ASPIRIN AS A QUANTITATIVE ACETYLATING REAGENT
FOR THE FATTY ACID OXYGENASE THAT FORMS PROSTAGLANDINS

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ABSTRACT

    A selective acetylation of the prostaglandin-forming fatty acid
oxygenase (part of the prostaglandin "synthetase" system) occurs with
100 μM concentrations of aspirin (acetylsalicylic acid).  The amount of
acetylation, measured by counting the [$^3$H]acetyl-protein formed, was pro-
portional to the amount of active, functional oxygenase in a sample.
When samples were aged to allow spontaneous inactivation of the oxygenase,
the amount of acetylation was proportional to the remaining measurable
activity rather than the initial amount of oxygenase protein in the sample.

    Diethyl dithiocarbamate inhibited the oxygenase activity, but did
not interfere with the subsequent acetylation by aspirin.  Indomethacin,
on the other hand, appeared to inactivate the oxygenase in a manner that
interfered only partially with the action of aspirin as an acetylating
reagent.
    The amount of acetylation appeared to be dependent upon the amount
of native, undenatured enzyme.  The results suggest that the acetylation
may be dependent upon an essential functional group or conformation of
groups in the catalytic peptide chain(s) that can be destroyed during
spontaneous inactivation of the oxygenase, and altered by indomethacin.

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INTRODUCTION

Aspirin (acetylsalicylic acid) selectively acetylates a single particulate-fraction protein, molecular weight 85,000, in human platelets (1) and in sheep and bovine seminal vesicles (2). On the basis of substrate inhibition studies, the acetylated protein was proposed to be identical (2) to a catalytic portion of the fatty acid oxygenase (prostaglandin "synthetase", for review see ref. (3)) that forms prostaglandins (2). We now present further support for the hypothesis by correlation of the measured oxygenase enzyme activity in purified preparations of different specific activity with the [$^3$H]acetyl-protein content of these preparations after treatment with [acetyl-$^3$H] aspirin.

METHODS

[Acetyl-$^3$H] aspirin, 200 µCi/µmol, was synthesized from [$^3$H]acetic anhydride and salicylic acid as previously described (2). To assess [$^3$H]-acetyl-protein content, samples containing oxygenase activity were mixed with an equal volume of 200 µM [acetyl-$^3$H] aspirin solution and incubated (0.1 M sodium phosphate pH 7.4, 0.67 mM phenol, with or without 0.5 µM hemoglobin) until maximal acetylation had occurred (60-120 min at 37° C, 240 min at room temperature) or for the indicated times in other experiments. Acetylated samples were then boiled in sodium dodecyl sulfate (SDS) solution (4% SDS, 0.1 M β-mercaptoethanol) and subjected to SDS polyacrylamide gel electrophoresis (1).

The content of [$^3$H]acetyl-protein in each gel was determined by measuring [$^3$H]-acetate radioactivity in gel slices by liquid scintillation counting (2). Recovery of radioactivity from gel slices was estimated using [$^3$H]acetyl-albumin (200 µCi/µmole) (4). We estimate that 30% to 45% of the [$^3$H]acetyl-albumin applied to a gel is detected by liquid scintillation counting of gel slices as outlined above. Oxygenase activity was determined by adding aliquots of the enzyme preparation to 3.0 ml reaction mixtures containing 0.1 M sodium phosphate buffer (pH 7.4) with 0.67 mM phenol and 100 µM arachidonate. The oxygen content was monitored with a Yellow Springs Instrument Co. oxygen electrode by continuous recording of both the oxygen content with time and d$O_2$/dt as described earlier (5). Acetone powder preparations from sheep vesicular glands were prepared in accord with the method of Wallach and Daniels (6), and partially purified fractions were obtained as described recently (7).

RESULTS

Quantitative Correspondence of Acetylation with Enzyme Inactivation

When the oxygenase preparation was treated with 100 µM aspirin, aliquots removed at various times showed a progressively diminished activity compared to untreated controls. The inactivation of the enzyme followed apparent first order kinetics in a manner described earlier (2,5). Subsequent SDS gel electrophoresis of the samples indicated that the amount of

