Inhibition of 5-lipoxygenase by vitamin E

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Purified 5-lipoxygenase from potato tubers was inhibited strongly by vitamin E and its analogs. The inhibition by d-α-tocopherol was found to be irreversible and non-competitive with respect to arachidonic acid. An IC_{50} of 5 μM was calculated for d-α-tocopherol. The inhibition appears to be unrelated to its antioxidant function. Binding studies with ^{14}C-labelled d-α-tocopherol revealed that there is a strong interaction between vitamin E and 5-lipoxygenase. Tryptic digestion and peptide mapping of 5-lipoxygenase-vitamin E complex indicate that vitamin E binds strongly to a single peptide. These studies suggest that cellular vitamin E levels may have profound influence on the formation of leukotrienes.

5-Lipoxygenase *Vitamin E* *α-Tocopherol* *Enzyme inhibition* *Antioxidant* *Leukotriene*

1. INTRODUCTION

Vitamin E, as an integral part of membranes, is visualized as a biological antioxidant which by sequestering free radicals functions to terminate the propagation of autooxidation processes such as lipid peroxidation [1–3]. Important to this discussion is the fact that enzymatically regulated lipid peroxidation is associated with polyenoic fatty acid metabolism via the lipoxygenase(s) and cyclooxygenase pathways. The immediate oxygenation products of these pathways are hydroperoxides and cyclic endoperoxides respectively, which are further metabolized to various biologically active compounds like leukotrienes, prostaglandins, prostacyclins and thromboxanes. It is well recognized that free radicals are generated during enzymatic oxidation of arachidonic acid. Therefore, it is conceivable that modulation of radical production by antioxidants like vitamin E could serve as regulatory mechanism(s) in arachidonic acid cascade. Although the precise mechanism is still under investigation, recent reports indicate that vitamin E plays an important role in the modulation of enzyme activities as well as the product profile of arachidonic acid cascade [4–8]. More recently, Grossman and Waksman [9] have shown that 15-lipoxygenase is inhibited by vitamin E via a specific interaction with the enzyme. To our knowledge, such studies with 5-lipoxygenase, a pivotal enzyme in leukotriene biosynthesis, have not been undertaken. This may be partly due to stability problems associated with 5-lipoxygenase in the past. Here, we demonstrate that 5-lipoxygenase from potato tubers is inhibited strongly by vitamin E. Also, we provide some evidence to show that there might be a specific interaction of vitamin E with 5-lipoxygenase.

2. EXPERIMENTAL

2.1. *Materials*

Arachidonic acid (99% pure), nordihydroguaiaretic acid (NDGA) and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO); d-α-tocopherol, d-α-tocopherol acetate, d-γ-tocopherol and d-α-tocopherol quinone from Eastman Kodak, Rochester, NY; ^{14}C-labelled d-α-tocopherol acetate was kindly provided by Dr L.J. Machlin, Hoffman La Roche Inc., Nutley, NJ; the

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conversion of tocopherol acetate to free tocopherol was carried out by the method of Duggan [10] prior to its use; fresh red potato tubers were obtained from local farmer's markets.

2.2. *Purification of 5-lipoxygenase*

Arachidonate 5-lipoxygenase was purified from potato tubers by a modification of the procedure of Sekiya et al. [11]. A brief description of the purification is as follows: a protamine sulfate treatment (0.1%) step was included prior to the ammonium sulfate fractionation (15–45%). The 5-lipoxygenase was further purified by a combination of conventional chromatographic and high-pressure liquid chromatographic steps employing hydrophobic interaction and anion-exchange phenomena. The final lipoxygenase preparation thus obtained was found to be greater than 99% pure as judged by analytical and SDS gel electrophoresis as well as by analytical isoelectric focusing. Details of the purification procedure and molecular properties of the enzyme will be published elsewhere.

2.3. *Assay of 5-lipoxygenase*

The lipoxygenase activity was monitored polarographically by an oxygen monitor (YSI model 53, Yellow Springs Instrument, Yellow Springs, OH). The assay mixture consisted of 0.1 M potassium phosphate, pH 6.3, 10–100 μg enzyme and 0.13 mM arachidonic acid (in ethanol) in a total volume of 3.0 ml. The reaction was initiated by the addition of arachidonic acid and incubated at 30°C. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol 5-hydroperoxyeicosatetraenoic acid (5-HPETE) or that consumed 1 μmol oxygen per min at 30°C. The specific activity was expressed as number of units per mg protein. The protein concentration was determined either by the spectrophotometric method of Warburg and Christian [12] or by the method of Lowry et al. [13].

