The effects of selenium deficiency on hepatic type-I iodothyronine deiodinase and protein disulphide-isomerase assessed by activity measurements and affinity labelling

John R. ARTHUR,* Fergus NICOL,* Ewan GRANT† and Geoffrey J. BECKETT†

*Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, and †University Department of Clinical Chemistry, Royal Infirmary, Edinburgh EH3 9YW, Scotland, U.K.

We determined protein disulphide-isomerase (PDI) and iodothyronine deiodinase (ID-I) activities in liver homogenates from rats subjected to selenium (Se) and/or iodine deficiencies and food restriction. Additionally, the effects of propylthiouracil (PTU) on the enzymes were studied *in vivo* and *in vitro*. Selenium deficiency markedly inhibited ID-I activity, but had no significant effects on PDI. Iodine deficiency resulted in a 1.6-fold stimulation in ID-I and a 1.2-fold stimulation in PDI activities. ID-I was much more sensitive than PDI to the inhibitory effects of PTU both *in vitro* and *in vivo*. By using a 3,3',5'-tri[¹²⁵I]iodothyronine affinity label, two major protein bands were identified when hepatic microsomal fractions from Se-sufficient rats were subjected to SDS/PAGE and autoradiography. These bands had molecular masses of 55 and 27.5 kDa, which are similar to those of PDI and ID-I respectively. Selenium deficiency resulted in the loss of the 27.5 kDa band, but did not affect the intensity of the 55 kDa band. These results are consistent with the changes in PDI and ID-I enzyme activities. Previous studies have shown that ⁷⁵Se may be incorporated *in vivo* into the 27.5 kDa protein band. This, taken together with our observation that Se is required for the expression of ID-I and the 27.5 kDa protein band, strongly suggests that ID-I is a selenoprotein.

INTRODUCTION

All thyroxine (T₄) is synthesized by the thyroid gland but more than 90 % of plasma 3,3',5-tri-iodothyronine (T₃) and 3,3',5'-tri-iodothyronine (rT₃; reverse tri-iodothyronine) are produced by deiodination of T₄ in non-thyroidal tissues by the iodothyronine deiodinases (IDs) [1–4]. The liver, kidney and muscle contain a type I iodothyronine deiodinase (ID-I), which can perform both 5- and 5'-monodeiodination of T₄ to produce rT₃ and T₃ respectively. These tissues are quantitatively the most important for providing plasma T₃ [1–4]. The pituitary, brain, central nervous system and brown adipose tissue contain type II (ID-II) and type III (ID-III) ID which catalyse 5'- and 5-monodeiodination respectively. The tissues with ID-II and ID-III activity may contribute little to the plasma T₃ pool, but are thought to be the major source of plasma rT₃ [1–4].

Propylthiouracil (PTU), at low concentrations, inhibits ID-I, but not ID-II. Thiol reductants such as GSH or dithiothreitol (DTT) activate both enzymes, whereas selenium (Se) deficiency inhibits ID-I and ID-II [3–6]. The inhibition of ID-I and ID-II by Se deficiency cannot be reversed by addition of thiol reductants or homogenates from Se-replete animals to enzyme assay mixtures. However, a single intraperitoneal injection of 200 µg of Se/kg body weight (as Na₂SeO₃) in Se-deficient rats restored ID-I activity within 5 days [6]. The effects of Se deficiency on ID-I and ID-II activity have suggested that the IDs are selenoenzymes [5–7]. This is supported by the demonstration that a hepatic microsomal selenoprotein with the same molecular mass as ID-I can be labelled *in vitro* with an [¹²⁵I]rT₃ affinity label, which binds to ID-I [8].

Although the IDs have not yet been purified, Boada *et al.* [9] claimed to have isolated a near-full-length cDNA clone for rat

ID-I [9]. The predicted amino acid sequence from this clone showed 99 % sequence identity with protein disulphide-isomerase (PDI), prompting the suggestion that PDI and ID-I are the same protein [9].

