Boulanger, Y., Schreier, S., & Smith, I. C. P. (1981) *Biochemistry 20*, 6824–6830.
Chong, P. L.-G. (1988) *Biochemistry 27*, 399–404.
Chong, P. L.-G., & Weber, G. (1983) *Biochemistry 22*, 5544–5550.
Franks, N. P., & Lieb, W. R. (1982) *Nature (London) 300*, 487–493.
Gennis, R. B. (1989) in *Biomembranes: molecular structure and function*, Chapter 7, pp 235–269, Springer-Verlag, New York.
Halsey, M. J., & Wardley-Smith, B. (1975) *Nature (London) 257*, 811–813.
Kamaya, H., Suezaki, Y., Ueda, I., & Eyring, H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3572–3575.
Lakowicz, J. R., Cherek, H., Laczko, G., & Gratton, E. (1984) *Biochim. Biophys. Acta 777*, 183–193.
MacDonald, A. G. (1984) *Philos. Trans. R. Soc. London, B 304*, 47–68.
Macgregor, R. B., & Weber, G. (1981) *Ann. N.Y. Acad. Sci. 366*, 140–154.
Macgregor, R. B., & Weber, G. (1986) *Nature (London) 319*, 70–73.
Makriyannis, A., Siminovitch, D. J., Das Gupta, S. K., & Griffin, R. G. (1986) *Biochim. Biophys. Acta 859*, 49–55.
Massey, J. B., She, H. S., & Pownall, H. J. (1985) *Biochemistry 24*, 6973–6978.
Morrison, L. E., & Weber, G. (1987) *Biophys. J. 52*, 367–379.
Mushayakarara, E. C., Wong, P. T. T., & Mantsch, H. H. (1986) *Biochim. Biophys. Acta 857*, 259–264.
Parasassi, T., Conti, F., & Gratton, E. (1986) *Cell. Mol. Biol. 32*, 103–108.
Roth, S. H. (1979) *Annu. Rev. Pharmacol. Toxicol. 19*, 159–178.
Seeman, P. (1972) *Pharmacol. Rev. 24*, 583–655.
Siminovitch, D. J., Wong, P. T. T., & Mantsch, H. H. (1987) *Biophys. J. 51*, 465–473.
Snyder, R. G. (1961) *J. Mol. Spectrosc. 7*, 116–141.
Trudell, J. R., Hubbel, W. L., Cohen, E. N., & Kendig, J. J. (1973) *Anesthesiology 38*, 207–211.
Weber, G., & Farris, F. J. (1979) *Biochemistry 18*, 3075–3078.
Wong, P. T. T. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 1–24.
Wong, P. T. T. (1987a) in *High Pressure Chemistry and Biochemistry* (van Eldik, R., & Jonas, J., Eds.) pp 381–400, D. Reidel, Dordrecht, The Netherlands.
Wong, P. T. T. (1987b) *Vib. Spectra Struct. 16*, 357–445.
Wong, P. T. T., & Mantsch, H. H. (1988) *Chem. Phys. Lipids 46*, 213–224.
Wong, P. T. T., Moffatt, D. J., & Baudais, F. L. (1985) *Appl. Spectrosc. 39*, 733–735.

Formation of Free Radical Metabolites in the Reaction between Soybean Lipoxygenase and Its Inhibitors. An ESR Study

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ABSTRACT: Recent studies showed that soybean lipoxygenase inhibitors like phenidone and nordihydroguaiaretic acid (NDGA) reduce the catalytically active ferric lipoxygenase to its inactive ferrous form. Addition of 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD) regenerated the active ferric form. In this paper, it is shown that in such a system the inhibitors are oxidized to free-radical metabolites. Incubation of soybean lipoxygenase and linoleic acid with *p*-aminophenol, catechol, hydroquinone, NDGA, or phenidone resulted in the formation of the one-electron oxidation products of these compounds. Free-radical formation depended upon the presence of the lipoxygenase and 13-HPOD. The free radicals were detected by ESR spectroscopy, and their structure was confirmed by analysis of the spectra, using a computer correlation technique. These data support the proposed mechanism for the inhibition of lipoxygenase by phenolic antioxidants.

