OCCURRENCE OF α-TOCOPHEROL BINDING PROTEIN IN RAT LIVER CELL SAP

O.V. Rajaram, P. Fatterpaker and A. Sreenivasan
Biochemistry & Food Technology Division
Bhabha Atomic Research Centre
Bombay-400 085, India

Received March 28, 1973

SUMMARY

An α-tocopherol binding lipoprotein, from rat liver cell sap has been isolated and purified. Its possible involvement in the intracellular transport and distribution of the vitamin is discussed.

INTRODUCTION

Binding of some of the fat soluble vitamins to specific serum and intracellular proteins has been well documented (1-5). However, information is lacking in respect of vitamin E binding to specific proteins *in vivo*, though it is reported that it can bind to various native proteins like serum albumin and egg albumin under *in vitro* conditions (6). Occurrence of a bound form of α-tocopherol *in situ* is inferable from observations on the presence of this vitamin in low density lipoproteins of rat serum (7, 8). The finding that vitamin E deficiency is greatest in β-lipoproteinemia (9) would also suggest the importance of binding proteins in determining the availability of the vitamin. The present work relates to the isolation and purification of an α-tocopherol binding lipoprotein in rat liver cell sap and its possible involvement in the intracellular transfer of this vitamin.

MATERIALS AND METHODS

Acrylamide, NN'methylene bisacrylamide and NNN'N' tetramethyl ethylene diamine were purchased from Koch-Light Laboratories. Sephadex G-200 was obtained from Pharmacia Fine Chemicals and (5-methyl-^{3}H) DL- α-tocopherol (4.6 mCi/mg) from the Radiochemical Centre, Amersham. All other chemicals used were either AnalaR grade from BDH or Proanalyst quality from E. Merck A.G.

Male albino rats (180-200 g) of the Wistar strain were injected 100 μCi of (5-methyl-^{3}H) DL- α-tocopherol emulsified with 0.5 ml of 0.9% saline containing 1% Tween 80 through the tail vein and were killed 30 min.

later. Livers were removed after perfusion with chilled 0.9% NaCl and were homogenised in 2 volumes of chilled 0.25 M sucrose containing 0.001 M EDTA and 0.01 M Tris-HCl, pH 7.4. Clear cell sap was obtained after centrifuging an aliquot of this homogenate at 105,000 x g for 60 min. in a Beckman $L_2$65 B ultracentrifuge. Subcellular fractions were isolated from the remaining homogenate after diluting it to 10% with the homogenising medium (10).

Sephadex chromatography and polyacrylamide gel electrophoresis (11) were employed for the isolation and purification of the tocopherol binding protein of liver cell sap.

In vitro exchange of α-tocopherol between the subcellular organelles of livers of rats injected with ^{3}H- α-tocopherol was carried out according to the method described by Wirtz et al (12).

Protein was estimated by the method of Lowry et al (13). Lipids were extracted by chloroform-methanol (2:1) mixture and aliquots of extract used for lipid analysis (14, 15).

RESULTS AND DISCUSSION

In preliminary experiments, the intracellular distribution of α-tocopherol in rat liver cell was studied. The α-tocopherol content was found to be 48-56 µg/g of fresh liver, of which 30-34% was localised in mitochondria. Microsomes and cell sap contained about equal amounts of 24-27%, the remainder being present in nuclei. In mitochondria, 80-85% of the vitamin was found to be membrane bound. In separate experiments, it was also observed that, when large doses of the vitamin (up to a maximum of 0.75 mg of α-tocopherol/100 g body wt.) were administered to rats, there was a corresponding rise in the liver uptake, without altering the subcellular distribution. These results probably indicate that the cell sap is not a storage site of the vitamin, unlike in case of vitamin D where administration of large doses causes increased accumulation of the vitamin in the cell sap (16).

