FEBS Letters 436 (1998) 424–426 FEBS 20936

Binding of α-tocopherylquinone, an oxidized form of α-tocopherol, to glutathione-*S*-transferase in the liver cytosol

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Received 24 August 1998

Abstract **α-Tocopherol (vitamin E) is an important fat-soluble antioxidant in biological systems and, as a result of scavenging reactive oxygen, it is converted to α-tocopherylquinone. α-Tocopherol binds to α-tocopherol transfer protein (αTTP) in the liver cytosol, whereas α-tocopherylquinone does not. We found that α-tocopherylquinone binds to a liver protein with a molecular mass of about 40 kDa that is distinct from αTTP. This α-tocopherylquinone binding protein was purified further by multiple-step column chromatography. Sodium dodecylsulfate-polyacrylamide gel electrophoresis of the final preparation yielded a single band with an apparent molecular mass of 25 kDa, which microsequencing revealed was identical to glutathione-*S*-transferase (GST). The GST activity was inhibited in the presence of α-tocopherylquinone, as it is by other non-substrate ligands for GST, confirming that GST and α-tocopherylquinone interact directly. α-Tocopherylquinone binds to GST and may be transported to the site of metabolism or excreted in the bile as other non-substrate ligands for GST.**

Key words: α-Tocopherol; Antioxidant; α-Tocopherylquinone; Binding protein; Glutathione-*S*-transferase; Rat liver

1. Introduction

α-Tocopherol (vitamin E) is an important anti-oxidant in biological systems [1]. It inhibits peroxidation of membrane lipids by scavenging lipid peroxyl radicals, as a consequence of which it is converted into a tocopheroxyl radical. This radical is thought to be either recycled to α-tocopherol by interacting with soluble antioxidants, such as ascorbic acid, or irreversibly oxidized to α-tocopherylquinone. In fact, α-tocopherylquinone has been detected in a variety of tissues [2,3], and it may act as a potent anticoagulant [4], and as an antioxidant through its reduction to hydroquinone [5]. Therefore, it is important to understand the mechanism responsible for the metabolism of α-tocopherylquinone in the body, although little is known about this at present.

The liver cytosol contains a specific α-tocopherol binding protein, which is referred to as the α-tocopherol transfer protein (αTTP). We purified this protein and isolated its cDNA from rats and humans [6–8], and demonstrated that αTTP is a product of the causative gene of familial isolated vitamin E deficiency [9–11]. These studies established that αTTP in the liver cytosol stimulates the secretion of α-tocopherol taken up by the liver into the general circulation.

αTTP binds specifically to α-tocopherol and enhances its transfer between membranes, but it does not bind to α-tocopherylquinone to any appreciable extent [12]. In this study, we found that α-tocopherylquinone binds to a liver cytosolic protein distinct from αTTP, and identified this protein as glutathione-*S*-transferase (GST).

2. Materials and methods

2.1. Materials

D-[^{3}H]α-Tocopherol (9.8 Ci/mmol) was purchased from Amersham International. D-α-Tocopherol and α-tocopherylquinone were kindly provided by Eisai (Tokyo). Sephacryl S-200, DEAE Sepharose CL-6B, CM Sepharose CL-6B, PBE118 and Pharmalyte were purchased from Pharmacia Biotech. Hydroxyapatite and goat anti-mouse IgG monoclonal antibody conjugated with alkaline phosphatase were purchased from Bio-Rad. Bromosulfophthalein and pregnenolone were purchased from Sigma.

2.2. Preparation of [^{3}H]α-tocopherylquinone

D-[^{3}H]α-Tocopherol (1 Ci/mmol, 400 μg) was dissolved in diethyl ether (400 μl), 100 mg $FeCl_3$ in 400 μl acetone was added and the mixture was stirred at room temperature for 30 min. Diethyl ether (750 μl) was added to the reaction mixture, which was washed twice with water (750 μl), the ether layer was dried over Na_2SO_4 and concentrated by evaporation. The crude product was purified by preparative thin-layer chromatography with chloroform, and the compound at R_f 0.25 was scratched off the plate, extracted with ethyl acetate, and its radioactivity was counted using a liquid scintillation counter.