Lipoxygenases are dioxygenases that catalyze the hydroperoxidation of polyunsaturated lipids containing a *cis*-1,*cis*-4-pentadiene moiety (Vliegenthart & Veldink, 1982). For example, linoleic acid is converted by molecular oxygen and soybean lipoxygenase to 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-HPOD).[1] Interest in the mechanism and inhibition of lipoxygenases has been stimulated by the observation that 5-lipoxygenase has a pivotal role in the biosynthesis of leukotrienes. Leukotrienes play an important role in inflammation and immediate hypersensitivity reactions (Samuelsson, 1983).

Recent studies showed that incubation with NDGA, one of the most efficient inhibitors of lipoxygenases, reduces the catalytically active ferric soybean lipoxygenase to the inactive ferrous form (Kemal et al., 1987). Ferric lipoxygenase exhibits a characteristic ESR signal near $g = 6$, while the ferrous form of the enzyme is ESR silent (Slappendel et al., 1982). Treatment of the enzyme with NDGA resulted in the disappearance of the ESR signal, indicating that the iron is converted to its inactive ferrous state. This reaction, however, was reversible as addition of 13-HPOD to the reaction mixture regenerated the ferric state and restored catalytic activity. The oxidation of ferrous to ferric iron by 13-HPOD results in the

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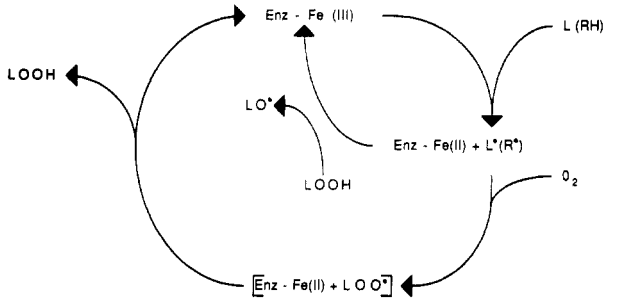
[1] Abbreviations: 13-HPOD, 13(*S*)-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; NDGA, nordihydroguaiaretic acid; DETAPAC, diethylenetriaminepentaacetic acid; BW 755C, 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline; phenidone, 1-phenyl-3-pyrazolidinone.

Scheme I: Catalytic Cycle of the Dioxygenation of Linoleic Acid (LH) by Soybean Lipoxygenase [Enz–Fe(III) or Enz–Fe(II)] and Catalytic Cycle of the Oxidation of Lipoxygenase Inhibitors (RH) by Soybean Lipoxygenase and 13-HPOD (LOOH)



homolytic cleavage of the hydroperoxide and the subsequent disappearance of its UV chromophore. The oxidation of ferrous to ferric iron can thus be conveniently followed by monitoring the disappearance of the 234-nm chromophore of 13-HPOD. Incubation of soybean lipoxygenase and 13-HPOD with lipoxygenase inhibitors like NDGA, phenidone, or BW 755C resulted in the disappearance of the 234-nm chromophore. The mechanism shown in Scheme I was proposed for the catalytic cycle of soybean lipoxygenase and its inhibition by catechols and *N*-alkylhydroxylamines (Clapp et al., 1985; Kemal et al., 1987; Mansuy et al., 1988). The mechanism implies the oxidation of the inhibitors to free radicals, but so far no data have been published demonstrating that free radicals are formed in this system. On the contrary, Nelson (1988) was unable to detect the formation of the semiquinone radical from catechol with ESR, in a system containing catechol and lipoxygenase. In order to further elucidate the mechanism in Scheme I, we decided to search with ESR spectroscopy for the formation of the free radical metabolites from these inhibitors.

Materials and Methods

Soybean lipoxygenase 1 (type IV, 440 000 units/mg of protein) was purchased from Sigma. 13-HPOD was obtained from Cayman Chemical Co. and stored at −70 °C. All other chemicals were purchased from Sigma and were of the highest grade available.

Incubations were performed in 0.1 M sodium phosphate buffer, pH 7.4 or 6.5, in the presence of 1 mM DETAPAC, which helps to inhibit autoxidation of the substrates. Incubations containing linoleic acid were prepared as follows: lipoxygenase and linoleic acid were added to the buffer and mixed vigorously for 15 s to allow for the formation of 13-HPOD prior to the addition of phenidone or one of the other compounds. When 13-HPOD was used, phenidone was added immediately after the enzyme and 13-HPOD.

