Phytochemistry, 1978, Vol 17, pp 41-44. Pergamon Press. Printed in England

KINETICS OF WHEAT GERM LIPOXYGENASE ADSORBED TO HYDROPHOBIC SURFACES

EDWARD L. WHEELER and JOAN M. WALLACE

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710, U.S.A.

(*Revised received* 10 *June* 1977)

Key Word Index—*Triticum vulgare*; Gramineae; wheat germ; lipoxygenase kinetics; hydrophobic interaction; linoleate hydroperoxide; Teflon; epoxy.

Abstract—Lipoxygenase binds to Teflon or epoxy coated surfaces, presumably through a hydrophobic interaction. The kinetics of bound enzyme differ from the kinetics of free enzyme both in the effect of substrate concentration on velocity and in the dependence of the induction time on substrate concentration. The binding site for the product hydroperoxide appears to be masked when the enzyme is bound to hydrophobic surfaces. *In vivo* kinetic behavior of the enzyme may be more closely approximated by that of adsorbed enzyme than by free enzyme.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12) catalyzes the oxygenation *cis, cis*, methylene interrupted diene compounds, such as linoleic acid forming primarily conjugated *cis, trans* diene hydroperoxides. The kinetics of the reaction can be conveniently studied with an oxygen electrode. This paper describes experiments in which the oxygen uptake was initiated by injecting substrate into buffer containing an aliquot of stock enzyme solution. By this procedure, we found that lipoxygenase can adsorb to Teflon and epoxy surfaces, resulting in a marked change in enzyme kinetic behavior compared to free lipoxygenase. Other enzymes have been found to exhibit altered kinetics when bound to solid surfaces [1].

RESULTS AND DISCUSSIONS

Early in our work with substrate-initiated lipoxygenase reactions, we had difficulty obtaining reproducible steady state rates of oxygen uptake. Also, an apparent pre-steady state 'burst' of oxygen uptakes sometimes occurred, as opposed to the lag period usually associated with lipoxygenase. (For this discussion the enzymatic reaction induction time, τ, is defined as the extrapolation of the steady state straight line on the time axis. It can be positive (lag), or negative (burst). We found that the steady state rate and induction time were functions of the time that the stock enzyme solution was pre-incubated in the buffer before linoleate was added to initiate reaction.

The data in Fig. 1 are typical for experiments in which the enzyme was pre-incubated for various times before substrate was added. The induction time was positive (lag) when the enzyme was preincubated for only 5 sec before substrate was added, and negative (burst) when pre-incubated for 1 min and 3 min. A closer inspection of the results, however, showed that reproducible bursts were obtained only when the electrode was immersed in the enzyme solution during the pre-incubation period. By placing 1 µl of concentrated stock enzyme solution on the Teflon membrane covering the electrode, and then

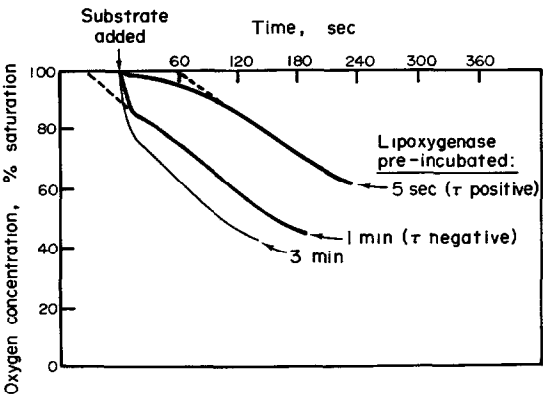

Fig. 1. Progress curves of oxygen consumption for lipoxygenase (2 µl) pre-incubated in 3 ml of 0.052 M, pH 6.9 phosphate buffer for 5 sec, 1 min, and 3 min before initiation of reaction by the addition of 100 µl of linoleate substrate. Final linoleate concn of 1.2 mM, temp. = 25°.

immersing the electrode into buffer, a typical burst occurred when linoleate was subsequently added, indicating that it was enzyme adsorbed on the Teflon membrane that was causing the apparent burst. Confirming evidence for this hypothesis was obtained by rinsing the membrane with 95% ethanol after it had been in contact with an enzyme pre-incubation mixture for 3 min. The lag typical of enzyme pre-incubated for only 5 sec was observed when linoleate was added after re-insertion of the ethanol rinsed electrode into the same pre-incubation mixture. Thus, ethanol had removed or denatured any enzyme adsorbed to the membrane. If water was used to rinse the membrane, a burst still occurred after reinsertion of the electrode into the pre-incubation mixture. We concluded that the apparent burst of oxygen uptake was an experimental artefact caused by some of the enzyme binding to the Teflon membrane covering the electrode and bringing about a rapid local depletion of oxygen around or in the sensors upon addition of substrate.

