was calculated for T_4 inhibition.

in the kidney tissue system with both rT_3 and T_4 at nanomolar substrate concentrations (results not shown).

Inhibitory effects of various other compounds on 5′-deiodination activated by partially purifed DF_A and DF_B

The effects of other substrates (T_4 and T_3) and various compounds known to be potent inhibitors of 5′-DI activity, or of the activity of the CCS, were also studied at nanomolar substrate concentrations (Table 4). T_4 inhibited hepatic deiodination of rT_3 significantly at concentrations greater then 0.33 μM, with an I_{50} of 3.3 μM in the presence of 0.2 nM-rT_3 (Table 4). Kinetic analysis of inhibition by T_4 showed it to be competitive with respect to rT_3 concentration, with a K_i of 1.3 μM (Fig. 3). These findings also indicated that this enzymic deiodinating system utilizes both iodothyronines as substrates but deiodinates rT_3 much more rapidly than it does T_4. Similar inhibition kinetics and substrate preferences were also found when inhibition of the rT_3 5′-DI activity by T_3 was studied (K_i 3.0 μM). Iopanoic acid strongly inhibited hepatic rT_3 5′-DI activity, producing 31% inhibition at 1 nM, and complete inhibition at concentrations greater then 10 μM, with an I_{50} of 0.09 μM. Dicoumarol inhibited the activity at concentrations greater than 1 μM with an I_{50} of 4.6 μM, whereas diamide required higher concentrations to produce significant inhibition, with an I_{50} of 0.25 mM. Sodium arsenite, a specific inhibitor of dithiol compounds [22], including reduced DF_B [8], also inhibited 5′-DI at concentrations greater than 1 μM, with an I_{50} of 2.5 μM. Similar effects of these inhibitors were also found in the renal rT_3 5′-DI studies (Table 4), although the relative order of potency differed from that found with liver. Both the low-K_m 5′-DI activated by CCS in the present study and the high-K_m 5′-DI activated by DTT show similar sensitivities to several of these inhibitors [1,23,24].

DISCUSSION

Our previously reported investigations of the 5′-DI cofactor system of rat liver cytosol utilized partially purified chromatographic Fractions A and B, together with NADPH [7–9,25], and indicated that this system resembled the thioredoxin system in the following respects: (*a*) NADPH-dependence, (*b*) requirement for a reductase of $M_r > 60000$ plus a dithiol of M_r approx. 13000 and pI < 6, (*c*) sensitivity to arsenite and iodoacetamide, (*d*) resistance to thermal inactivation, (*e*) enhancement by DTT and (*f*) inhibition of 5′-DI-supportive activity of Fractions A and B by anti-(thioredoxin reductase) IgG and by anti-thioredoxin IgG respectively. Substitution experiments [25] showed that *Escherichia coli* thioredoxin could replace Fraction B. Highly purified thioredoxin reductase and thioredoxin of rat liver cytosol, together with NADPH, were shown by others to support 5′-deiodination of rT_3 [25]. These various findings strongly suggested that the stimulatory activity that we reported in rat liver [7–9] could be attributed to the thioredoxin system. There remained the possibility, however, that another cofactor system with closely similar properties might also have been present in cytosol but remained undetected. We therefore measured the 5′-DI-supportive activity of all subfractions of cytosolic Fractions A and B obtained by using a modified version of the methods used by Luthman & Holmgren for the purification of thioredoxin reductase and thioredoxin [11]. The distributions of DF_A and DF_B activities closely followed those of thioredoxin reductase and thioredoxin respectively. Identity was confirmed by the appearance of major single bands (with additional faint bands, as also reported by others [11,26]) on SDS/polyacrylamide-gel electrophoresis for highly purified

DF_A or thioredoxin reductase and DF_B or thioredoxin. Furthermore, at each stage of the procedure, which achieved purification to near homogeneity in two steps, the specific activities of DF_A and DF_B paralleled those of thioredoxin reductase and thioredoxin respectively (Tables 1 and 2). From these observations, we conclude that activation by the previously identified non-GSH NADPH-dependent cytosolic cofactor system (CCS) that supported a low-K_m 5′-deiodination of rT_3 can be attributed primarily to cytosolic thioredoxin system components.