2.3. Preparation of rat liver cytosol

The pooled supernatant fraction obtained after centrifuging liver homogenates at 100 000×*g* was precipitated with 90% (w/v) ammonium sulfate, the precipitate was dissolved in a small volume of SET buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and dialyzed against 20 mM Tris-HCl, 1 mM EDTA, pH 7.2.

2.4. Assay of [^{3}H]α-tocopherylquinone and [^{3}H]α-tocopherol binding activity

Binding activities of [^{3}H]α-tocopherylquinone and [^{3}H]α-tocopherol by rat liver cytosol were evaluated by the method of Sato et al. [6], with slight modifications. [^{3}H]α-Tocopherylquinone or [^{3}H]α-tocopherol (2 μCi/number of rats) in 10 μl ethanol per ml liver cytosol sample, was added to aliquots of the sample, the mixtures were incubated at room temperature for 30 min and subjected to Sephacryl S-200 column chromatography, as described below.

2.5. Purification procedures

Four-milliliter aliquots of the samples described above were applied to a Sephacryl S-200 column (2.5×95 cm) equilibrated with 10 mM KH_2PO_4, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, pH 6.8 (buffer A) and eluted with this buffer at a flow rate of 10 ml/h. The active fraction was applied to a DEAE Sepharose CL-6B column (1.5×14 cm) equilibrated with buffer A and the flow-through fraction eluted with buffer A was collected, its pH was adjusted to 6.5 with HCl and it was applied to a CM Sepharose CL-6B (1.5×12 cm) column equilibrated with buffer A, pH 6.5. The protein was eluted with a linear gradient of 0–150 mM NaCl in buffer A, pH 6.5. The active fraction

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Abbreviations: αTTP, α-tocopherol transfer protein; GST, glutathione-*S*-transferase

PII: S0014-5793(98)01176-4

from the CM Sepharose CL-6B column was applied to a hydroxyapatite column (1.5×6 cm) equilibrated with buffer A, and the protein was eluted with a linear gradient of 10–200 mM KH_2PO_4 in buffer A.

2.6. Amino acid sequence

The amino-terminal sequence of the purified protein (40 μg) was analyzed using a gas-phase sequenator model 470A (Applied Biosystems).

2.7. Western blot analysis

Proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13], then transferred to a nitrocellulose membrane using a Bio-Rad blotting apparatus and Western blotting using the anti-αTTP monoclonal antibody AT-R1 (see [7]) was carried out after blocking the nitrocellulose membrane with 3% (w/v) gelatin overnight. After incubation with the antibody for 1 h, the membrane was washed and incubated with goat anti-mouse IgG monoclonal antibody conjugated with alkaline phosphatase for 1 h at room temperature. After washing, the blot was developed in a buffer (100 mM $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8) containing 300 μg/ml nitroblue tetrazolium and 150 μg/ml 5-bromo-4-chloro-3-indolyl phosphate.

2.8. Assay of glutathione-S-transferase activity

GST activity was measured as described elsewhere [14] with slight modifications. The reaction was performed using 1 mM glutathione and 2 mM 1-chloro-2,4-dinitrobenzene in 100 mM sodium phosphate, pH 6.5, at 37°C, and was monitored spectrophotometrically by recording the increase in absorbance at 340 nm. α-Tocopherylquinone in 10 μl ethanol per 2 ml reaction mixture was added to determine its effect on GST activity. Bromosulfophthalein and pregnenolone were used as positive and negative controls, respectively.