ESR spectra were obtained on an IBM ER-200 spectrometer operating at 9.7 GHz with 100-kHz modulation frequency and equipped with an ER-4103 TM cavity. The solutions were transferred to the quartz flat cell by means of a rapid sampling device, and ESR spectra were recorded (Mason, 1984). All the experiments were performed at room temperature. The spectra were analyzed by a computer correlation technique, and hyperfine splitting constants were obtained from spectra simulated on a HP 9000 computer system (Duling et al., 1988).

Results and Discussion

Soybean lipoxygenase-catalyzed oxidation of linoleic acid is most effective at pH 9.0–9.5 (Axelrod et al., 1981). However, the compounds used in this study are subject to base-catalyzed autoxidation, and when these compounds were dissolved in 0.1 M Tris-HCl buffer, pH 9.0, free radicals could be detected with ESR spectroscopy quite easily. Therefore, experiments were performed at pH 7.4 or 6.5, and 1 mM DETAPAC was added to chelate adventitious transition metals and thereby possibly inhibit autoxidation.

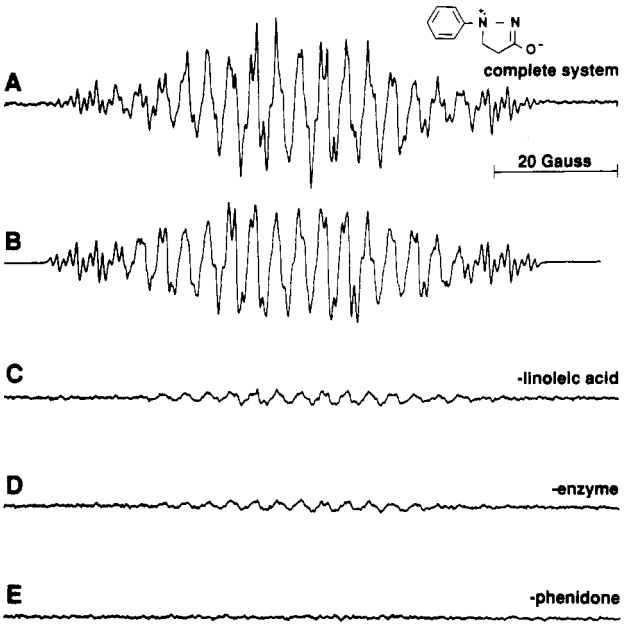


FIGURE 1: Oxidation of phenidone. (A) The incubation mixture contained phenidone (0.52 mM), lipoxygenase (57 μg/mL), and linoleic acid (0.86 mM) in 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM DETAPAC. Instrumental conditions were as follows: microwave power, 20.9 mW; modulation amplitude, 0.5 G; time constant, 0.66 s; scan width, 100 G; scan time, 5 min. (B) Simulation of spectrum in (A). Hyperfine splitting constants were a_1^N = 14.9 G, a_2^N = 7.42 G, $a_{CH_2}^H$ = 11.5 G (2 H), a_{ortho}^H = 3.17 G (2 H), a_{meta}^H = 1.05 G (2 H), and a_{para}^H = 3.47 G. Line width = 0.4 G. (C) As (A), but no linoleic acid. (D) As (A), but no lipoxygenase. (E) As (A), but no phenidone.

In all experiments radical formation depended upon the presence of lipoxygenase and 13-HPOD. The hydroperoxide of linoleic acid was added directly to the incubation mixture, or it was formed by incubating the enzyme and linoleic acid for 15 s prior to addition of one of the inhibitors. Incubation of phenidone (0.5 mM) with preincubated linoleic acid (0.86 mM) and lipoxygenase (57 μg/mL) resulted in the spectrum shown in Figure 1A. Phenidone readily autoxidizes, and even at pH 6.5, the radical could be detected at low levels in the control experiments (Figure 1C,D).