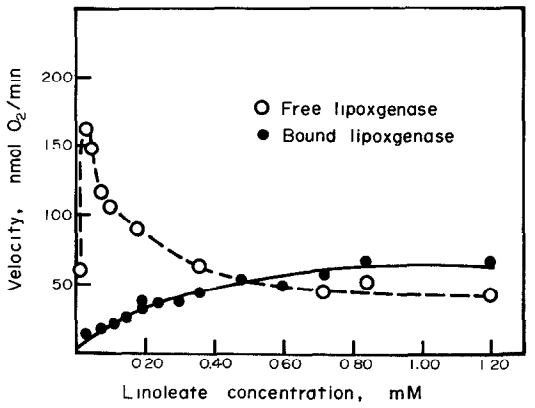

Fig. 2. Velocity vs substrate concn curves for bound and free lipoxygenase (definition in Experimental section) at 25°, pH 6.9 The concentration of free enzyme was chosen to give rates similar to bound enzyme rates at the higher linoleate concns.

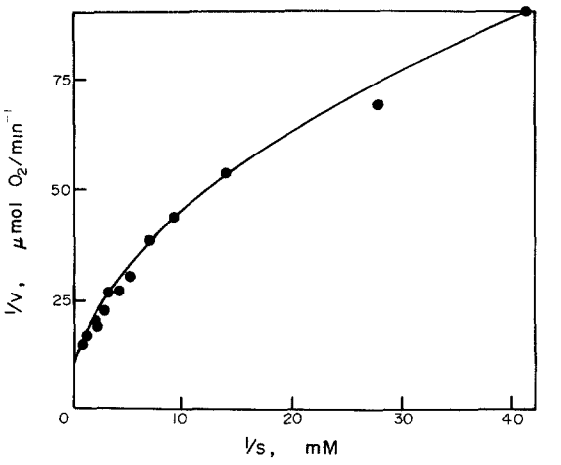

Fig. 3. Reciprocal plot of the data in Fig. 2 for bound lipoxygenase

We reasoned, however, that if lipoxygenase could bind to the hydrophobic Teflon membrane and remain active, it might also bind to the hydrophobic surface of the epoxy stirring bars supplied with the oxygen monitor. If so, we would have an ideal system for studying the kinetics of adsorbed lipoxygenase without the possibility of spurious results due to enzyme adsorption on the membrane covering the electrode sensors. Evidence that lipoxygenase in an active form does indeed bind to the epoxy coated stirring bars is shown in Fig. 2. Velocity vs linoleate concentration curves for the free and the bound enzyme were obtained as described in the Experimental. The linoleate concentrations giving the maximum velocity for the adsorbed enzyme (procedure A in Experimental) was highly inhibitory for the free enzyme (procedure B), while the adsorbed enzyme displayed no obvious substrate inhibition. Furthermore, the dependence of the lag period on substrate concentration was reversed in the case of the adsorbed enzyme as compared to the free enzyme (Table 1). No spurious bursts were observed (τ was always positive) in either case.

A reciprocal plot of the velocity vs linoleate concentration data for the bound enzyme gave a curve that was concave downwards, indicating negative cooperativity (Fig. 3). The data points in Fig. 2 for the free enzyme were mostly in an inhibitory range of substrate concentrations, but data obtained at higher enzyme concentration and lower linoleate concentrations (data not shown) gave a reciprocal plot that was concave upwards, indicating positive cooperativity, in agreement with Egmond *et al.* for soybean lipoxygenase-1 [2]. Approximate Michaelis constants were 500 μM for the adsorbed enzyme and 5 μM for the free enzyme, indicating a much greater affinity for linoleate by free enzyme than by adsorbed enzyme.

