THE OXIDATION OF CHALCONE CATALYSED BY PEROXIDASE

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SUMMARY

The studies recorded in this thesis began with the aim of elucidating the enzymology of the recently reported oxidation of 2',4,4'-trihydroxychalcone (isoliquiritigenin) to a hydrated aurone product, isolated as the diastereoisomers Y_1 and Y_2. On partial purification of this activity from cell-free extracts of seedlings of garbanzo bean (Cicer arietinum) it emerged that a peroxidase in the extract was responsible for the catalysis initially observed. Of even greater eventual significance, however, was the concurrent finding that the peroxidase-catalysed oxidation of isoliquiritigenin (chalcone) led mainly to products other than Y_1 and Y_2. Much of the work recorded here is consequently concerned with the elucidation of these major products of the reaction, which are new compounds, and with studies of the biochemistry of their formation. From the results of these chemical and biochemical studies a scheme which rationalises the enzymic reaction is presented.

Preliminary studies on enzyme purification are described which led to the finding that a peroxidase in cell-free extracts of garbanzo was responsible for Y_1 and Y_2 formation from added chalcone in the crude extract. A further finding was that when certain soluble substances were removed from the enzyme preparation, the reaction of chalcone became much more extensive and the major product was then a hitherto unknown compound which was isolated chromatographically and referred to as OC. Under these

conditions, Y_1 and Y_2 production decreased considerably. The soluble substances were thus seen to exert the dual effect of inhibiting OC formation and promoting Y_1 and Y_2 formation. Dialysis of the crude enzyme or precipitation of the protein (enzyme) therein by ammonium sulphate or acetone treatment effectively removed the soluble substances and wrought the changes noted in the extent of reaction and product pattern. The reaction was found to be accelerated by the addition of hydrogen peroxide.

Following the identification of peroxidase as the enzyme responsible for the activities of interest, a procedure for the partial purification of this enzyme from the cell-free extract was developed as a prelude to more detailed biochemical studies.

With the availability of purified enzyme, a system was developed for the successful production and isolation of preparative amounts of the major product, OC, hitherto isolated solely by chromatography. In handling this OC, evidence of its unstable nature was obtained. This led to spectrophotometric study of the reaction in an attempt to obtain quantitative estimates of product formation by eliminating as far as possible any product breakdown.

Spectrophotometric monitoring of the reaction product formed in a rapid complete consumption of the added chalcone revealed surprisingly a spectrum other than that of OC in the reaction buffer. Thus the first evidence was obtained that the initial reaction product consisted mainly of a precursor of OC. This initial product rapidly gave

rise to mainly OC on careful treatment with dilute alkali. Further, it could be recovered unchanged by solvent extraction from weakly acidic media. Chromatography then revealed that it consisted largely of two compounds each giving rise to OC on treatment with dilute alkali. These compounds which had chromatographic properties that overlapped with those of Y_1 and Y_2, are referred to as $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$. These studies also revealed that the initial product contained small amounts of Y_1 and Y_2 (converted to the aurone, hispidol, on alkali treatment). Further, they showed that small amounts of the known flavonoid, 4',7-dihydroxyflavonol (flavonol) were also formed on treatment of the initial product or of chromatographically isolated $\not{\alpha}Y_1$ and $\not{\alpha}Y_2$ preparations with dilute alkali. Thus the initial product was identified as consisting of precursors of three distinct compounds; the new compound OC and the recognised flavonoids 4',7-dihydroxyflavonol and hispidol.

Additional studies showed how the chromatographic product pattern changed according to the treatment the initial product received at various stages prior to or during 2-D chromatography. The highly unstable nature of the $\not{\alpha}Y$ precursors of OC was clearly evident. This work uncovered another precursor of OC, referred to as $\not{\alpha}OC$, which arose apparently from the $\not{\alpha}Y$ compounds under acidic conditions. This extensive array of products arising from the enzymic reaction was simplified somewhat when it was shown that the $\not{\alpha}Y$ compounds were common precursors of OC and flavonol.

