Communications

Stimulation and Blockade of Prostaglandin Biosynthesis*

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SUMMARY

An enzymic system of sheep vesicular gland which forms prostaglandins showed a time-dependent, concentration-dependent activation by phenol before full dioxygenase activity could be manifested. The activation process could be reversibly inhibited by o-phenanthroline.

Aspirin and indomethacin did not instantly inhibit the dioxygenase, but acted in a time-dependent, concentration-dependent manner to block full activity of the synthetic system in an irreversible manner. The enzyme preparation was protected from the inhibitory action of these drugs by the presence of o-phenanthroline.

Prostaglandins have been reported to occur in a wide variety of tissues (1). However, the recent assays of Jouvenaz et al. (2) suggest that little prostaglandin is actually present in most tissues in vivo, and that many of the earlier assay results reflect a rapid biosynthetic action of various tissue after homogenization. We must then consider factors that can change the apparently latent activity into a hormone-producing system. For example, if the enzymes responsible for prostaglandin synthesis were fully active intracellularly, then the supply of the free acid substrate from precursors (3) may be the initiating or rate-limiting event. Recently, however, we have found evidence that the initial enzyme in the synthetic system that forms prostaglandins may have inactive and active states (4) so that the activation of the oxygenation system can also be considered as a possible rate-limiting step in hormone production.

Using acetone powder preparations of sheep vesicular gland, we observed that oxygen consumption (as measured with an oxygen electrode) was dependent upon the presence of added arachidonate. For example, a control reaction mixture without substrate acid showed a consumption rate of 1 μM O₂ per min which changed to 45 μM per min after addition of arachidonate. Additional studies (4) indicated that 2 moles of oxygen were consumed for each mole of arachidonate oxidized and thin layer chromatographic separations showed the same relative abundances of prostaglandin derivatives that were noted in earlier studies with homogenates (5).

In examining the well known requirement for antioxidant (6, 7) or closely related phenolic compounds (8, 9), we observed that there was a time dependent, concentration dependent activation of the sheep vesicular gland dioxygenase activity. Fig. 1 shows the velocities of oxygen consumption attained after treating a vesicular gland acetone powder preparation for varying lengths of time with 0.06 mM phenol. The concentration of phenol was selected after experiments with varied amounts of phenol showed a hyperbolic dependence upon concentration with an apparent Kₘ value near 0.1 mM phenol. Although some discussions have ascribed an antioxidant role to phenolic compounds in later stages of the biosynthesis, the requirement for an activating phenolic compound is shown to apply prior to the initial oxygenation processes. This suggests that such an activation step prior to any oxidation of substrate could be a regulatory feature of prostaglandin formation.

The dioxygenase activity of both the activated and untreated enzyme was inhibited by 10 mM o-phenanthroline. Dilution of the o-phenanthroline to 0.5 mM or less, however, provided full restoration of the original activity indicating a reversibility of this inhibitor’s action on the dioxygenase. Interestingly, these levels of inhibitor (10 mM) also prevented the activation process induced by phenol. Removal of o-phenanthroline from unactivated preparations did allow subsequent phenol activation to occur, however. The o-phenanthroline is capable of two separate types of inhibition and may prevent the phenol activation by a type of mechanism separate from that involved in inhibiting the dioxygenase activity. For example, o-phenanthroline has been found capable of reversibly inhibiting enzymes by either preventing pyridine nucleotide binding (10) or by directly binding Fe⁺ (11).

Recently, another type of inhibition of prostaglandin formation has been described in which aspirin-like drugs inhibited prostaglandin formation in guinea pig lung homogenates (12), human platelets (13), and dog spleen (14). We have found that with sheep vesicular gland preparations indomethacin is a much more effective inhibitor than aspirin (about 100-fold in this instance, see Table I) as also reported for those tissues above. In addition to confirming the concentration dependence of the inhibition (Table I) we also noted that the inhibition was time-dependent and could not be reversed by dilution of the inhibitor. Indomethacin had no immediate effect upon the rate or on the subsequent time course of the reaction when it was added to an activated preparation that was in the process of oxidizing arachidonate. However, treatment of the enzyme for different times prior to the addition of substrate did lead to progressive, irreversible inhibition (Fig. 2). These results suggest that indomethacin may take part in some time-dependent reaction before it can produce its inhibitory effect.

When the acetone powder preparation of vesicular gland was treated with 10 mM o-phenanthroline prior to and during exposure to indomethacin, the irreversible inhibitory action of indomethacin was prevented, and the enzyme could later be substantially activated by phenol after diluting the inhibitors to low concentrations (Table II). Apparently, o-phenanthroline binds an active component that is also involved in the process of indomethacin inhibition. The antagonism of indomethacin action by an inhibitor of the activation process supports the above suggestion that the irreversible blockade of prostaglandin formation by aspirin and indomethacin is dependent upon a reactive system.
FIG. 1. The effect of preliminary incubation of vesicular gland acetone powder with phenol on the rate of oxygenation of 5,8,11,14-eicosatetraenoic acid. Rates were obtained by continuous measurement of oxygen uptake with an O2 electrode. Measurement of the activity of the untreated preparation was performed by adding a buffered suspension of the powder to the sample chamber at a final concentration of 0.8 mg per ml at the indicated times before addition of fatty acid substrate (O-O). Measurements of rates during phenol activation (△-△) were made with preliminary incubations of an over-all total of 5 min (in- cluding the time of incubation with phenol) in the reaction chamber at 30°C. Phenol (0.66 μm, final concentration) was added to the enzyme sample at various times before addition of 5,8,11,14-eicosatetraenoic acid (60 μm, final concentration) to initiate the reaction. Final volumes in the reaction mixtures were 3.0 ml of 0.1 M Tris-HCl (pH 8.5).