Table 1. Induction times for bound (procedure A) and free (procedure B) lipoxygenase as a function of substrate concentration Determined in the same reactions shown in Fig. 2

Linoleate concentration (mM)	(sec)	
	Bound enzyme	Free enzyme
0 024	36	22
0·036	32	30
0.072	26	54
0.36	12	94
0.84	5	114

Smith and Lands [3], and Cook and Lands [4], have proposed a kinetic model for soybean lipoxygenase-1 in which two binding sites are postulated, one to which only substrate can bind, and another for which product and substrate compete, with no activity resulting unless hydroperoxide product is bound to the second site. The increase in the induction time with increasing substrate concentration, inhibition by excess substrate, and the decrease of the induction time by the initial presence of hydroperoxide are explained by this model. Egmond *et al.* [2] however, proposed that a regulatory site for product exists, but that it is not necessary for hydroperoxide to be bound in order to have activity. In addition, they proposed a third binding site, for oxygen, and suggested that the oxygen may also bind to more than one site. We have found, in agreement with Lands *et al.* [3, 4] that the initial presence of hydroperoxide product can eliminate the dependence of the lag period on substrate concentration and can overcome substrate inhibition in free lipoxygenase reactions (procedure B). However, for reactions in which the enzyme had been previously bound to a surface (experimental procedure A), the presence of hydroperoxide prior to the addition of linoleate had no noticeable effect, either on the steady state rate of oxygen uptake, or on induction time (Table 2). These data, and the data in Figs 2 and 3, suggest that a regulatory binding site for which product and substrate compete does exist, but that it is masked when the enzyme is bound. Thefore, because bound enzyme reactions do occur, hydroperoxide is not a compulsory activator of lipoxygenase (at least type 2 lipoxygenase). The binding of hydroperoxide to free enzyme (either added initially or formed during a reaction) does, however, greatly increase the affinity of lipoxygenase for linoleate, considering the large difference in K_m between the bound and the free enzyme mentioned earlier.

Finally, we have found that if an excess of hydro-

Table 2. Steady state velocities and induction times for bound and free lipoxygenase as a function of linoleate concentration with and without hydroperoxide initially present. The one, relatively low hydroperoxide concentration used does not completely overcome substrate inhibition in free enzyme reactions

Linoleate concentration (mM)	Hydroperoxide concentration (mM)	Free enzyme		Bound enzyme	
		rate (μmol O_2/min)	(sec)	rate (μmol O_2/min)	(sec)
1.2	0	0.045	145	0.068	5
	0.15	0.096	12	0.076	5
0.60	0			0.050	8
	0.15			0.045	10
0.12	0	0.110	44	0.024	22
	0.15	0.148	10	0.026	25
0.036	0	0.147	30		
	0.15	0.165	10		

peroxide is incubated with lipoxygenase before it has absorbed to a surface, the enzyme does not bind (very low or zero rates by procedure A) as it does in the absence of hydroperoxide: furthermore, once an excess of linoleate has bren added to free enzyme (procedure B) to a reaction, the enzyme does not adsorb. This suggests that the same functional groups involved in binding linoleate and hydroperoxide at the regulatory site may also be the groups involved in binding of the enzyme to hydrophobic surfaces.

In Table 1, it was noted that the lag period in adsorbed enzyme reactions decreased with increasing linoleate concentration, and high linoleate concentration was not inhibitory (Fig. 3), while the lag period in free enzyme reactions increased with increasing linoleate concentration and excess substrate was highly inhibitory. If 3 binding sites are assumed for the wheat germ type-2 lipoxygenase, as proposed by Egmond *et al.* for soybean lipoxygenase-1 [2], an explanation for our results suggests itself. With the product (regulatory) site masked and the affinity of the enzyme for linoleate consequently greatly reduced, competition between oxygen and linoleate for one or both of the remaining binding sites may become an important rate determining factor. A constant oxygen concentration of 228 μM was used throughout this work, and oxygen was in excess initially over linoleate in the reactions at lower linoleate concentrations. Thus, oxygen, and not linoleate, may have been the inhibiting substrate in adsorbed enzyme reactions, whereas linoleate was the inhibiting substrate in free enzyme reactions because of competition between linoleate and product hydroperoxide for the regulatory site.