In the biochemical studies the peroxidase preparation from garbanzo was compared with the action of commercial

horseradish peroxidase (HRP). Very similar results were obtained throughout with these two enzymes.

Both enzymes showed a pH optimum for catalysis of chalcone oxidation at slightly above neutrality (garbanzo, pH 7.5 and horseradish, pH 7.9). The rate of reaction was found to be dependent on the concentration of added hydrogen peroxide in a manner indicative of a substrate requirement for this compound. Under the conditions employed, the concentration of hydrogen peroxide giving maximum rate with garbanzo enzyme (0.15 mM) was about half that required with HRP (0.30 mM). Typical substrate concentration curves were obtained on varying the concentration of chalcone, both enzymes being apparently saturated at concentrations above about 45 μM. An oxygen requirement for the reaction was demonstrated manometrically with a chalcone:oxygen stoicheiometry of approximately 1:1. This result was independent of the concentration of hydrogen peroxide tested and indicated that this compound did not substitute for oxygen. This was demonstrated by inhibition of the reaction in the absence of oxygen but in the presence of substrate amounts of hydrogen peroxide. Direct measurement showed only a very small (catalytic) net consumption of hydrogen peroxide in the course of the aerobic reaction, in keeping with the stoicheiometric oxygen requirement. The reaction was found to be strongly inhibited by manganous ion, mercaptoethanol and diethyldithiocarbamate and by the redogenic donors, hydroquinone, catechol, pyrogallol and p-phenylenediamine. The oxidogenic donors tested were not found to be stimulatory. Qualitative studies indicated the

effect of substitution pattern on the enzymic reaction of a range of chalcones; for both enzymes a free phenolic group in the B ring, preferably in the 4-position, was required.

While the enzymic reaction was treated, for the purposes of the quantitative biochemical studies, as a $\not{a}Y_1$ and $\not{a}Y_2$ (CC)-forming reaction, additional chromatographic studies of Y_1, Y_2 and flavonol formation were made. Thus Y_1 and Y_2 were found to be ubiquitous minor products of the aerobic reaction; the combined yield was measured (as aurone) at approximately 1% of the chalcone consumed under typical reaction conditions with purified systems. As expected, Y_1 and Y_2 formation was inhibited by redogenic donors yet with diethyldithiocarbamate, an inhibitor of the main ($\not{a}Y$-forming) reaction, a relative increase was noted. The promotor(s) of Y_1 and Y_2 formation in the cell-free extract were found to be substances with hydrophilic sugar-like properties. They were considered to have the added capacity to simultaneously inhibit the formation of $\not{a}Y$ compounds (observed as OC).

The source of flavonol noted in small amounts on all chromatograms of reaction products was found to be the $\not{a}Y$ compounds. When treated with alkali these compounds gave rise to both OC and flavonol. A competitive formation of OC and flavonol occurred and at higher concentrations of alkali (0.1-0.5 N) the flavonol product predominated. A more facile reaction was observed with $\not{a}Y_1$ than with $\not{a}Y_2$. An apparently specific inhibition of flavonol formation in the enzymic reaction by cupric ion was initially observed chromatographically and on the identification of the

precursors of flavonol, it was shown that cupric ion caused extensive degradation of ØY compounds and could effectively inhibit flavonol formation at low alkali concentrations.

The finding that the ØY compounds were the precursors of flavonol in the peroxidase-catalysed system was in contrast to the accepted role of the dihydroflavonol as precursor of flavonol in flavonoid biosynthesis. Consequently the place of the specific dihydroflavonol (garbanzol) in the peroxidase reaction was investigated. Garbanzol was found not to be oxidised to flavonol in the enzymic system. Further, with the aid of labelled chalcone, evidence was obtained for the production of traces of garbanzol in the products of the enzymic reaction.