The ESR spectrum of the phenidone radical was previously published. It was first obtained by autoxidation in aqueous solution at pH 10 (Lee & Miller, 1966), and it was also detected in a system containing horseradish peroxidase and H_2O_2 at pH 10 (Marnett et al., 1982), but no hyperfine splitting constants were given. The one-electron oxidation of phenidone results in the formation of the 1-phenyl-3-pyrazolidinone free radical with the lone electron on the nitrogen attached to the phenyl ring (Lee & Miller, 1966). Computer analysis of the hyperfine pattern confirmed the formation of this radical in the lipoxygenase/linoleic acid system. Splitting constants were obtained for two nitrogens, a_1^N = 14.9 G and a_2^N = 7.42 G, for two equivalent protons at C(5) with $a_{CH_2}^H$ = 11.5 G, and for the protons in the phenyl ring, with a_{ortho}^H = 3.17 G (2 H), a_{meta}^H = 1.05 G (2 H), and a_{para}^H = 3.47 G. When BW 755C, an analogue of phenidone, was incubated with lipoxygenase and linoleic acid, no signal could be detected, presumably due to the instability of the BW 755C radical (data not shown).

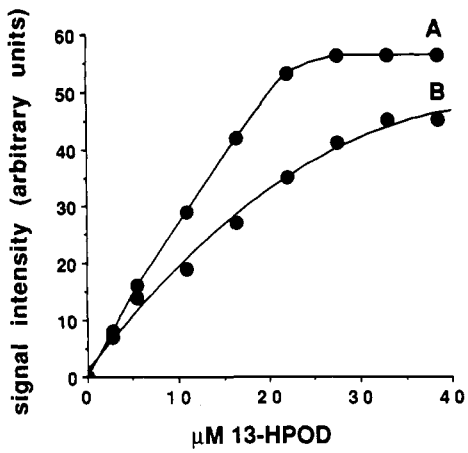


FIGURE 2: Formation of the phenidone radical by soybean lipoxygenase: 13-HPOD dependence. The incubation mixture consisted of soybean lipoxygenase (55 μg/mL), phenidone (0.5 mM), and 13-HPOD (0–40 μM). Values were corrected for the signal intensity detected without 13-HPOD (n = 2). Instrumental conditions were as described under Figure 1A. (A) Under air; (B) under nitrogen atmosphere.

Similar results were obtained when phenidone was incubated with lipoxygenase and 13-HPOD in 0.1 M sodium phosphate buffer, pH 6.5 (results not shown). Formation of the phenidone radical clearly depended on the presence of 13-HPOD, as is illustrated by the results depicted in Figure 2. In this case the intensity of the phenidone radical signal was determined in a system containing soybean lipoxygenase (55 μg/mL), phenidone (0.5 mM), and 13-HPOD (concentration varied from 0 to 40 μM). The signal intensity increased with increasing 13-HPOD concentration until approximately 25 μM, where maximum signal intensity was reached. When phenidone was incubated with lipoxygenase and 13-HPOD under a nitrogen atmosphere, the signal intensity was 20–30% lower. This effect might be due to the fact that oxygen is involved in the decomposition of lipid hydroperoxides. It has been proposed that this decomposition proceeds through an alkoxy radical and epoxidation, followed by incorporation of molecular oxygen to form another peroxyl radical (Gardner et al., 1975, 1978, 1981). The presence of more hydroperoxides will stimulate the oxidation of the inhibitors and, in this case, will cause a higher phenidone signal (Figure 2).

Another efficient inhibitor of soybean lipoxygenase is NDGA. Oxidation of NDGA (1.1 mM) with lipoxygenase (45 μg/mL) and preincubated linoleic acid (0.43 mM) in 0.1 M sodium phosphate buffer, pH 7.4, resulted in the formation of a semiquinone free radical (Figure 3A). When linoleic acid or the enzyme was omitted from the incubation mixture, a weak signal was detected due to autoxidation of NDGA (Figure 3C,D). Incubation of NDGA at pH 6.5 prevented this autoxidation, but when NDGA was incubated with lipoxygenase and linoleic acid at this pH, only a very weak signal was obtained. Therefore, experiments were performed at pH 7.4. The semiquinone radical obtained was very unstable, and the signal intensity decreased rapidly during the scan unless a short scan time was used (Figure 3A). Unexpectedly the lines in the center part of the spectrum were broadened as compared to the two doublets at the outside. These alternating line-width effects might be due to rotation of the two methylene hydrogens of the side chain (Sullivan & Bolton, 1970). Similar problems have been encountered by Pilař (1970) in the analysis of the spectrum of the 4-ethyl-benzosemiquinone radical.