TABLE I
Concentration-dependent inhibition of vesicular gland dioxygenase activity by aspirin and indomethacin

Phenol-activated acetone powder (2.5 mg) was added to an assay mixture containing 0.66 mm phenol and the indicated concentrations of acetyl salicylate or indomethacin at 30°C. After 1-min exposure to the inhibitors, 5,8,11,14-eicosatetraenoic acid (final concentration, 60 μm) was added and the reaction rate (μm/min = nanomoles/ml/min) determined from continuous measurements of oxygen uptake with an oxygen electrode.

| Indomethacin | Rate | Acetyl salicylate | Rate |
|--------------|------|------------------|------|
| μm/min       | μm/min | μm/min |
| 0            | 20    | 0                | 20   |
| 1.4          | 24    | 1,200            | 19   |
| 7            | 18    | 4,500            | 14   |
| 10           | 8.8   | 9,000            | 10   |
| 70           | 4.4   | 12,000           | 9.8  |
| 105          | 2.0   | 18,000           | 5.0  |

Thus, both of these previously unrecognized time-dependent processes affecting the prostaglandin synthetic system are reversibly blocked by o-phenanthroline. The need for an activation step to achieve full dioxygenase activity and the irreversibility of the indomethacin action provides a new basis for examining not only the mechanism of action of analgesic and anti-inflammatory drugs but also the physiological imbalances that lead to their use. The fact that the sheep vesicular gland system resembles preparations from several different tissues in being inhibited by aspirin-like drugs suggests that the detailed knowledge obtained from this enzymic system might be applicable to all tissues which form prostaglandins.

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Nanossecond Time-resolved Proton Transfer Studies with Dehydroluciferin and Its Complex with Luciferase*

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SUMMARY

Fluorescence lifetime measurements have been used to obtain nanosecond time-resolved emission spectra of dehydroluciferin in various solvents and when bound to luciferase. The blue fluorescence caused by the phenol decreases with decay time relative to the green emission caused by the phenolate. The time course of excited state ionization may thus be measured directly. The rate of proton transfer is very fast in aqueous solution but slower in 80% ethanol. Addition of imidazole increases the rate of proton transfer. Dehydroluciferin when bound to luciferase shows a slow rate of proton transfer, suggesting that the binding site is hydrophobic.

Nanossecond time-resolved emission spectroscopy is a technique capable of providing dynamic information about reactions involving excited states. The method has already been used to investigate the relaxation of polar residues around excited chromophores (1, 2). The basic approach is to obtain fluorescence decay curves at various wave lengths in the spectral range of interest. From this data it is possible to reconstruct fluorescence emission spectra corresponding to any time window during the decay. A variety of excited state reactions can then be studied in terms of a nanosecond time-resolved spectral shift, provided that the reaction takes place during the time span of the fluorescence decay.

These experiments show with dehydroluciferin that nanosecond time-resolved fluorescence spectroscopy can be used to investigate the kinetics of excited state ionization. The results indicate that this approach has merit as a probe for obtaining information regarding the active sites of enzymes.

It is well established that ionization constants of organic acids differ significantly in excited states from those in the ground state (3). Perhaps the best studied case is that of β-naphthol (4) which has a ground state pKₐ of 4.6 but an excited state pKₐ close to 2. Rate constants for proton transfer depend on the solvent, being less in nonpolar than polar solvents, and on the nature of the proton acceptor (5).

In firefly bioluminescence, luciferin reacts with Mg²⁺ATP to form an enzyme-bound luciferyl adenylate with the release of pyrophosphate. Subsequent oxidation of this enzyme-bound intermediate leads to light emission (6). Dehydroluciferin (Structure I), an analog of luciferin with a double bond between carbon 4 and carbon 5, is a competitive inhibitor of luciferase. Although dehydroluciferin can be activated by the enzyme to form the luciferase dehydroluciferyladenylate complex, there is no production of light (7).

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Denburg, Lee, and McElroy (8) have shown that there are two dehydroluciferin binding sites per mole of luciferase, assuming a molecular weight of 100,000 for the native enzyme. Morton, Hopkins, and Seliger (9) have investigated the acid-base properties of dehydroluciferin in both the ground and excited states using absorption and steady state fluorescence. The dissociation of the 6'-hydroxyl group has a pKₐ of 8.7 in the ground state and a pKₐ of -1 in the excited state. Thus, in the pH range of 3 to 7, absorption will be by the protonated species but the equilibrium, if established during the excited state lifetime, will favor emission due to the ionized form. The emission resulting from the ionized species is at longer wavelength than that resulting from the protonated molecule and the two are readily distinguish.

Fluorescence decay curves of dehydroluciferin in 80% ethanol-water are shown in Fig. 1. The decay at 435 nm (uncorrected) is caused by the protonated species and the decay at 530 nm (uncorrected) is caused by the ionized species. This solvent was chosen since in water the rate of equilibrium formation is so fast that little emission resulting from the protonated molecule is observed. Approximate decay times of 1 nsec (protonated) and 3.6 nsec (ionized) were obtained by the best fit method (Handley et al. (14)) assuming a single exponential. Decay curves were computed using trial constants, convoluted with the lamp flash, and compared in the least square sense to the actual decay curve. Steady state fluorescence excitation spectra of this solution showed that all of the absorption was due to the phenol form. In 100% ethanol the fluorescence decay time of the blue fluorescence increased to 2 nsec. This increase in lifetime with decreased polarity would be expected if proton transfer is considered as a quenching mechanism in 80% ethanol.

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