2.4. *Inhibition studies*

Freshly prepared d-α-tocopherol and its analogs in ethanol were used in these experiments. The enzyme was preincubated with the inhibitor for 1 min prior to initiation of the reaction by the addition of arachidonic acid. The lipoxygenase reaction was monitored as described earlier. For all kinetic studies of inhibition, the initial reaction velocities were determined in the presence of varying concentrations of inhibitors while the concentration of arachidonic acid was held constant. Identical determinations were made in the presence of fixed concentrations of inhibitors with varying concentrations of arachidonic acid. Data were analyzed by double-reciprocal plots. The IC_{50} was determined by linear-regression analysis.

2.5. *d-α-Tocopherol binding experiments*

For binding studies, 5-lipoxygenase (3 mg) was incubated with 100 μg d-α-tocopherol containing ^{14}C-labelled d-α-tocopherol (1.5–1.7 $\times$ 10^6 cpm) for 10 min at 25°C in a total volume of 3.0 ml of 25 mM potassium phosphate buffer, pH 6.3. At the end of incubation, unbound d-α-tocopherol was removed by gel filtration on a Sephadex G-15 column.

Tryptic digestions were carried out in 3-ml aliquots consisting of 0.5 mg protein-vitamin E complex recovered from Sephadex G-15 column chromatography, 100 μg trypsin and 50 mM Tris-HCl, pH 8.0 for 24 h at 37°C. Primary peptide separation was achieved by gel filtration on a Sephadex G-15 column. Absorbance of the effluent was monitored at 220 nm. Radioactivity of individual fractions was determined by liquid scintillation spectrometry. The fractions containing radioactivity were pooled and subjected to HPLC analysis on a C-18 μBondapak reversed-phase column (10 μm, 4 mm $\times$ 30 cm) employing a Waters Associates (Milford, MA) 272 microprocessor-controlled gradient system consisting of an M720 system controller along with M6000A pumping, and U6K universal injection system. Elution of peptides was achieved by the use of a linear gradient, from 0.1% trifluoroacetic acid (TFA) in water to 0.1% TFA and 70% acetonitrile in water at a constant flow rate of 1 ml/min. The radioactivity of the effluent fractions was determined by liquid scintillation spectrometry.

3. RESULTS AND DISCUSSION

The effect of vitamin E and its analogs on 5-lipoxygenase was studied with a purified enzyme (90–95% pure) from potato tubers. For comparison, the effect of BHT and NDGA, known inhibitors of lipoxygenases, was also investigated. As

shown in table 1, d-α-tocopherol was found to be a potent inhibitor of 5-lipoxygenase. The inhibition was concentration dependent with an IC_{50} value of 5 μM. A similar inhibitory pattern was observed with d-γ-tocopherol which suggests that the inhibition is not specific for natural isomer of vitamin E. The inhibition by d-α-tocopherol was found to be irreversible and non-competitive with respect to arachidonic acid. Interestingly, d-α-tocopherol acetate and d-α-tocopherol quinone exhibited an inhibitory effect on 5-lipoxygenase, although to a lesser extent. This is somewhat surprising because the latter 2 vitamin E analogs are known to be poor antioxidants. These results suggest that the inhibition of 5-lipoxygenase by vitamin E and its analogs is probably not related to their antioxidant function. Our observations with 5-lipoxygenase are quite agreeable with the recent findings of Grossman and Waksman [9] on 15-lipoxygenase. However, our results differ from theirs in that d-α-tocopherol and d-α-tocopherol acetate do not inhibit 5-lipoxygenase to the same extent whereas they were found to be equipotent on 15-lipoxygenase. It should be noted that many of these inhibitors are not water soluble, therefore, ethanol was employed as a vehicle. However, ethanol by itself caused significant inhibition (~50%) of 5-lipoxygenase activity at concentrations of 5% and above. Therefore, ethanol concentrations were not allowed to be higher than 1% in our reaction mixtures.