These results illustrate that the lipoxygenase inhibitors phenidone and NDGA are converted to their one-electron

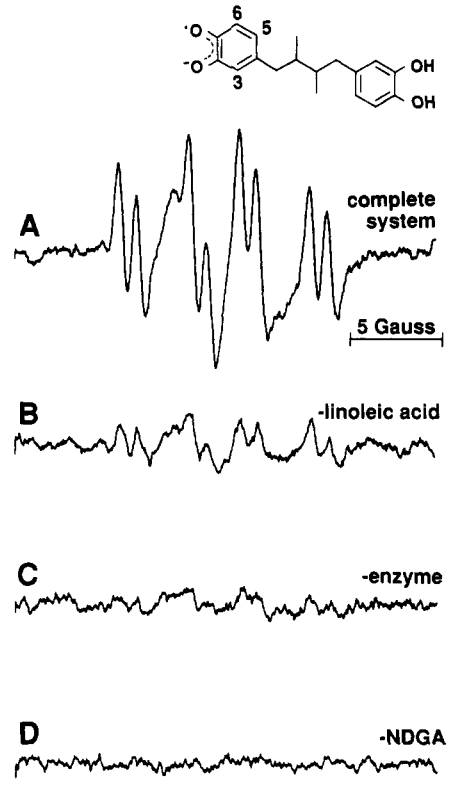


FIGURE 3: Oxidation of NDGA. (A) The incubation mixture contained NDGA (1.1 mM), lipoxygenase (45 μg/mL), and linoleic acid (0.43 mM) in 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM DETAPAC. Instrumental conditions were as follows: microwave power, 20.9 mW; modulation amplitude, 0.5 G; time constant, 0.66 s; scan range, 40 G; scan time, 90 s. (B) As (A), but no linoleic acid. (C) As (A), but no lipoxygenase. (D) As (A), but no NDGA.

oxidation products when they reduce the ferric lipoxygenase to its ferrous form. Several other compounds have been studied for their ability to reduce the active ferric form of the enzyme to the inactive ferrous form by measurement of the disappearance of 13-HPOD (Kemal et al., 1987; Mansuy et al., 1988). These compounds included *p*-aminophenol, catechol, and hydroquinone. The oxidation of these compounds has been studied in a variety of systems, and their radicals are well characterized (Josephy et al., 1983; Pedersen, 1985). We therefore decided to study the formation of the radicals of these compounds in the lipoxygenase/linoleic acid system as well. In the case of *p*-aminophenol (2 mM), no detectable signal was observed in freshly prepared solutions at pH 6.5. Oxidation with lipoxygenase (55 μg/mL) and preincubated linoleic acid (0.83 mM) resulted in a short-lived ESR spectrum (Figure 4A). The signal was dependent on the presence of both the enzyme and linoleic acid (Figure 4C,D). Computer simulation of the hyperfine pattern confirmed the identification of the paramagnetic species as the one-electron oxidation product of *p*-aminophenol. Hyperfine splitting constants were a^N = 5.4 G, $a^H_{NH_2}$ = 5.25 G (2 H), a^H_{ortho} = 2.77 G (2 H), and a^H_{meta} = 1.74 G (2 H); the simulation is shown in Figure 4B. The oxidation product of *p*-aminophenol was studied previously by ESR with horseradish peroxidase and H_2O_2 as the oxidizing system. These splitting constants are in agreement with the published values (Josephy et al., 1983).

Incubation of catechol (2 mM) or hydroquinone (2 mM) with lipoxygenase (57 μg/mL) and preincubated linoleic acid (0.83 mM) resulted in the formation of the 1,2-benzosemiquinone radical and the 1,4-benzosemiquinone radical, respectively (Figure 5A,C). When lipoxygenase or linoleic acid was omitted from the incubation mixture, no ESR signal was

