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PROTECTION OF PROSTAGLANDIN H SYNTHASE FROM TRYPSIN UPON BINDING OF HEME

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The sensitivity of the apo- and holoenzyme forms of prostaglandin H synthase to trypsin have been investigated. Both the cyclooxygenase and peroxidase activities associated with the synthase were rapidly lost from the apoenzyme when incubated with trypsin. However, both activities were resistant to trypsin in the holoenzyme, suggesting that some structural change accompanies heme binding. Inactive protein present in some holoenzyme preparations, although indistinguishable from the synthase subunit by polyacrylamide gel electrophoresis, was also hydrolyzed by trypsin.

Prostaglandin H (PGH) synthase is a microsomal glycoprotein which catalyzes the oxidation of arachidonic acid to prostaglandin G_2 and the subsequent peroxidative reduction of prostaglandin G_2 to H_2. Both reactions are dependent upon heme (1). The enzyme appears to be dimeric when solubilized with detergent (2,3), and is reported to have identical polypeptide subunits with a molecular weight of 72,000 each (2,4).

The presence of two distinct catalytic activities in the same polypeptide has raised many questions about the relationship between the two catalytic sites, the number of heme molecules present per subunit, and the possibility of conformational changes associated with binding of cofactor, substrate, activator, or inhibitor molecules.

As one approach to answering questions about the structural arrangement of PGH synthase, we studied limited digestion of the enzyme with trypsin. In this communication we report results which indicate that a change occurs in the synthase upon heme binding, conferring resistance to proteolytic attack.

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EXPERIMENTAL PROCEDURES

Materials: Trypsin, N-benzoyl-L-arginine ethyl ester, soybean trypsin inhibitor, octyl glucoside, N,N,N',N' - tetramethyl-p-phenylenediamine, and hematin were from Sigma Chemical Company, St. Louis, Missouri. Arachidonic acid was obtained from Nu-Chek Preparations, Elysian, Minnesota. All other chemicals were of reagent grade or better.

Prostaglandin synthase was purified as the holoenzyme from sheep vesicular glands by the procedures described previously (4,5). Holoenzyme was converted to apoenzyme by repeated anaerobic reduction with excess sodium dithionite and treatment with pure oxygen gas. This procedure has been found to selectively degrade the heme (6); enzymatic activity is restored upon addition of hematin. Apoenzyme preparations used in this study expressed more than twenty times as much activity in the presence of saturating concentrations of hematin as in its absence.

Assays of Enzymatic Activity: Cyclooxygenase activity was measured by monitoring oxygen consumption as described (7). The reaction mixture contained 3 ml of 100 mM Tris/HCl, pH 8.5, 100 μM arachidonic acid, 0.67 mM phenol, and, for assay of total activity, 1 μM hematin. One unit of cyclooxygenase activity is that amount of enzyme which catalyzes the consumption of 1 nmole of oxygen per minute at 30°C.

Peroxidase activity was measured colorimetrically with N,N,N',N'-tetramethyl-p-phenylenediamine as described by Van der Ouderaa et al. (2). Hematin (1 μM) was added to assay activity of the apoenzyme, and the nonenzymatic peroxidase activity of the cofactor was subtracted.

Other Assays: Protein was determined by the method of Lowry et al. (8). Heme concentrations were determined with the reduced pyridine hemochrome method, using $\Delta\varepsilon(557\text{-}540) = 24.5\ mM^{-1}\ cm^{-1}$ (9).

Analysis of proteins by gel eletrophoresis was done after denaturation at 100° for 2 min in 1% SDS, 40 mM DTT, 10 glycerol and 2 M urea using 7.5% polyacrylamide gels as described by Banker and Cotman (10). Protein bands were stained with Coomassie Blue.

RESULTS AND DISCUSSION

In preliminary experiments, the absorbance of the holo-PGH synthase at 408 nm was found to be essentially unchanged after incubation at room temperature for 1 hr with 0.1 mg of trypsin per mg of holoenzyme. This apparent resistance to trypsin was surprising given the large number of basic amino acid residues in the protein (2), and it led us to examine the effect of trypsin on the cyclooxygenase and peroxidase activities of holoenzyme and apoenzyme. As shown in Figure 1, trypsin causes rapid loss of both cyclooxygenase and peroxidase activities only with apoenzyme, not holoenzyme. The loss of cyclooxygenase activity in apoenzyme could be rapidly halted by the addition of hematin (Figure 1C). Thus, the sensitivity to trypsin is associated only with a lack of heme in the apoenzyme and does not reflect any irreversible change caused by the procedure used to convert holoenzyme to apoenzyme. Hematin at 10