The new compounds which were isolated from the enzymic reaction product, namely $ØY_1$, $ØY_2$, OC and ØOC, were obtained in preparative amounts and characterised using u.v., i.r., NMR and mass spectral techniques. From these data the structures of these closely related compounds were formulated as the benzoxepin derivatives given in Figure 61. Thus $ØY_1$ and $ØY_2$ are diastereoisomeric diols and OC is the enolic form of the α-diketone (ØOC) derived from the diols. Schemes are presented to rationalise these transformations and also the formation of flavonol in competition with OC from the ØY compounds. Base-catalysed elimination of the elements of water occurs in the conversion of ØY compounds to OC and to flavonol; in the latter case skeletal rearrangement also occurs. A pinacolic rearrangement accounts for the generation of ØOC from the ØY compounds under acidic conditions and enolization for the conversion of ØOC to the more stable enol, OC. A very significant facet of the

characterisation studies was the resolution of $\not{\alpha}Y_2$ into enantiomers by paper chromatography as confirmed by optical rotation and spectral data. No similar resolution of the virtually optically inactive $\not{\alpha}Y_1$ was observed.

The chemical oxidation of chalcone with hydrogen peroxide was also studied. At room temperature and slightly alkaline pH (7-11) it was found that the products isolated were chiefly OC and flavonol. Direct evidence that the precursor relationships of the enzymic reaction also applied to the chemical reaction was obtained with the isolation of $\not{\alpha}Y$ compounds as the only significant product under carefully controlled conditions. The product of the chemical reaction under Algar-Flynn-Oyamada (A.F.O.) conditions (high pH, low temperature - 0^{o}) was found, however, to be flavonol with no trace of OC. It is nevertheless proposed that under A.F.O. conditions, the intermediates ($\not{\alpha}Y$ compounds) of both the enzymic and the room temperature, lower pH, chemical reaction are still produced and that they rearrange spontaneously at the high pH and low temperature to flavonol. This conclusion is in contrast to the role normally ascribed to dihydroflavonol as the precursor of flavonol in the A.F.O. reaction.

Schemes are presented and discussed to explain the enzymic and chemical reaction of chalcone to form $\not{\alpha}Y$ compounds. A central feature of these schemes is the postulated formation of an enantiomeric epoxide as the true initial reaction product. Hydrolytic opening of the epoxide ring of this structure would give rise to the $\not{\alpha}Y$ (diol) structure. Since $\not{\alpha}Y_2$ occurs in enantiomeric form, it is also proposed that both isomers of $\not{\alpha}Y_1$ similarly occur and therefore that

all four isomeric diols occur in the product. The formation of these four diols from the epoxide enantiomers is discussed. An attractive feature of the epoxide as true initial product is that it permits a scheme for the enzymic reaction to be advanced based on a chain reaction which utilises only known redox states of peroxidase and which accommodates all features of the biochemical data.

The enzymic reaction of chalcone is thus a four-equivalent oxidation (dehydrogenation plus oxygenation), the postulated epoxide precursor, the ØY structure, OC and ØOC all being at the one oxidation level (4 equivalents higher than chalcone). The proposed oxygenating species of the enzyme (formally Compound III) is discussed in relation to other examples of this activity of peroxidase and also with respect to selected examples of oxygenation by mixed function oxidases.

This new reaction of chalcone is held to provide a particularly clear-cut example of the incompletely understood oxygenating activity of peroxidase which occurs in this case without detectable requirement for any organic cofactors or of metal ions. In relation to the original starting point of flavonoid biosynthesis, the reaction accounts for the formation of Y_1 and Y_2 initially observed in the crude system and may account for the biosynthesis of garbanzol also previously recorded in that system. Significantly, the enzymic reaction provides the first example of an *in vitro* system leading to flavonol formation through the hitherto unknown ØY structure rather than by way of the anticipated dihydroflavonol. The question of the *in vivo* significance of this complex activity of peroxidase remains open.

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