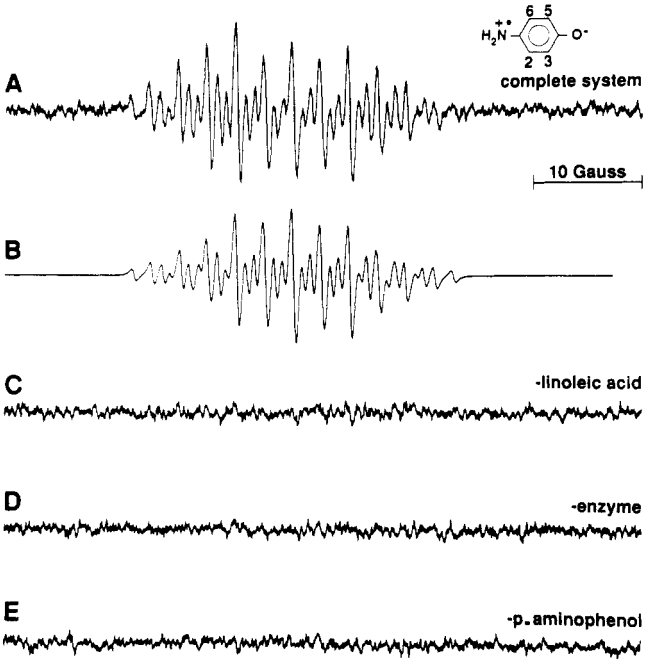


FIGURE 4: Oxidation of *p*-aminophenol. (A) The reaction mixture contained *p*-aminophenol (2 mM), lipoxygenase (55 μg/mL), and linoleic acid (0.83 mM) in 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM DETAPAC. Instrumental conditions were as follows: microwave power, 20.9 mW; modulation amplitude, 0.5 G; time constant, 0.66 s; scan range, 60 G; scan time, 5 min. (B) Simulation of spectrum in (A). Hyperfine splitting constants were a^{N} = 5.4 G, $a^{H}_{NH_2}$ = 5.25 G (2 H), a^{H}_{ortho} = 2.77 G (2 H), and a^{H}_{meta} = 1.74 G (2 H). Line width = 0.4 G. (C) As (A), but no linoleic acid. (D) As (A), but no lipoxygenase. (E) As (A), but no *p*-aminophenol.

observed (results not shown). The spectrum of the 1,2-benzosemiquinone radical consists of a triplet of triplets, due to two sets of equivalent protons. Hyperfine splitting constants were $a^{H}_{4,5}$ = 3.68 G (2 H) and $a^{H}_{3,6}$ = 0.77 G (2 H); the simulation is shown in Figure 5B. The 1,4-benzosemiquinone radical spectrum consists of a quintet, due to four equivalent protons with hyperfine splitting constant $a^{H}_{2,3,5,6}$ = 2.36 G; the simulation is shown in Figure 5D. These values are in accordance with those previously published (Pedersen, 1985).

As was mentioned previously, the compounds used in this paper are easily oxidized at pH 8–9 without enzyme or linoleic acid present. Kemal et al. (1987) already observed that the behavior of ferric soybean lipoxygenase toward catechols was similar to that of ferric iron in aqueous solution at pH 1–2. Our observations indicate that, in general, compounds that easily autoxidize in solutions of pH 7–10 are good substrates for the lipoxygenase. We therefore studied the oxidation of *N,N,N′,N′*-tetramethyl- and *N,N′*-dimethylphenylenediamine, two compounds that easily autoxidize in aqueous solution and form very stable radicals. Incubation of either compound with soybean lipoxygenase and linoleic acid, under conditions where autoxidation was minimal, resulted in the formation of nitrogen-centered cation free radicals (not shown). These compounds are not very effective inhibitors of the lipoxygenase activity in comparison with NDGA (data not shown), but since they form very stable radicals, they can be observed by ESR spectroscopy quite easily.