The in vivo binding of α-tocopherol to cell sap proteins was next investigated by assessing the intravenously administered ^{3}H- α-tocopherol in the cell sap proteins/lipoproteins by Sephadex gel chromatography. On gel filtration, the cell sap proteins resolved into four fractions (Fig.1). Most of the radioactive α-tocopherol was associated with the first protein peak which comes immediately after the void volume. This fraction had a specific radioactivity of 3.1 x 10^4 cpm/OD; radioactivity in other fractions

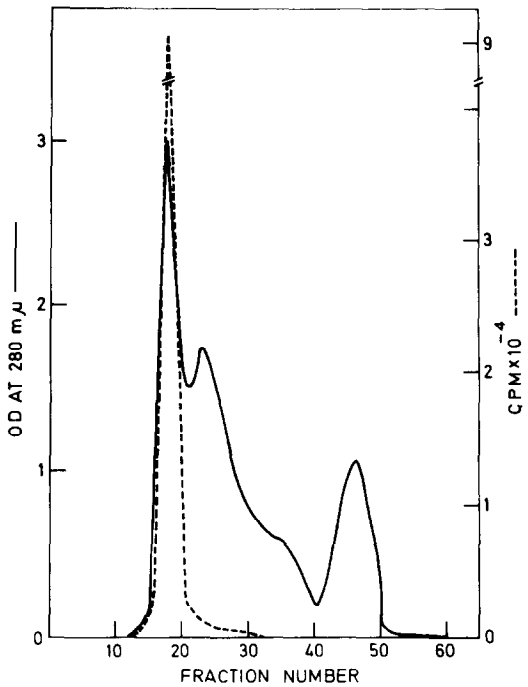

Fig. 1 : Association of intravenously administered ^{3}H- α-tocopherol with liver cell sap proteins.

Gel filtration was carried out in a Sephadex G-200 column (1.8 x 50 cm) equilibrated with sodium phosphate buffer (0.05 M, pH 7.4). 2 ml of cell sap equivalent to 100 mg protein was loaded and fractions, each of 2.5 ml, were collected in cold (4°C). Radioactivity in each fraction was measured in a Beckman LS-100 liquid scintillation spectrometer.

---

was insignificant. This fraction carrying ^{3}H- α-tocopherol also showed the presence of lipids (235 μg/mg of protein) when analysed, suggesting that the α-tocopherol binding protein could be a lipoprotein.

Further purification of the tocopherol binding protein fraction was carried out by polyacrylamide gel electrophoresis. The proteins resolved into six bands (Fig.2A) of which the first slow moving band was a lipoprotein (Fig.2B). The recovery of about 80% of the total radioactivity from this lipoprotein band (Table 1) indicated that α-tocopherol is carried by this lipoprotein. The pattern of emergence of this protein fraction from the Sephadex column and its electrophoretic mobility on a 3.5% running gel suggests the molecular size of the binding protein to be very high.

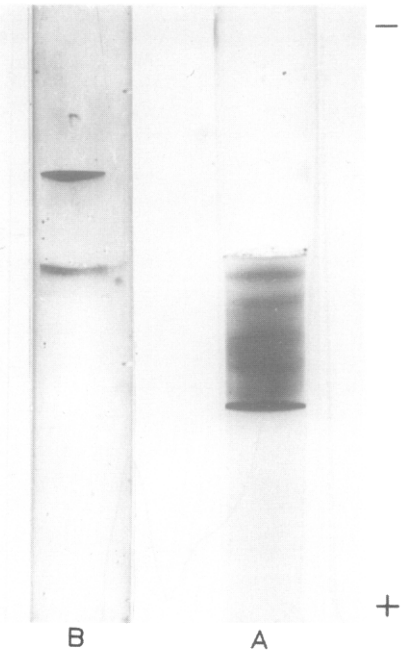

Fig. 2 : Polyacrylamide gel electropherogram of liver cell sap protein fraction.

The combined fractions of the first peak, obtained after Sephadex gel filtration of cell sap (Fig. 1) were concentrated by reverse dialysis against polywax 6000 and electrophoresed in a 3.5% running gel for 90 min. (in cold). A. Protein pattern : stained with Amido Schwarz. B. Lipoprotein pattern : a simultaneous run after loading the sample, prestained with Sudan Black B.