[$^3$H]acetyl-protein formed tended to correspond to the amount of inactivated oxygenase. In many cases, the acetylation was almost complete in 60 min at 37°C, but the reaction was slower, and incomplete at room temperature (28°). Results for two separate series of measurements at the higher temperature are shown in Figure 1. Although some variance was apparent, the inactivation with 100 μM aspirin had a half-life of 8 to 10 min at 37° in contrast to values of about 17 min observed earlier (2). The acetylation, however, appeared to proceed relatively rapidly initially with the final extent being reached more slowly than the enzyme inactivation. Two considerations of the apparent difference in rates are important. First, the percent of unlabeled protein plotted in Figure 1, was calculated on the basis of three averaged values for the 2- and 4-hour extent of acetylation as full labeling. In such an instance, the slope of the line depends on this estimate of 100%, and is extremely sensitive to small differences in that value. If the 60 min value were selected as 100%, the acetylation would appear to have a half-life of 10-20 min and resemble the enzyme inactivation. A second aspect is the possibility of slow continued acetylation of material that does not exhibit oxygenase activity during enzyme assays. Such additional acetylation with a half-life of 1-2 hours could tend to obscure the more rapid acetylation with the active oxygenase.
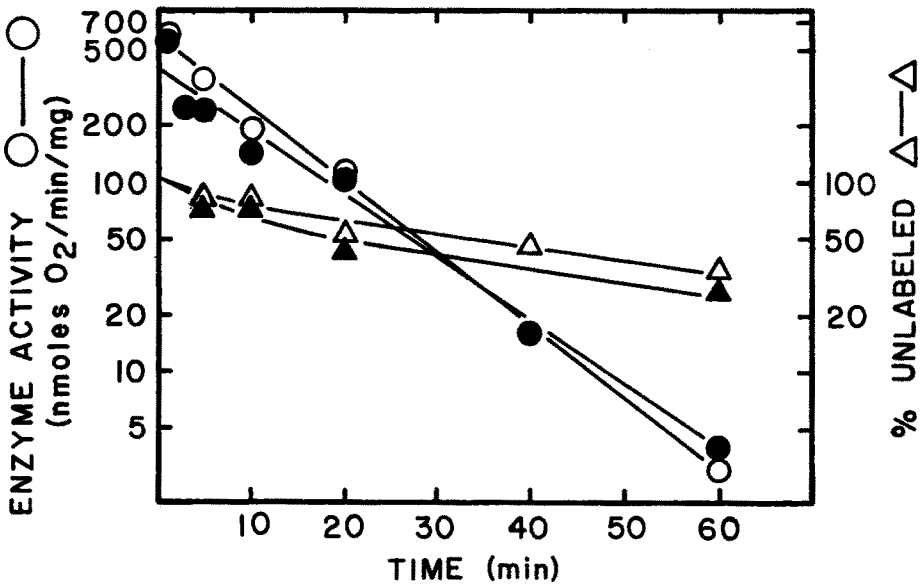


Figure 1. Comparison of the rate of formation of [$^3$H]acetyl-protein with loss of oxygenase activity. - Radioactive aspirin (100 μM) was added to enzyme preparations at 37° at zero time. Oxygenase activity was recorded as nmoles $O_2$/min/mg protein at the times indicated, and the percent of unlabeled protein was based on full labeling (0 percent unlabeled) for the samples treated for 2-4 hours. Open symbols, Expt. 1; closed symbols, Expt. 2.

Quantitative correspondence of acetylation with available native enzyme.

To see whether other contaminating proteins would interfere with the acetylation reaction, preparations of different specific activity were treated with radioactive aspirin. The acetylation mixtures were sampled at various times to confirm complete loss of oxygenase in the different samples within 4 hours at 37° (results not shown). Table I shows that the amount of acetylated protein migrating to the characteristic location on the gels (∿85,000 daltons) was generally proportional to the amount of measurable oxygenase activity in a wide variety of preparations. Acetone powder preparations (XII, XIII, XIV) made at different times during the past two years had specific activities of 23, 190 and 530 units per mg and gave 158, 18 and 15 cpm per unit oxygenase. Enzyme samples from different stages of purification (7) had specific activities of 380 (X) and 6790 (V). When these samples were stored under different conditions, the spontaneous instability of the oxygenase activity (7) enabled us to secure added preparations with apparently identical protein content, but different specific activities. For example, the value of 6790 (V) reduced to 4580 (III) when stored at -10° and to 2470 (II) when stored at 0°C.