The enzymic functions of PDI and its isoforms include oxidation, isomerization and reduction of disulphide bonds; PDI is also a subunit of prolyl 4-hydroxylase (for a review, see [10]). In addition to its catalytic properties, PDI also binds thyroid hormones. A protein with molecular mass 55 kDa, named P55, was isolated by Cheng *et al.* [11] and shown to be identical with PDI. The importance of P55 is not known, but the protein is situated both on the cell surface and in the endoplasmic reticulum and is the major non-nuclear T₃-binding protein [12].

The suggested identity of ID-I with PDI [9] has recently been questioned by Schoenmakers *et al.* [13]. By using bromoacetyl-[¹²⁵I]T₃ as an affinity label, they demonstrated labelling of two proteins (molecular mass 27 kDa and 56 kDa) in rat hepatic microsomes (microsomal fractions). Through a series of experiments using inhibitors of PDI and ID-I, they concluded that the two protein bands corresponded to PDI and ID-I (or a subunit of ID-I) respectively [13]. Although these data suggest that PDI and ID-I are not identical, Schoenmakers *et al.* [13] noted that, since PDI contains two domains which show high sequence identity with thioredoxin, PDI might interact with the active site of ID-I [13]. If this were the case, our observations of the inhibitory effects of Se deficiency on ID-I activity could reflect changes in PDI activity rather than direct effects on ID-I.

Here we report experiments which addressed three questions. (1) Are PDI and ID-I identical enzymes? (2) Is PDI the Se-sensitive factor in the inhibition of hepatic T₃ production in Se-deficient animals? (3) What is the mechanism whereby Se deficiency inhibits hepatic T₃ production?

Abbreviations used: PDI, protein disulphide-isomerase; ID, iodothyronine deiodinase; rT₃, reverse tri-iodothyronine (3,3',5'-tri-iodothyronine); PTU, propylthiouracil; T₃, 3,3',5-tri-iodothyronine; T₄, thyroxine; DTT, dithiothreitol.

METHODS

Reagents

Antisera for T_3 measurement were from the Scottish Antibody Production Unit (Carlisle, Lanarkshire, Scotland, U.K.). ^{125}I -labelled rT_3 and Na^{125}I (sp. radioactivity $> 1200 \mu\text{Ci}/\mu\text{g}$) were from Amersham International, Amersham, Bucks., U.K. All other reagents were from Sigma Chemical Co. or BDH (both of Poole, Dorset, U.K.).

Animals

Weanling male Hooded Lister rats of the Rowett strain, 21 days old, were used in all experiments. The animals were individually housed in plastic cages with stainless-steel-grid tops and floors; distilled water was available *ad libitum*. Food intake was monitored daily, and body weight was monitored weekly.

Diets

Expt. 1: selenium deficiency, food restriction and PTU treatment. Two groups each of six animals were fed *ad libitum* for 5 weeks on a synthetic diet containing less than 0.005 mg of Se/kg (Se-deficient group) or the same diet supplemented with 0.1 mg of Se/kg as Na_2SeO_3 (control group) [5]. Two further groups of animals were fed either the Se-deficient or Se-supplemented diets *ad libitum* for 3 weeks, after which time the diets were given at 75% of the *ad libitum* food intake for 2 weeks. A further two groups of animals were fed either the Se-supplemented or Se-deficient diets *ad libitum* for 5 weeks, with PTU added to their drinking water (0.05%, w/v) and given *ad libitum*.

Expt. 2: Se and iodine deficiency. Four groups, each of six rats, were given one of the following diets *ad libitum* for 7 weeks: (i) Se- and iodine-supplemented, (ii) Se-deficient, (iii) iodine-deficient and (iv) Se- and iodine-deficient. The basal diet [5] contained less than 0.005 mg of Se and less than 0.1 mg of iodine/kg. Where appropriate the basal diet was supplemented with 0.1 mg of Se/kg as Na_2SeO_3 and/or 1.0 mg of iodine as KIO_3 .

In both experiments Se deficiency was confirmed by determination of hepatic glutathione peroxidase activity [5]; this was less than 1% of the activity found in Se-supplemented rats (results not shown).