The in vitro uptake and efflux of α-tocopherol by liver subcellular fractions were studied to know the significance of the vitamin E carrying lipoprotein of cell sap. The uptake of radioactivity by mitochondria from the labeled cell sap was 28% and remained constant throughout the incubation period of 120 min. (Table 2). Neither exogeneously added ATP nor 2,4-dinitrophenol (DNP) had any effect on the uptake. The uptake by microsomes on the other hand was 2.9% at 90 min. and reached a maximum of 15% at 60 min. after which it remained constant. The efficiency of the microsomes to take up the label was about half that of mitochondria. The efflux of radioactivity from mitochondria was about 5% (Table 3) and was almost constant throughout the incubation up to 60 min. with a tendency to rise thereafter. The increase in the efflux of the label observed after 60 min. of incubation may possibly be due to mitochondrial swelling which is

TABLE 1

^{3}H-α-TOCOPHEROL IN PROTEIN BANDS OF THE ELECTROPHEROGRAM

Band No.	Radioactivity (CPM)			
	Protein	% Recovery	Lipoprotein	% Recovery
Origin	174 ± 20	5.7	510 ± 40	7.2
1	2480 ± 180	82.0	5670 ± 370	80.0
2	155 ± 17	6.2		
3	75 ± 8	3.0		

Radioactivity in each band (Fig. 2A) was measured in a liquid scintillation spectrometer after treating the gel slices with H_2O_2 (17). Values in the bands are expressed as per cent of total radioactive CPM of all protein fractions (minus blank) in the gel. Results are averages for four observations; there was negligible activity in bands 4-6.

TABLE 2

IN VITRO UPTAKE OF ^{3}H-α-TOCOPHEROL FROM LIVER CELL SAP BY UNLABELED MITOCHONDRIA OR MICROSOMES

Time (min)	Radioactivity (CPM) after incubation			
	Mitochondria	% uptake	Microsomes	% uptake
30	4862 ± 305	28.4	496 ± 38	2.9
30 + ATP	4948 ± 340	28.9	-	-
30 + DNP_a	5393 ± 272	31.5	-	-
30 + DNP_b	5051 ± 381	29.5	-	-
60	4280 ± 350	25.0	2568 ± 151	15.0
120	4392 ± 295	25.6	2395 ± 123	14.6

Labeled liver cell sap was obtained from rats sacrificed 30 min. after administration of 50 µCi of ^{3}H-α-tocopherol/100 gm body wt. 1.5 ml of unlabeled mitochondrial or microsomal suspension (≡ 30 mg protein) in 0.25 M sucrose-Tris-HCl, pH 7.4 was incubated with 1 ml of liver cell sap (≡ 25 mg protein) having 17120 CPM. The incubations were carried out at 37°C for the time periods indicated, after which the particulate fractions were reisolated and the incorporated radioactivity measured by liquid scintillation spectrometer. 2 µmoles of ATP/mg of mitochondrial protein was added to the system. DNP solution was adjusted to pH 7.4 and added to the incubation medium such that its concentration was 1 µM (a) or 10 µM (b). The values are corrected for 0 h uptake and are averages of four experiments ± SE.

TABLE 3

IN VITRO EFFLUX OF ^{3}H- α-TOCOPHEROL FROM MITOCHONDRIA OR MICROSOMES TO UNLABELED CELL SAP

Time (min)	Radioactivity (CPM) in cell sap after incubation with			
	Mitochondria	% efflux	Microsomes	% efflux
30	6695 ± 397	4.2	540 ± 49	0.52
30 + ATP	7173 ± 515	4.5	-	-
30 + DNP_a	6536 ± 434	4.1	-	-
30 + DNP_b	6376 ± 332	4.0	-	-
60	6853 ± 380	4.3	1834 ± 105	1.7
120	11158 ± 830	7.0	1942 ± 113	1.8

Labeled mitochondria and microsomes were obtained after administration of ^{3}H- α-tocopherol as under Table 2. 1.5 ml of mitochondrial (159400 CPM) or microsomal (107900 CPM) suspension, containing 30 mg proteins, was incubated with 1 ml of unlabeled cell sap (25 mg protein). Other details as in Table 2. Values are averages of four experiments ± SE.