TABLE I.  Acetylation of Different Oxygenase Preparations

| Sample number | Specific activity | Units on gel | CPM in peak | CPM per unit | nmoles acetyl per mg. protein† | Estimated purity* |
|---|---|---|---|---|---|---|
| II | 2470 | 99 | 1000 | 10.1 | 0.398 | 3.4% |
| III | 4580 | 100 | 1065 | 10.6 | .774 | 6.6 |
| V | 6790 | 119 | 1050 | 8.9 | .954 | 8.1 |
| VII | 620 | 54 | 2025 | 37.5 | .373 | 3.2 |
| VIII | 70 | 21 | 179 | 8.5 | .009 | 0.1 |
| X | 380 | 38 | 563 | 15.0 | .090 | 0.76 |
| XI | 4290 | 123 | 1439 | 11.7 | .797 | 6.8 |
| XII | 23 | 17 | 2690 | 158.0 | .059 | 0.5 |
| XIII | 190 | 159 | 2854 | 18.0 | .054 | 0.5 |
| XIV | 530 | 80 | 1194 | 14.9 | .127 | 1.1 |
| XVI | 90 | 67 | 1075 | 16.6 | .023 | 0.2 |

†We estimated the number of acetyl groups per mg. protein from the known specific activity of the $^3$H-acetylsalicylate, the counting efficiency of 45%, and by assuming the recovery of radioactivity on SDS-gels was 30%.
*Purity was estimated by regarding one mole of acetyl per 85,000 mg protein (11.7 nmole per mg) as 100% pure.

The amount of acetylation observed with these samples was generally related (see Fig. 2) to the measurable enzyme activity at the time of acetylation rather than the amount of oxygenase protein initially present in the sample, although one old preparation (XII) was much less active in the enzyme assay than predicted by the acetylation result. This may reflect some interference of enzyme activity by other proteins in this very crude preparation.

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Another possible exception to the correlation may be sample VII in which the purified oxygenase protein was not supplemented with any hemoglobin to serve as activator (7,8). In this case, the acetylation of oxygenase occurred to a greater extent than would be predicted by the measured enzymic activity, although it was much less than expected on the basis of oxygenase protein present in the sample. In addition, the acetylation reaction appeared to proceed more slowly for VII than for samples containing added hemoglobin or for crude preparations that still contained endogenous hemoproteins.
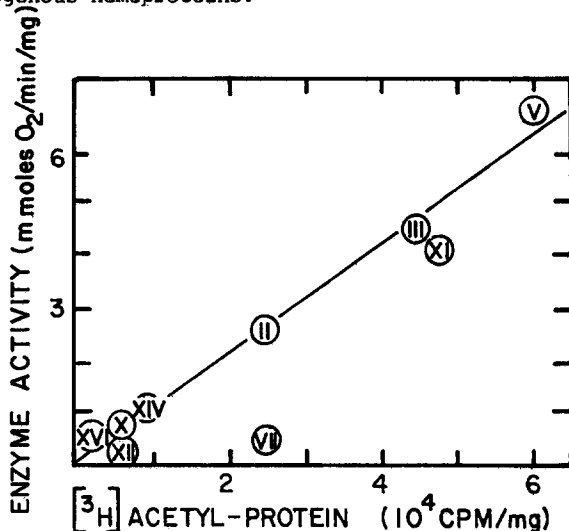


Figure 2. Comparison of the amount of [³H]acetyl-protein with the amount of active oxygenase.

Acetylation of Inhibited Oxygenase Preparations

Added indomethacin (1 µM final concentration) caused progressive loss of oxygenase in a manner described previously (5). The inactivation did not however fully prevent acetylation of the oxygenase preparation by aspirin. When an oxygenase preparation was incubated for 60 min at 37° with 100 µM aspirin 5350 cpm were incorporated into oxygenase protein. In contrast, in the presence of 1 µM indomethacin only 2370 cpm were incorporated after 60 min confirming some interference with acetylation as described in a previous report (2). However, when indomethacin was incubated with the oxygenase at room temperature for 4 and 20 min prior to aspirin addition, the extent of acetylation observed subsequently (see Fig. 3) was greater than would have been predicted by the measured enzyme activity at the time of aspirin addition. After 4 min, there was 50% loss of oxygenase activity with little effect on acetylation. At 20 min, when 95% enzyme inactivation had occurred, acetylation (1050 cpm) was inhibited by only 50% compared to the value at 0 time (2370 cpm). An apparent half-life of 17 minutes for the ability to react with aspirin is several-fold greater than the 4 minute half-life observed for the oxygenase activity.