Preparation of liver homogenates and microsomal fraction

Rats were anaesthetized with diethyl ether and their livers perfused with KCl (150 mmol/l) and frozen in liquid N_2 before storage at -85°C . For the determination of ID-I and PDI activity, a portion of liver was thawed and homogenized in 3 vol. of sodium phosphate buffer (100 mmol/l)/EDTA (5 mmol/l), pH 7.4, using a Teflon-pestle/glass-body homogenizer. The homogenates were centrifuged at 1500 g for 10 min at 4°C and the supernatants were used in the assays.

For the preparation of the microsomal fraction, portions of perfused livers were immediately homogenized (1:10; w/v) in potassium phosphate (0.125 mmol/l)/EDTA (1 mmol/l), pH 7.4, by using four passes in a Teflon-pestle/glass-body homogenizer. Homogenates were centrifuged at 12000 g for 20 min at 4°C ; thereafter the supernatant was collected and centrifuged at 105000 g for 60 min at 4°C . The microsomal pellet was re-suspended in homogenization buffer at a final protein concentration of approx. 20 g/l.

Measurement of hepatic ID-I activity

The rate of T_3 production from added T_4 was determined by radioimmunoassay based on the method of Visser *et al.* [14],

modified as described previously [7]. Briefly, DTT was added to homogenates to a final concentration of 20 mmol/l, and 0.45 ml of homogenate was preincubated at 37°C for 5 min. The reaction was started by addition of T_4 in buffer (50 μl) at a final concentration of 100 nmol/l, and after 10 min the reaction was stopped by addition of 1 ml of ethanol. Duplicate incubations of homogenate, previously heated to 80°C for 10 min, were performed to allow for correction for a small amount of cross-reactivity of the added T_4 in the T_3 radioimmunoassay.

Measurement of hepatic PDI activity

PDI activity was measured by using a modification of the procedure described by Carmichael *et al.* [15]. The method is based on the ability of PDI to generate a trichloroacetic acid-soluble fraction of insulin by reduction of disulphide bonds.

^{125}I -Insulin (bovine) was prepared by the chloramine-T reaction [15] and 50 μCi was added to 2 ml of a stock solution of unlabelled bovine insulin (1 mg/ml). In order to remove endogenous ^{125}I -labelled trichloroacetic acid-soluble insulin fragments, 2.5 ml of trichloroacetic acid (150 g/l) was added to the stock solution and the precipitate recovered by centrifugation at 1000 g for 10 min at 4°C . The pellet was washed once with 2.5 ml of trichloroacetic acid (150 g/l) and then dissolved in 20 ml of homogenization buffer containing BSA (30 g/l) and GSH (1 mmol/l) to provide a working ^{125}I -insulin solution.

For the assay, 0.25 ml portions of homogenates containing DTT (20 mmol/l) were preincubated for 5 min at 37°C and the reaction was started by addition of 0.25 ml of ^{125}I -insulin solution to a final concentration of 50 mg/l of insulin. After 10 min the reaction was stopped by addition of 0.5 ml of trichloroacetic acid (150 g/l) at 4°C and the trichloroacetic acid-insoluble material was separated by centrifugation at 3000 g for 15 min at 4°C . Portions (250 μl) of the supernatants were then taken in duplicate and ^{125}I radioactivity was determined in a γ -radiation counter.

Samples containing ^{125}I -insulin and homogenate heated to 80°C for 10 min were included, and corrected results were expressed as insulin solubilized/min per mg of homogenate protein.

Under the conditions described, the acid solubilization of ^{125}I -insulin was linear over the 10 min of the incubation.

Effect of addition of PTU *in vitro* on ID-I and PDI activity

Increasing concentrations of PTU were added to a liver homogenate obtained from Se-supplemented animals. The activities of both PDI and ID-I were measured in the presence of DTT (20 mmol/l) and PTU from 0.1 to 10 mmol/l. The concentrations of PTU required to inhibit PDI and ID-I to 50% of the activities in the homogenate in the absence of PTU (I_{50}) were calculated.