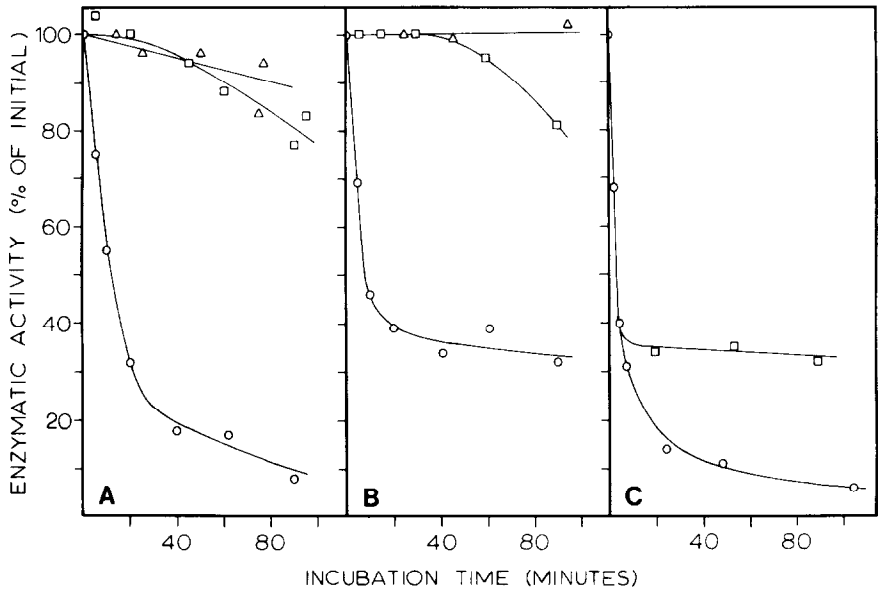

Figure 1: A. Effect of trypsin on cyclooxygenase activity of holoenzyme and apoenzyme. Holoenzyme and apoenzyme (1.4 mg protein/ml of 20 mM sodium phosphate 30% glycerol, pH 7.0) were incubated with trypsin (5.5 mg enzyme/mg trypsin) for the indicated times at 27°. Cyclooxygenase activity was assayed in the presence of hematin as described in Experimental Procedures. Apoenzyme with trypsin, o; holoenzyme with trypsin, □ , apoenzyme without trypsin, Δ. B. Effect of trypsin on peroxidase Activity of holoenzyme and apoenzyme. Holoenzyme and apoenzyme (1.8 and 1.4 mg protein, respectively, per ml of 20 mM sodium phoshate, 30% glycerol, pH 7.0) were incubated with trypsin (final concentration of 0.5 mg/ml) for the indicated times at 27°. Peroxidase activity was assayed as described in Experimental Procedures. Apoenzyme with trypsin, o; holoenzyme with trypsin, □ ; apoenzyme without trypsin, Δ. C. Reversibility of trypsin-sensitivity of apoenzyme. Apoenzyme (1.4 mg/ml of 20 mM sodium phosphate, 30% glycerol, pH 7.0) was incubated with trypsin (final concentration 0.5 mg/ml) at 27°. After 5 min, a portion was removed and mixed with 27 μM hematin (final hematin concentration was 0.5 μM), and the incubation countinued. Aliquots were taken periodically for assay of cyclooxygenase activity in the presence of hematin as described in Experimental Procedures. Apoenzyme with trypsin, o; apoenzyme with trypsin after addition of hematin, □ .

μM did not inhibit the hydrolysis of N-benzoyl-L-arginine ethyl ester, a synthetic substrate for trypsin, demonstrating that its inhibition results from binding the synthase rather than trypsin.

The coordinate loss of both cyclooxygenase and peroxidase activities from the apoenzyme and the retention of both with the holoenzyme offer additional evidence that the two enzymatic activities reside in one polypeptide (1,2).

More cyclooxygenase activity remained when smaller amounts of trypsin were employed, with over 43% of the initial activity remaining after 1