accompanied by altered membrane permeability. As in the case of uptake, the efflux of label from mitochondria to cell sap was not influenced by addition of either ATP or DNP. It therefore appears that the processes of uptake and efflux are energy independent. The efflux from microsomes on the other hand, was about 2%. The results indicate an appreciable exchange of the label between labeled cell sap and unlabeled mitochondria the efflux being comparatively low. The uptake and efflux of tocopherol between cell sap and microsomes is less than in mitochondria. The varying ability of mitochondria and microsomes to take up the label from cell sap *in vitro* in turn is reflected in the subcellular distribution of the vitamin *in vivo*. The vitamin, which is a constituent of membranes, is more in mitochondria than in microsomes. Wirtz (12) has studied extensively the intracellular transfer of membrane phospholipids both *in vivo* and *in vitro*. His results give evidence for a protein factor involved in such transfer processes. This, together with our observations on the present *in vitro* exchange experiments, suggests that the binding of α-tocopherol with cell sap lipoprotein is a prerequisite for the

solubilisation of the fat soluble vitamin and its subsequent intracellular transport and incorporation into subcellular membranes. Tinberg and Barber (18) have examined the binding *in vitro* of α-tocopherol to structural protein (SP)-lipid micelle complexes from microsomes and related this binding to its antioxidant action. Their results indicate a close relationship between the extent of inhibition of lipid peroxidation in SP-lipid micelle and the amount of α-tocopherol bound. According to them, the interaction of vitamin E with membrane structural protein could lead to decreased removal of the vitamin from the cell with concomitant increase in antioxidant property. Although the question of α-tocopherol solely acting as a lipid antioxidant *in vivo* still remains open, these studies nevertheless emphasise the importance of protein bound vitamin. Preliminary experiments from our laboratory point to the existence of specific vitamin E binding lipoproteins in the small intestinal mucosa and serum of rat. These lipoproteins may possibly help in the intestinal absorption and transfer of the vitamin to different organs.

REFERENCES

1. J. Ganguly, J. Sci. Ind. Res. 26, 110 (1967).
2. M. Kanai, A. Raz and D.S. Goodman, J. Clin. Invest. 47, 2025 (1968).
3. H. Rikkers, R. Kletziens and H.F. DeLuca, Proc. Soc. Exptl. Biol. Med. 130, 1321 (1969).
4. H.R. Adhikari, U.K. Vakil and A. Sreenivasan, Indian J. Biochem. Biophys. 9, 94 (1972).
5. P.A. Peterson and I. Berggard, J. Biol. Chem. 246, 25 (1971).
6. O.L. Voth and R.C. Miller, Arch. Biochem. Biophys. 77, 191 (1958).
7. E.C. McCormic, D.G. Cornwell and J.B. Brown, J. Lipid Res. 1, 221 (1960).
8. T. Davies, J. Kelleher and M.S. Losowsky, Clin. Chim. Acta, 24, 431 (1969).
9. H.J. Kayden and R. Silber, Trans. Amer. Ass. Physicians, Philadelphia, 78, 334 (1965).
10. W.C. Schneider, J. Biol. Chem. 176, 259 (1948).
11. S.V. Koppikar, P. Fatterpaker and A. Sreenivasan, Biochem. J. 121, 643 (1971).
12. K.W.A. Wirtz, L.M.G. Van Golde and L.L.M. Van Deenen, Biochim. Biophys. Acta, 218, 176 (1970).
13. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193, 265 (1951).
14. J. Folch, M.M. Lees and G.H. Sloane Stanley, J. Biol. Chem. 226, 497 (1957).
15. J.H. Bragdon, J. Biol. Chem. 190, 513 (1950).
16. P.P. Nair and C. Bucana, Biochim. Biophys. Acta, 124, 254 (1966).
17. R.W. Young and H.W. Fulhorst, Anal. Biochem. 11, 389 (1965).
18. H.H. Tinberg and A.A. Barber, J. Nutr. 100, 413 (1970).