Since many of the compounds used in this study are also good peroxidase substrates, we were interested in comparing the lipoxygenase activity with the peroxidase activity. However, good peroxidase substrates like *N,N,N′,N′*-tetramethylbenzidine and aminopyrine, compounds that form stable and well-characterized radicals, were not oxidized by the

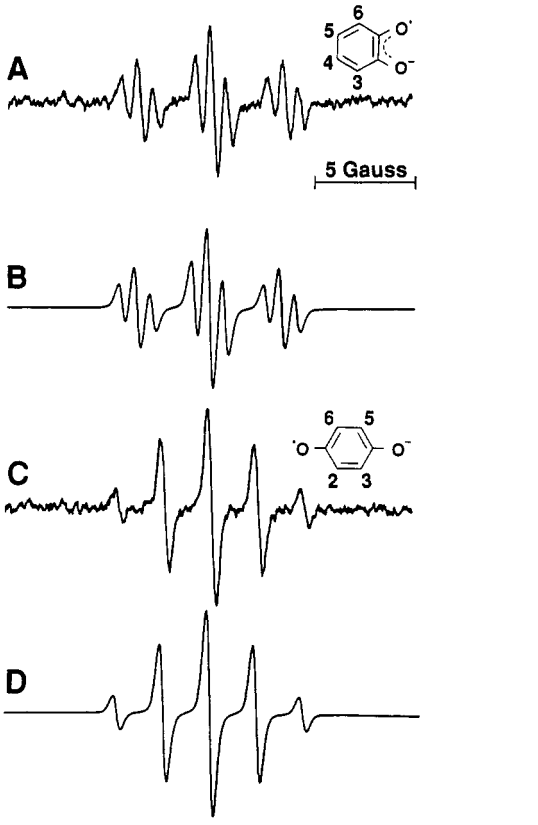


FIGURE 5: Oxidation of catechol and hydroquinone. (A) The reaction mixture contained catechol (2 mM), lipoxygenase (57 μg/mL), and linoleic acid (0.83 mM) in 0.1 M sodium phosphate buffer, pH 6.5, containing 1 mM DETAPAC. Instrumental conditions were as follows: microwave power, 20.9 mW; modulation amplitude, 0.5 G; time constant, 0.66 s; scan range, 40 G; scan time, 5 min. (B) Simulation of spectrum in (A). Hyperfine splitting constants were $a^{H}_{4,5}$ = 3.68 G (2 H) and $a^{H}_{3,6}$ = 0.77 G (2 H). Line width = 0.3 G. (C) As (A), but with hydroquinone (2 mM). (D) Simulation of spectrum in (C). Hyperfine splitting constant was $a^{H}_{2,3,5,6}$ = 2.36 G (4 H). Line width = 0.3 G.

soybean lipoxygenase/linoleic acid system, clearly indicating that the lipoxygenase activity is different from a peroxidase activity (data not shown).

In conclusion, in this paper it is shown that incubation of *p*-aminophenol, catechol, hydroquinone, NDGA, or phenidone with lipoxygenase and linoleic acid leads to the formation of free radical metabolites of these compounds. Previously, either the reduction of the enzyme or the oxidation of 13-HPOD was monitored (Kemal et al., 1987; Mansuy et al., 1988). It was proposed that free radicals would be formed from compounds that were able to reduce the catalytically active ferric soybean lipoxygenase to its inactive ferrous form (Scheme I). By using ESR spectroscopy, we were able to directly detect the formation of these free radicals and thus confirm, at least in part, the proposed mechanism.

REFERENCES

Axelrod, B., Cheesbrough, T. M., & Laakso, S. (1981) *Methods Enzymol. 71*, 441–451.

Clapp, C. H., Banerjee, A., & Rotenberg, S. A. (1985) *Biochemistry 24*, 1826–1830.

Duling, D. R., Motten, A. G., & Mason, R. P. (1988) *J. Magn. Reson. 77*, 504–511.

Funk, M. O., Isaac, R., & Porter, N. A. (1976) *Lipids 11*, 113–117.

Gardner, H. W. (1975) *J. Agric. Food Chem. 23*, 129–136.

Gardner, H. W., & Kleiman, R. (1981) *Biochim. Biophys. Acta 665*, 113–125.