By using double-reciprocal plots of data to characterize the kinetics of this 5′-deiodinative system in the presence of purified thioredoxin cytosolic cofactor components, data consistent with a sequential, rather than a ping-pong [18,27], mechanism were obtained. Similar observations have been reported for PTU-insensitive 5′-DI enzymes of brain, pituitary and kidney activated by DTT [28–30]. The K_m value obtained for rT_3 (1.4 nM) agrees reasonably well with the value of 2.5 nM previously reported by others for a purified rat thioredoxin system [26]. The calculated K_m value [1.8 μM for thioredoxin (DF_B)] suggests that, if the intracellular concentration of thioredoxin is 12 μM, as estimated by others [11,26], the microsomal 5′-DI could be operating *in vivo* with respect to this activator at nearly $V_{max.}$.

The kinetic data on inhibition of the thioredoxin-activated system by PTU showed that this inhibitor was non-competitive with rT_3, with a K_i value intermediate between those reported by others [26,31], but competitive with activator (thioredoxin), as reported for type I 5′-DI [32,33]. Unlike others [26], we were able to detect significant deiodination with a partially purified activator system in which the $V_{max.}$ for rT_3 was approx. 200 times that for T_4 for both kidney and liver cofactor systems, whereas the K_m for rT_3 in liver was half that in kidney. Deiodination of T_4 could also be measured with the use of the highly purified DF_A/DF_B (thioredoxin) system (results not shown), but, in comparison with the conditions required to demonstrate rT_3 deiodination, longer incubation times and higher concentrations of microsomes were required to detect a significant rate of reaction in this low-K_m system.

T_4 was found to be a competitive inhibitor of rT_3 deiodination (K_i 1.3 μM) by the thioredoxin-activated microsomal system (Fig. 3). Various other well-known inhibitors of 5′-DI activation also showed strong inhibition of 5′-deiodination activated by the partially purified CCS (Table 4) in a manner similar to their inhibition with DTT as activator [1,23,24]. In some cases inhibition could have resulted from interaction with either microsomal 5′-DI or the components of a cytosolic activating system. We previously demonstrated that sodium arsenite, in the absence of microsomes, strongly inhibited the reduction of DF_B by DF_A and NADPH, whereas iopanoic acid had no such inhibitory effect, indicating that it acted directly with the 5′-DI [8].

In agreement with other investigators [34], we could not detect any stimulation of 5′-DI by the thioredoxin endogenous cofactor system when high (micromolar) iodothyronine concentrations were employed. This effect may be due to inhibition of the reduction of the oxidized enzyme by reduced DF_B (thioredoxin) through steric hindrance of the 5′-DI active site by bound iodothyronine, which prevents the close approach of the activating polypeptide. DTT, having a much lower M_r than thioredoxin, would be less impeded by bound substrate and kinetically more mobile. Other workers [5] could not detect any stimulator effect of GSH on renal 5′-DI at high (micromolar) iodothyronine concentrations. No studies to date, except for one [34], have identified an endogenous cofactor system for 5′-DI that functions at high (micromolar) substrate concentrations in the absence of DTT. Cytosolic soluble protein factor was reported [34] to stimulate 5′-DI at high concentrations of iodothyronine in the presence of both 1 mM-NADPH and 5 mM-GSH, but the physiological significance of this system is unclear, since it was relatively insensitive to PTU and thus differs in this respect from the previously described characteristics of type I 5′-DI.

The present study has demonstrated that in normal fed rats a non-GSH NADPH-dependent cytosolic cofactor system (CCS) that activated hepatic and renal microsomal 5′-DI is present not only in liver but also in several other tissues such as kidney, brain and heart.

Since the thioredoxin-mediated system is operative at substrate concentrations in the physiological range, activation of this low-K_m rT_3 5′-DI could result in a decrease in the intracellular concentration of rT_3, known to be a potent inhibitor of the conversion of T_4 into T_3. Thus the thioredoxin system, which is apparently ubiquitious in mammalian tissues [35], may play a significant role in the intracellular regulation of iodothyronine metabolism.

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