Table 1

Effects of vitamin E and its analogs on 5-lipoxygenase activity[a]

Inhibitor	Concentration (μM)	Specific activity	% inhibition
None	–	11.0	0
Ethanol[b]	1	12.0	0
	3	10.0	10
	5	6.0	50
d-α-Tocopherol	1	9.2	17.0
	5	6.1	45.0
	10	3.9	65.0
	100	0.6	95.0
d-α-Tocopherol acetate	1	10.5	5.0
	5	9.2	17.0
	10	8.3	25.0
	100	5.7	48.0
d-γ-Tocopherol	1	7.7	30.0
	5	3.5	68.0
	10	3.1	72.0
	100	0.8	92.0
d-α-Tocopherol quinone	1	9.9	10.0
	5	8.4	23.0
	10	8.3	25.0
	100	4.8	57.0
BHT	1	6.1	45.0
	5	3.0	73.0
	10	2.9	73.0
	100	2.0	82.0
NDGA	1	3.7	67.0
	5	2.2	80.0
	10	1.7	85.0
	100	0.7	93.0

[a] Values given for specific activity are the averages of 3 separate experiments. Individual values among the 3 experiments were within 5% variation

[b] Concentration of ethanol in the reaction mixtures are given as percentage solutions

As expected, BHT and NDGA inhibited 5-lipoxygenase very potently. But the interesting aspect of our studies is the fact that the concentrations of vitamin E required to inhibit the enzyme are within 1/10 of BHT and NDGA. Furthermore, these values appear to be within the physiological range of cellular vitamin E levels. For example, in certain tissues like lung, heart, testes and liver, the local concentrations of vitamin E within the subcellular organelles of cell are reported to be in the micromolar range [14]. Therefore, it is quite conceivable that the 5-lipoxygenase activity in those tissues, particularly lung, might be under check by vitamin E. Thus, tissue vitamin E levels may exert profound influence on the formation of leukotrienes, main products of 5-lipoxygenase pathway. However, experimental evidence relating vitamin E levels and leukotriene formation needs to be generated before one can draw definite conclusions on the modulatory role of vitamin E in leukotriene biosynthesis.

Grossman and Waksman [9] have reported that vitamin E inhibits 15-lipoxygenase via a specific interaction with the enzyme protein. We have investigated the possibility that vitamin E might be inhibiting 5-lipoxygenase by such an interaction with the protein. This was tested by incubating ^{14}C-labelled d-α-tocopherol with 5-lipoxygenase and the unbound radioactive d-α-tocopherol was separated by gel filtration. The results of d-α-