Another oxygenase inhibitor, diethyldithiocarbamate (DDC), also failed to interfere with acetylation by aspirin (data not shown) even though the oxygenase is completely inactive in the presence of the 10 mM

inhibitor.that was used. Such a result confirms that DDC does not inter-
fere with the progressive inhibitory reaction of the oxygenase with
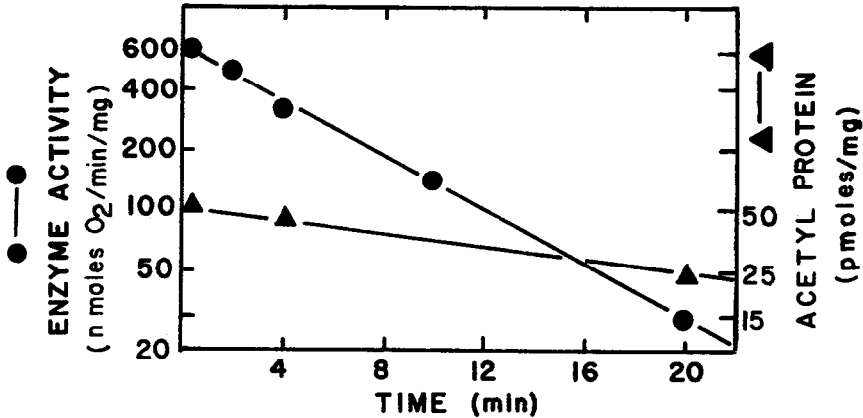aspirin (9).



Figure 3. Lack of correlation of acetylation with measurable activity
after indomethacin treatment. - An oxygenase preparation was adjusted
to contain 1 μM indomethacin at 0 time and oxygen uptake was assayed at
the indicated times: 0-0-0. Aliquots removed at 0 time, 4 min, and 20
min were adjusted to contain 100 μM [acetyl-$^3$H]-aspirin and incubated
for 60 min at 37°. [$^3$H]acetyl-protein (cpm) was determined as described
in Methods: Δ-Δ-Δ.

## DISCUSSION

Our initial plan to propose selective acetylation by aspirin as a
quantitative method for determining the amount of oxygenase protein in
different preparations must be modified in light of the present results.
It now appears that acetylation will measure only certain forms of the
oxygenase protein. Acetylation may prove to be useful in quantitating
active oxygenase (or potentially active (see below)) in crude systems
for which oxygen consumption cannot be reliably determined. The combina-
tion of SDS gel electrophoresis with quantitative isotope measurements
does provide minimal estimates of oxygenase purity (based on a minimal
molecular weight) that are not possible with only kinetic measurements
of oxygen consumption. Such estimates may be useful in more detailed
studies of the nature of the isolated catalytic protein(s).

The kinetic similarity in the irreversible inactivation of oxygenase
by indomethacin and aspirin (10) could lead to consideration that both
inactivations occur by acylation of an essential group on the enzyme pro-
tein. Transfer of the acyl group from the indole ring in indomethacin is
not as probable as that from the phenolic group of aspirin, but might be
possible. Our results make such a possibility seem less likely, If
indomethacin was inactivating by acylation, we would not expect the 50%
inactivated preparation to be capable (95%) of acetylation by aspirin (or
the 95% inactivated still able to be acetylated 45%). Other reactions or
rearrangements of the oxygenase must be considered in explaining the ir-

reversible inactivation by indomethacin. Indomethacin and aspirin appear to compete with substrate (5) and perhaps each other for a common binding site on the oxygenase enzyme. Some possible explanations for the incomplete interference with acetylation are: 1) indomethacin binding may be partly reversible in the presence of aspirin thus resulting in greater than predicted protein labeling; 2) indomethacin may act at some site distinct from its binding site to inactivate the oxygenase but still allow protein acetylation by aspirin; 3) indomethacin inhibition may destabilize the active site so that it is not only enzymically inert, but also slowly rearranges further to a configuration that can no longer be acetylated.

Previous studies have indicated that DDC does not interfere with binding at the fatty acid substrate site, but rather seems to prevent the interaction of oxygen with the enzyme (5). Thus DDC treatment gives reversible loss of oxygenase action comparable to anaerobic conditions (11), that does not, in itself, interfere with the interaction of the oxygenase with indomethacin and aspirin (5). Since acetylation proceeded without apparent interference, the acetylation does not seem to require an actively functioning oxygenase, but merely a potential for functioning.

Acetylation of proteins by transacetylation from a phenyl ester has been described for many different situations (*e.g.* review (12)). Apparently the fatty acid oxygenase can be acetylated more rapidly than most other proteins at low aspirin concentrations (1,2) suggesting some selective interaction occurs with that protein. Results in this report give further evidence for the selectivity of the acetylation in that the aged, spontaneously inactivated enzyme did not react as rapidly. Not only did functionally competent oxygenase protein seem needed, but optimal acetylation also seemed dependent upon the presence of hemeprotein activators that give optimal enzymic activity of the oxygenase protein. Acetylation by acetylsalicylate may therefore be dependent on an essential functional group or conformation of groups in the catalytic peptide chains of the oxygenase enzyme, and the existence of the oxygenase protein *per se* is not sufficient to secure acetylation.

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