Affinity labelling of microsomes with N -bromoacetyl[^{125}I] rT_3

N -Bromoacetyl[^{125}I] rT_3 was synthesized using the method of Nikodem *et al.* [16]. Thereafter solvent was evaporated from affinity label (0.2 μCi), which was then allowed to react with 60 μg of microsomal protein in the presence of DTT (3 mmol/l)/EDTA (3 mmol/l)/Tris (50 mmol/l), pH 7.4, in a final volume of 50 μl . After 15 min the samples were mixed with glycerol, SDS and DTT to give final concentrations of 20% (v/v), 4% (w/v) and 50 mmol/l respectively. The mixtures were then boiled for 2 min and thereafter subjected to PAGE (12% acrylamide; 1.5 mm-thick gels) [17]. Fixed and dried gels were autoradiographed at -70°C for 4 h to locate ^{125}I -containing proteins.

Table 1. Effects of Se and iodine deficiency, food restriction and PTU administration on ID-I activity and activity in rat liver homogenates

For Expts. 1 and 2, animals were fed for 5 and 7 weeks respectively before they were killed. There were six animals in each group. Significant differences in ID-I and PDI activity from control animals are shown by * $P < 0.05$ and ** $P < 0.001$.

(a) Expt. 1. Effect of Se deficiency, food restriction and PTU administration on ID-I and PDI activity

Parameter	Control		Food-restricted		PTU-treated	
	Se +	Se -	Se +	Se -	Se +	Se -
ID-I activity [T ₃ production of (fmol/min per mg of protein)]	98.0 ± 35.2	2.2 ± 5.3**	70.3 ± 29.8	0.5 ± 1.2**	1.8 ± 4.5**	0**
PDI activity [insulin solubilized (ng/min per mg of protein)]	86.7 ± 12.3	84.0 ± 12.6	98.4 ± 16.4	97.3 ± 10.9	40.0 ± 12.6**	48.7 ± 24.1**

(b) Expt. 2. Effect of Se, I and combined Se and I deficiency on ID-I and PDI activity

Parameter	Se + I + (Control)	Se - I +	Se + I -	Se - I -
ID-I activity [T ₃ production (fmol/min per mg of protein)]	71.6 ± 24.7	4.6 ± 4.7**	116.5 ± 30.5*	13.3 ± 9.4**
PDI activity [insulin solubilized (ng/min per mg of protein)]	67.2 ± 12.7	63.0 ± 12.6	78.6 ± 9.4*	64.1 ± 13.4

Fig. 1. Autoradiograph of microsome fractions from Se-sufficient and Se-deficient rats after separation of proteins by SDS/PAGE

Hepatic microsome fractions were prepared from Se-supplemented and Se-deficient rats (*ad libitum*-fed groups; expt. 1) and rats of the same strain and age, maintained on a standard laboratory diet (Labsure, Cambridge, U.K.). The microsomes were then allowed to react with *N*-bromoacetyl[¹²⁵I]rT₃ as described in the Methods section. Thereafter, the microsome proteins were separated by SDS/PAGE and the gel was autoradiographed. Lanes 1 and 4, rats receiving standard laboratory diet; lanes 2 and 5; Se-supplemented rats; lanes 3 and 6, Se-deficient rats.

RESULTS

Effect of Se and iodine deficiencies on ID-I and PDI (expts. 1 and 2; Table 1)

In expts. 1 and 2, ID-I activities in liver homogenates from Se-deficient rats were only about 2% of those in the livers from Se-supplemented rats. In contrast, Se deficiency had no significant effect on PDI activity.

Iodine deficiency increased ID-I and PDI activity by 1.6 and 1.2-fold respectively. With combined iodine and Se deficiencies, PDI activity was significantly ($P < 0.05$) lower than in the group deficient only in iodine, but not significantly different from that in the other groups. ID-I activity in rats with combined iodine and Se deficiencies was substantially less than that in iodine- and

Se-supplemented or iodine-deficient rats, but was significantly ($P < 0.05$) greater than that in the Se-deficient group.

Effect of food restriction on ID-I and PDI activities (Expt. 1; Table 1)

Restriction in food intake for 2 weeks to 75% of normal had no significant effect on ID-I or PDI activity, but Se deficiency in the food-restricted rats caused a marked decrease in ID-I activity, but had no effect on PDI activity.

Effect of PTU on ID-I and PDI activities (Expt. 1; Table 1)

Administration of PTU to Se-supplemented rats completely abolished ID-I activity in five out of six rats. PDI activity was diminished by PTU in both Se-supplemented and Se-deficient rats to approx. 50% of that found in rats that did not receive PTU.