Gardner, H. W., Weisleider, D., & Kleiman, R. (1978) *Lipids 13*, 246–252.
Josephy, P. D., Eling, T. E., & Mason, R. P. (1983) *Mol. Pharmacol. 23*, 461–466.
Kemal, C., Louis-Flamberg, P., Krupinski-Olsen, R., & Shorter, A. L. (1987) *Biochemistry 26*, 7064–7072.
Lee, W. E., & Miller, D. W. (1966) *Photogr. Sci. Eng. 10*, 192–201.
Mansuy, D., Cucurou, C., Biatry, B., & Battioni, J. P. (1988) *Biochem. Biophys. Res. Commun. 151*, 339–346.
Marnett, L. J., Siedlik, P. H., & Fung, L. W. M. (1982) *J. Biol. Chem. 257*, 6957–6964.
Mason, R. P. (1984) *Methods Enzymol. 105*, 416–422.
Nelson, M. J. (1988) *Biochemistry 27*, 4273–4278.
Pederson, J. A. (1985) *Handbook of EPR Data from Quinones and Quinols*, CRC Press, Boca Raton, FL.
Pilař, J. (1970) *J. Phys. Chem. 74*, 4029–4037.
Samuelsson, B. (1983) *Science 220*, 568–575.
Slappendel, S., Aasa, R., Malmström, B. G., Verhagen, J., Veldink, G. A., & Vliegenthart, J. F. G. (1982) *Biochim. Biophys. Acta 708*, 259–265.
Sullivan, P. D., & Bolton, J. R. (1970) *Adv. Magn. Reson. 4*, 39–85.
Vliegenthart, J. F. G., & Veldink, G. A. (1982) in *Free Radicals in Biology* (Pryor, W. A., Ed.) Vol. V, pp 29–64, Academic Press, New York.

Identification of Phosphorylation Sites for Adenosine 3′,5′-Cyclic Phosphate Dependent Protein Kinase on the Voltage-Sensitive Sodium Channel from *Electrophorus electricus*†

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ABSTRACT: The voltage-sensitive sodium channel from the electroplax of *Electrophorus electricus* is selectively phosphorylated by the catalytic subunit of cyclic-AMP-dependent protein kinase (protein kinase A) but not by protein kinase C. Under identical limiting conditions, the protein was phosphorylated 20% as rapidly as the synthetic model substrate kemptamide. A maximum of 1.7 ± 0.6 equiv of phosphate is incorporated per mole. Phosphoamino acid analysis revealed labeled phosphoserine and phosphothreonine at a constant ratio of 3.3:1. Seven distinct phosphopeptides were identified among tryptic fragments prepared from radiolabeled, affinity-purified protein and resolved by HPLC. The three most rapidly labeled fragments were further purified and sequenced. Four phosphorylated amino acids were identified deriving from three consensus phosphorylation sites. These were serine 6, serine 7, and threonine 17 from the amino terminus and a residue within 47 amino acids of the carboxyl terminus, apparently serine 1776. The α-subunits of brain sodium channels, like the electroplax protein, are readily phosphorylated by protein kinase A. However, these are also phosphorylated by protein kinase C and exhibit a markedly different pattern of incorporation. Each of three brain α-subunits displays an ~200 amino acid segment between homologous repeat domains I and II, which is missing from the electroplax and skeletal muscle proteins [Noda et al. (1986) *Nature (London) 320*, 188; Kayano et al. (1988) *FEBS Lett. 228*, 1878; Trimmer et al. (1989) *Neuron 3*, 33]. Most of the phosphorylation of the brain proteins occurs on a cluster of consensus phosphorylation sites located in this segment. This contrasts with the pattern of highly active sites on the amino and carboxyl termini of the electroplax protein. The detection of seven labeled tryptic phosphopeptides compared to the maximal labeling stoichiometry of ~2 suggests that many of the acceptor sites on the protein may be blocked by endogenous phosphorylation.

Second messenger systems involving protein phosphorylation play prominent roles in regulating many aspects of excitable cell physiology (Walaas & Greengard, 1987; Levitan, 1985). Several lines of evidence now demonstrate that the α-subunits of neuronal voltage-sensitive sodium channels are subject to enzymatic phosphorylation and suggest that this modification may play a role in modulating sodium channel gating or permeability (Rossie & Catterall, 1988b). The α-subunits of brain sodium channels differ from those of mammalian skeletal muscle or eel electroplax in possessing a specialized cytoplasmic segment which is enriched in consensus phosphorylation sites. This domain, which is the major site of phosphorylation both in vitro and in vivo, has been proposed to play a regulatory role unique to neuronal sodium channels. In this paper we examine the enzymatic phosphorylation of the muscle-type electroplax sodium channel, which lacks this segment and which displays a markedly different pattern of consensus

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