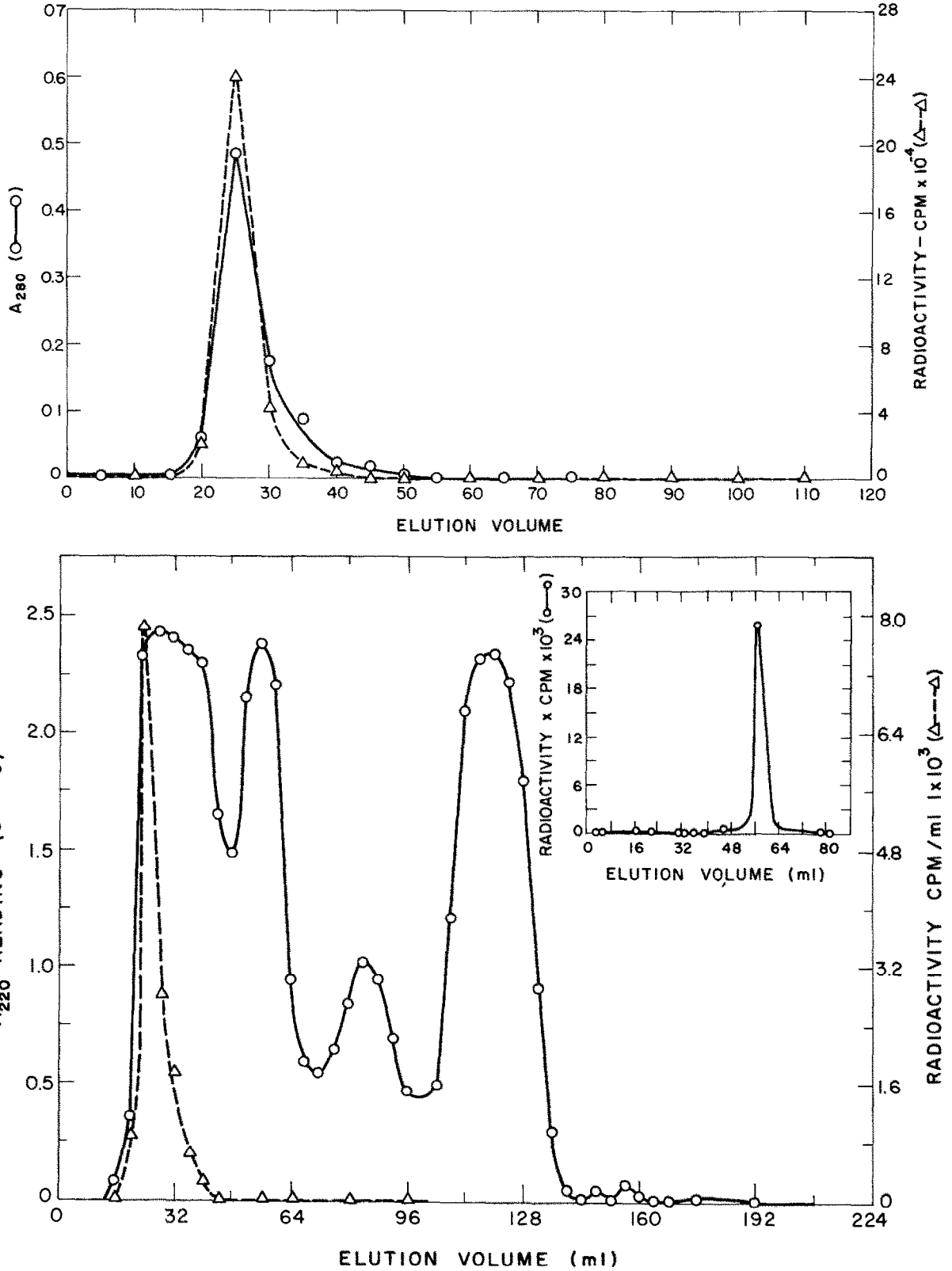
A280 (○—○)
RADIOACTIVITY – CPM x 10⁻⁴ (△--△)
ELUTION VOLUME
A220 READING (○—○)
RADIOACTIVITY CPM/ml 1x10³ (△--△)
RADIOACTIVITY x CPM x10³ (○—○)
ELUTION VOLUME (ml)
ELUTION VOLUME (ml)

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Fig.1. Gel filtration of 5-lipoxygenase-d-α-[^{14}C]tocopherol mixture on Sephadex G-15 column.

tocopherol binding studies are illustrated in fig.1. It is evident from the figure that the radioactivity peak and protein peak were co-eluted in the void volume of the Sephadex G-15 column which indicated that there was formation of a complex between vitamin E and 5-lipoxygenase. The nature of binding of vitamin E to the enzyme was further tested by repeated extraction of the pooled fraction between elution volumes 20 and 35 (fig.1) with ethyl acetate. Most of the radioactivity was recovered in the aqueous phase suggesting that vitamin E binds strongly to 5-lipoxygenase. To analyze further the binding pattern; the vitamin E-enzyme complex was subjected to tryptic digestion for 24 h and the resulting mixture of peptides was analyzed by a combination of gel filtration on Sephadex G-15 and HPLC on a reversed-phase column (fig.2). As shown in fig.2, all the radioactivity was recovered in a larger M_r peptide. Upon HPLC analysis (inset, fig.2), it was found that the entire radioactivity was associated with one peptide only. Studies are currently in progress in our laboratory to investigate the specific amino acid with which vitamin E is binding.

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Fig.1. Gel filtration of tryptic digestion products of 5-lipoxygenase-vitamin E complex on Sephadex G-15 column. Inset: further analysis of radioactivity containing peptide fraction on a reversed-phase HPLC column.