3. Results

The [^{3}H]α-tocopherylquinone binding activity of rat liver cytosol was determined by Sephacryl S-200 column chromatography (Fig. 1). Peak α-tocopherylquinone binding activity was detected in the fractions corresponding to a molecular mass of 40 kDa, clearly different from the fraction showing peak α-tocopherol binding activity (30 kDa). The α-tocopherylquinone binding activity was eluted as a single peak by subsequent sequential chromatography using DEAE-Sepharose CL-6B and CM-Sepharose CL-6B columns. Subsequent

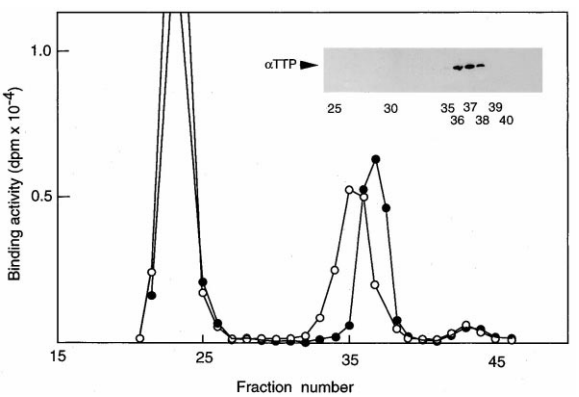

Fig. 1. Sephacryl S-200 column chromatographic elution profiles of rat liver cytosolic [^{3}H]α-tocopherol and [^{3}H]α-tocopherylquinone binding activities. Rat liver cytosol samples were incubated for 30 min at room temperature in the presence of [^{3}H]α-tocopherol or [^{3}H]α-tocopherylquinone (2 μCi/number of rats in 10 μl ethanol per ml sample) eluted from a Sephacryl S-200 column with buffer A. The fractions were collected, examined for α-tocopherol (●) and α-tocopherylquinone (○) binding activities and then subjected to SDS-PAGE, followed by immunoblotting with the anti-rat αTTP monoclonal antibody AT-R1.

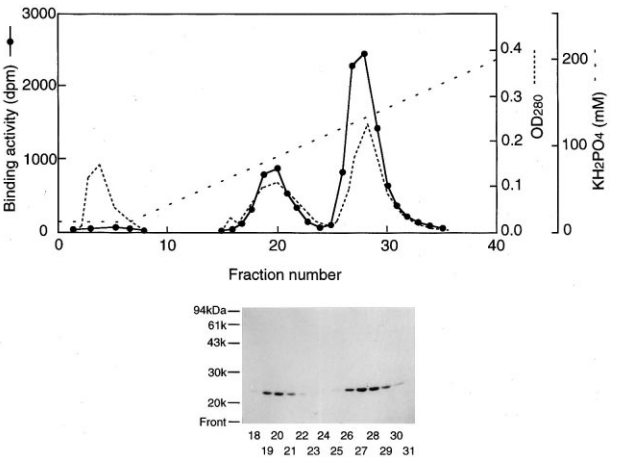

Fig. 2. Hydroxyapatite column chromatographic elution profile of [^{3}H]α-tocopherylquinone binding activity. The active fractions eluted from the CM-Sepharose CL-6B column were pooled and then applied to a hydroxyapatite column equilibrated with buffer A, the column was washed with this buffer and elution was carried out with a linear gradient of KH_2PO_4 (10–200 mM) in this buffer. The fractions were collected, examined for [^{3}H]α-tocopherylquinone binding activity and then subjected to SDS-PAGE, after which the gel was stained with Coomassie Brilliant Blue.

hydroxyapatite column chromatography separated the [^{3}H]α-tocopherylquinone binding activity into two peaks. SDS-PAGE of these fractions revealed that peak I yielded a 23-kDa band and peak II yielded a 25-kDa band (Fig. 2) and the activities of these peaks paralleled the intensities of these bands.

The N-terminal amino acid sequence of the purified protein (peak II) was determined and found to be PM(I/T)LGYW(N/D)(V/I)RGL(T/A)H(P/A)IRL(L/F)L. A search using a protein sequence data bank indicated that these sequences were identical to those of a mixture of N-terminal amino acid sequences of the Yb1 and Yb2 subunits of GST. The GSTs are homo- or heterodimers comprising multiple subunits and GST class *mu*, which consists of homo- and heterodimers of Yb1 and Yb2 subunits, is present at a high concentration in the liver (up to 5% of the total soluble protein). Peak I was identified as a GST of class *alpha* (data not shown).