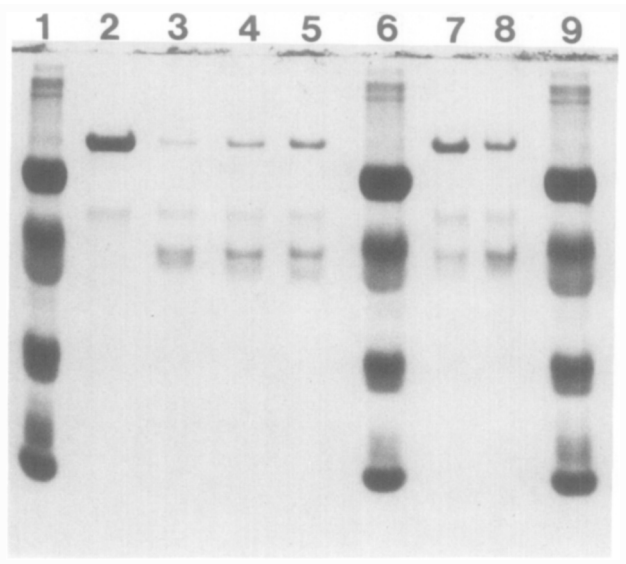

Figure 2: SDS-polyacrylamide gel analysis of synthase fragments. Apoenzyme (80 µl, 2.3 mg protein/ml of 20 mM sodium phosphate, pH 7.0, 30% glycerol) was mixed with 10 µl of solutions of trypsin in 1 mM HCl to give a final concentrations of 200, 67, or 20 µg trypsin/ml. To assess the effect of hematin, the same apoenzyme solution (90 µl) was mixed with 40 µl of 20 mM sodium phosphate, pH 7.0, 30% glycerol containing sufficient hematin to give a final concentration of 3.4 or 2.6 µM. Trypsin was added after the hematin in 10 µl of 1 mM HCl to a final concentration of 130 µg/ml. After incubation for 1 hr at 27°, cyclooxygenase activity was assayed in the presence of hematin, and an aliquot analyzed by polyacrylamide gel electrophoresis as described in Experimental Procedure. Lanes 1, 6, 9: molecular weight markers (bovine serum albumin, ovalbumin, pepsin, trypsinogen, β-lactoglobulin, lysozyme); Lanes 2-5: apoenzyme with 0, 200, 67, or 20 µg trypsin/ml; Lanes 7 and 8: apoenzyme with 3.4 or 2.6 µM hematin and 130 µg trypsin/ml. Aliquots applied to the gel all initially contained 20 µg apoenzyme.

hr incubation with a trypsin: apoenzyme ratio of 1:100, while only 21% remained when the ratio was 1:10. Analysis of the incubation mixtures by polyacrylamide gel electrophoresis also showed a larger amount of the original polypeptide (78K) remained when less trypsin was present (Lane 5 in Figure 2). Interestingly, the intensity of the band corresponding to the remaining intact PGH synthase subunit was much less than would be expected from the remaining level of cyclooxygenase activity (43% of initial). A similar discrepancy was seen when hematin was included in the incubation with trypsin (lanes 7 and 8, Figure 2), where 99% and 50%, respectively, of initial activity was retained. Similar results were also obtained when the holoenzyme preparation was incubated with trypsin

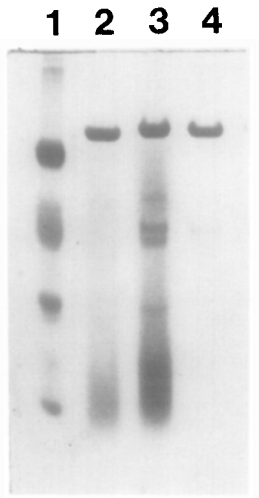

Figure 3: Further purification of holoenzyme by trypsin treatment. Holoenzyme (5.5 mg protein) was incubated with 10 μM hematin and 0.18 mg of trypsin in 1.0 ml of 20 mM sodium phosphate pH 7.0, containing 30% glycerol and 1 mg/ml octyl glucoside. After 2 hr at room temperature 0.18 mg soybean trypsin inhibitor was added and holoenzyme was reisolated by chromatography on Sephadex G-200 (1.2 x 18 cm). Lane 1, molecular weight markers; Lanes 2, holoenzyme before incubation (25 μg protein); Lane 3, incubation mixture after 2 hr (55 μg protein before incubation); Lane 4, repurified holoenzyme (8.4 μg protein).

(data not shown). The reason for the discrepancy between the molecular weight found for the subunit (78K) and that reported in the literature (72 K in reference 2) is not known.

Two large fragments, with molecular weights estimated at 40K and 44K, result from trypsin digestion of apoenzyme with and without hematin (Figures 2 and 3). About 84% of the cyclooxygenase activity present after trypsin treatment of holoenzyme resides in the 78K subunit of the holoenzyme reisolated by gel filtration. This purified holoenzyme showed little contamination with the 40 and 44 K fragments when examined by polyacrylamide gel electrophoresis (see below and Lane 4 in Figure 3).

The decrease in intensity of the 78K band of holoenzyme seen on polyacrylamide gel electrophoresis after incubation with trypsin while total activity remained unchanged suggested the the protease was digesting inactive protein with a molecular weight similar to that of the PGH synthase subunit. We exploited this sensitivity of the inactive

protein in designing a new step to purify PGH synthase of high specific activity from our holoenzyme preparations. Incubation with trypsin and gel filtration on Sephadex G-200 increased the specific activity of holoenzyme from 7100 to 26,000 units/mg protein. Analysis of the purified enzyme by polyacrylamide gel electrophoresis showed a major band at 78 K with faint bands at 40 K and 44 K (Figure 3).

The molecular basis for the selective apoenzyme and the resistance of holoenzyme to trypsin remains unclear. Resistance to the protease implies that all of the fifty-four basic amino acid residues are inaccessible. Bound detergent may contribute some resistance to trypsin by masking the exterior of the synthase. Purified synthase has been reported to bind 0.69 g of Tween-20 per g protein (2), and this detergent was used throughout the purification of the enzyme for these studies. The nature of the change which occurs upon heme binding to the apoenzyme may be a shift in conformations or perhaps heme simply obstructs any basic residues in the apoprotein which are not masked by detergent. Because apoenzyme is known to have multiple sites of varying affinities available for heme binding (11), the heme binding which confers resistance to trypsin may be at a site distinct from that involved in catalysis. Trypsin is a promising tool for structural studies of PGH synthase since this protease can distinguish not only between the apoenzyme and holoenzyme forms, but also between the holoenzyme and inactive protein which presumably originated from the synthase. We are pursuing the use of trypsin and other proteases for investigation of the interactions of the synthase with substrate, activators and inhibitors, in which a structural change is likely to have occurred in the synthase.

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