In preliminary work with more crude preparations of wheat germ lipoxygenase (such as simple ammonium sulfate fractions), procedure B was used to obtain velocity vs linoleate concentration curves. The shape of those curves and the dependence of the induction time on linoleate concentration were both qualitatively similar to the curves shown here for purified adsorbed enzyme (procedure A). Moreover, the more crude preparations did not adsorb to hydrophobic surfaces to the same degree as did purified preparations (unpublished results). This may indicate that association of lipoxygenase with proteins or other materials in the crude state at least partially masks the regulatory site. It is not surprising that lipoxygenase adsorbs to hydrophobic surfaces, considering that its natural substrates (linoleic, linolenic, and arachadonic acids) are also hydrophobic. It is possible, *in vivo*, where such hydrophobic surfaces abound, that the kinetic behavior of lipoxygenase is more closely approximated by the adsorbed enzyme than by the free enzyme.

EXPERIMENTAL

Linoleic acid (Applied Sci. Labs) was purified by TLC (hexane–Et_2O–HOAc, 70:30:1), and 36 mM stock substrate prepared as described in ref. [5], except that the linoleate was kept as a clear soln in pH 10.5 borate buffer. Linoleate hydroperoxide, prepared from linoleic acid by reaction with wheat germ lipoxygenase, was also purified by TLC using the same solvent system as used for linoleic acid. After elution from the TLC plate, the hydroperoxide was stored as a clear soln, under Ar, in a pH 10.5 borate buffer. Hydroperoxide concn was determined by *A* at 234 nm using a molar absorptivity of 25000 [6]. Wheat germ lipoxygenase was purified by modifications in the previously described method [5]. The isoenzyme used in this study, a soybean lipoxygenase-2 type with pH optimum near neutrality, was concd by a vacuum dialysis to 2.5 mg protein/ml. At this concn, the enzyme was stable indefinitely when stored at −10°. Activity was measured in 0.052 M Pi buffer, pH 6.85, air-equilibrated (228 μM O_2) at 25°. The electrode was pre-equilibrated in 3 ml of buffer, containing an aliquot of stock enzyme soln, or enzyme adsorbed to a surface. Linoleate substrate (1–100 μl) was injected to initiate reaction. Velocity vs linoleate concn curves were obtained in the following way: *Procedure A—for adsorbed enzyme.* An epoxy coated stirring bar (supplied with the O_2 monitor) was stirred in a 'pre-mix' soln consisting of 25 μl of enzyme in 3 ml of buffer. After 3 min, the bar was removed, rinsed well with H_2O so that only adsorbed enzyme would remain, and transferred to another reaction chamber containing 3 ml of buffer. The electrode was inserted and linoleate injected to initiate reaction. After each run, and prior to reuse, the stirring bar was rinsed with 95% EtOH, then H_2O, and rubbed dry in order to completely remove bound enzyme. This precaution was necessary to obtain good results. If the stirring bar were rinsed only with H_2O after a reaction, some active bound enzyme would remain on the bar and could be carried over to another reaction. The stirring bar became satd with lipoxygenase within 3 min of stirring in the enzyme 'pre-mix' soln, because reproducible rates of O_2 uptake were obtained when the entire procedure was repeated using the same pre-mix to coat the bar with enzyme. Longer stirring times did not affect rate of O_2 uptake. After 6–8 uses of a 'pre-mix' soln, a new pre-mix had to be prepared due to depletion of enzyme. *Procedure B—for free enzyme.* 2 μl of stock enzyme soln were injected into buffer equilibrated with the electrode, followed immediately by linoleate injection to initiate reaction. Because lipoxygenase did not adsorbe to hydrophobic surfaces in the presence of linoleate, the rates obtained by procedure B do not represent a mixture of bound and free enzyme kinetics.

Acknowledgement—The authors wish to express gratitude to Dr. Steven G. Platt for helpful discussions.

REFERENCES

1. McLaren, A. D and Packer, L. (1970) in *Advances in Enzymology* (Nord, F. F ed.) p 245. Wiley-Interscience, New York.
2. Egmond, M. R., Brunori, M. and Fasella, P M. (1976) *European J. Biochem* **61**, 93.
3. Smith, W. L. and Lands, W. E. M. (1972) *J. Biol. Chem.* **247**, 1038.
4 Cook, H. W. and Lands, W. E. M. (1975) *Can. J. Biochem* **53**, 1220.
5. Wallace, J. M. and Wheeler, E. L. (1975) *J Agric. Food Chem.* **23**, 146.
6. Johnston, A. E., Zilch, K. T., Selke, E. and Dutton, H. J. (1961) *J. Am. Oil Chem. Soc.* **38**, 367.