Addition of PTU to liver homogenates obtained from Se-supplemented rats caused a dose-dependent inhibition of both ID-I and PDI with I_{50} values of 0.7 mmol/l and 4 mmol/l respectively.

Affinity labelling of hepatic microsomes with *N*-bromoacetyl-[¹²⁵I]rT₃

Se deficiency caused a marked decrease in the intensity of the 27.5 kDa band in autoradiographs of hepatic microsomes treated with *N*-bromoacetyl[¹²⁵I]rT₃ (Fig. 1). The intensity of the 55 kDa band was relatively unaffected by Se deficiency.

DISCUSSION

We have demonstrated that variations in dietary iodine or Se supply cause differing effects on hepatic PDI and ID-I activity in the rat. The decreased ID-I activity (Table 1) in Se-deficient rats is consistent with our previous findings [5–8]. However, Se deficiency had no significant effect on PDI activity in the liver homogenates. Although iodine deficiency stimulated both ID-I

and PDI activity, the proportional increase was greater for ID-I than for PDI.

Food restriction had no effect on ID-I or PDI activities. Although food restriction can inhibit T_3 production in liver, with a resulting diminution in plasma T_3 , this effect only occurs when animals are fasted or food intake is severely restricted [18–20]. The absence of any effect of food restriction to 75% of normal food intake on hepatic ID-I (Table 1) is consistent with the normal plasma T_3 concentration found in these same rats (reported previously [21]).

The sensitivities of ID-I and PDI activities to the inclusion of PTU in the drinking water were clearly different, since ID-I activity was almost totally inhibited, but PDI was inhibited to 50% of values found in untreated rats. The study of the effects *in vitro* of PTU on ID-I and PDI confirmed that ID-I was more sensitive than PDI to inhibition by PTU.

The effects of Se deficiency on the affinity labelling with *N*-bromoacetyl[125 I]rT $_3$ of the 55 kDa protein observed on SDS/PAGE (Fig. 1) is consistent with the view that this band represents PDI [13]. Se deficiency did not affect either the activity of PDI or the intensity of labelling of the 55 kDa band. In contrast, Se deficiency produced marked decreases in both ID-I activity and the labelling of the 27.5 kDa protein band revealed by SDS/PAGE and autoradiography. These data show clearly that PDI and ID-I are not identical proteins.

We have shown previously that the 27.5 kDa protein labelled *in vitro* with *N*-bromoacetyl[125 I]rT $_3$ may also be labelled *in vivo* with Na $_2$ 75 SeO $_3$. In addition, partial purification of hepatic ID-I from rats treated with Na $_2$ 75 SeO $_3$ resulted in enzymic activity, 75 Se radioactivity and the 27.5 kDa protein co-purifying [8]. We have now shown that the biosynthesis of ID-I is blocked in the absence of adequate amounts of Se, showing that the trace element is required for expression of the enzyme. Taken together, the results of these experiments provide strong evidence that ID-I is a selenoprotein. Purification or cloning will be required to confirm this.

Recent studies have demonstrated that the selenocysteine of Se-containing glutathione peroxidase is incorporated into the polypeptide by translation of a UGA triplet which is normally a stop codon [22]. In selenium deficiency, loss of glutathione peroxidase activity is accompanied by decreased levels of glutathione peroxidase protein [23]. The parallel loss of the 27.5 kDa protein band and of ID-I activity in Se-deficiency suggests that a similar loss of protein may occur when Se is not available for ID-I synthesis.

The generally accepted mechanistic model for ID-I activity involves a thiol group at the active site [4]. However, Se and sulphur are chemically very similar, and selenocysteine has a functional role in the active centre of glutathione peroxidase. Since ID-I activity depends on an adequate dietary supply of Se, it is possible that selenocysteine may be involved at the active site of this enzyme also.

In conclusion, the present paper provides further evidence that ID-I is a selenoenzyme and that it is distinct from PDI. Furthermore, PDI is not the Se-sensitive factor involved in the inhibition of hepatic T_3 production in Se deficiency. Se deficiency probably decreases hepatic T_3 production by causing a fall in the amount of ID-I protein in the endoplasmic reticulum.

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