GSTs are a family of proteins that catalyze the conjugation of glutathione with various electrophils, many of which are toxic [15]. In addition, GSTs bind to a variety of hydrophobic compounds which do not undergo metabolism, such as bilirubin, bile acids and indocyanine green. Moreover, binding of these non-substrate ligands to GST results in inhibition of the catalytic activity of GST [16–18]. Therefore, we examined the effects of α-tocopherylquinone on the GST activities of peaks I and II. As shown in Fig. 3, the transferase activities of both peaks I and II were inhibited by α-tocopherylquinone in a concentration dependent manner. Bromosulfophthalein, a known non-substrate ligand of GST, was used as a positive control, and inhibited the GST activities of the peaks, whereas pregnenolone, which is not a ligand of GST, did not. These results confirm that GST and α-tocopherylquinone interact directly.

4. Discussion

In this study, we purified α-tocopherylquinone binding pro-

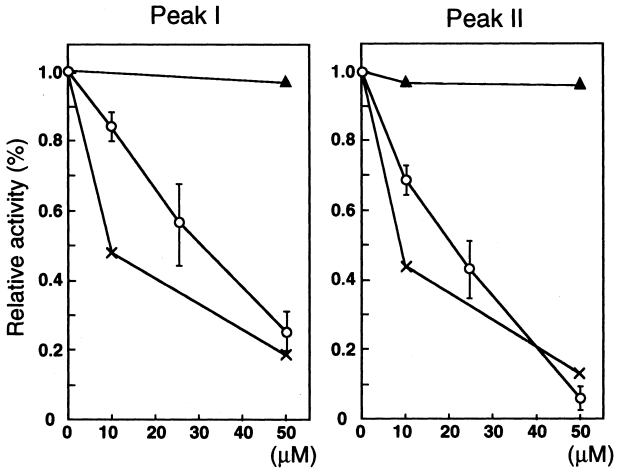

Fig. 3. Effect of α-tocopherylquinone on GST activity. The GST activity was measured by following the reaction between 1 mM glutathione and 2 mM 1-chloro-2,4-dinitrobenzene in 100 mM sodium phosphate, pH 6.5. α-Tocopherylquinone (○), bromosulfophthalein (×), or pregnenolone (▲) was added to determine their effects on GST activity. Each point denotes the mean ± S.E. of three experiments.

tein from rat liver cytosol and identified it as GST, which is a multifunctional protein that functions both as an enzyme that catalyzes glutathione conjugation and an intracellular binding protein of various non-substrate ligands. GSTs are homo- or heterodimers comprising multiple subunits and GST class *mu*, which consists of homo- and heterodimers of Yb1 and Yb2 subunits, represented the principal intracellular α-tocopherylquinone binding protein in the rat liver.

It has long been postulated that GST plays a role in cellular transport, carrying hydrophobic non-substrate ligands through the cytosol to intracellular sites of metabolism. There is evidence to suggest that GST facilitates the movement of heme out of mitochondria [19], GST class *mu* has been implicated in the transport of steroid hormones to the cell nucleus [20] and GST may deliver bilirubin to the site of metabolism within the cell [21]. α-Tocopherylquinone is the primary hepatic oxidation product of α-tocopherol and is present in rat liver at low concentrations. α-Tocopherylquinone is reduced to a hydroquinone, which may be conjugated with glucuronic acid and excreted in the bile [22]. However, neither the molecular mechanism responsible nor how it is regulated have been elucidated. In view of the results of this study, we speculate that GSTs are involved in the delivery of α-tocopherylquinone to the site of its metabolism.

In the liver, α-tocopherol and α-tocopherylquinone bind to two different cytosolic proteins. α-Tocopherol binds to αTTP, which is thought to be a critical factor in determining the plasma α-tocopherol level by virtue of its function of recycling α-tocopherol from the liver [23]. Recently, we demonstrated that αTTP stimulated the secretion of α-tocopherol by a hepatoma cell line [24]. In contrast, α-tocopherylquinone binds to GST and may be transported to the site of metabolism or excreted in the bile. Such a cellular mechanism for distinguishing between α-tocopherol and α-tocopherylquinone may exist in the liver and our findings may provide a clue for our understanding of the metabolic pathway of α-tocopherylquinone.

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