The applications of the fourth-derivative method can be extended to cases in which broad peaks overlap with those of interest. For example, ionized tyrosine has a broad absorption maximum at 295 nm, which obscures the bands arising from the un-ionized residues. The fourth-derivative operation cancels out the broad band, thus allowing the study of un-ionized tyrosine residues in the alkaline region[6]. On the other hand, when there is appreciable non-selective light-scattering, this technique still gives a horizontal baseline, thus permitting the study of moderately turbid samples.

Conclusions

The examples outlined in this article illustrate that fourth-derivative spectrophotometry can be used to monitor the environment of the aromatic residues in a variety of protein systems. The technique offers several advantages over classical absorption or difference spectrophotometry, such as the possibility of studying phenylalanine separately from tyrosine and tryptophan and, under certain conditions, tryptophan separately from tyrosine. Likewise, the method is capable of clearly revealing small peaks not readily apparent in the absorption or difference spectra and it is particularly useful when broad, perturbing bands are present.

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Open Question

Cytochrome P-450 and glutathione: what is the significance of their interrelationship in lipid peroxidation?

Aalt Bast and Guido R. M. M. Haenen

Both cytochrome P-450 and glutathione participate in the metabolism of xenobiotics. Their interrelationship is described here, as well as current findings indicating their mutual involvement in lipid peroxidation.

Lipid peroxidation is the process by which oxidative degradation of polyunsaturated fatty acids (LH) occurs. Because of the biomedical implications of lipid peroxidation (its toxicity), the process has been the subject of strenuous research. The effects of both enzymic and non-enzymic systems in lipid peroxidation have been studied.

Lipid peroxidation was first considered as two distinct sequential phases by Svingen *et al.*[1]: initiation and propagation. Initiation was defined as the formation of lipid hydroperoxides (LOOH) accompanied by a minimal formation of malondialdehyde (MDA). Propagation was defined as breakdown of LOOH to give reactive intermediates and products of lipid peroxidation (e.g. malondialdehyde) with reformation of LOOH. These definitions have been adopted frequently by others, with subsequent confusion. For, in general chemical terms, initiation means radical production and propagation means radical transfer. A more accurate representation of the reaction is shown in Fig. 1, with classification of LOOH-independent lipid peroxidation and LOOH-dependent lipid peroxidation. Both types of peroxidation have an initiation phase and a propagation phase. During the latter, reactants are converted to products with no net consumption of atoms or radicals. Malondialdehyde is formed during the propagation phase of both LOOH-independent and LOOH-dependent lipid peroxidation. The respective initiation processes differ and are not well understood.

A. Bast and G. R. M. M. Haeen are at the Faculty of Pharmacy, Dept. of Pharmacology and Pharmacotherapy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands.

Cytochrome P-450

Enzymically induced lipid peroxidation is frequently studied using NADPH and the microsomal membrane. The enzymes involved are components of the cytochrome P-450 system: NADPH cytochrome P-450 reductase and cytochrome P-450. Stimulation of lipid peroxidation by the reductase can occur via transfer of electrons, not to cytochrome P-450, but to iron or chelated iron (formation of reduced (chelated) iron) or to xenobiotics (with subsequent redox cycling)[2]. The role of cytochrome P-450 in lipid peroxidation is more controversial and is discussed here.

High concentrations of the cytochrome P-450 system are found in the microsomal membrane. The enzyme system has attracted researchers in the fields of biochemistry, pharmacology and toxicology. It plays a pivotal role in the metabolism of endogenous compounds (steroids, prostaglandins) and in the biotransformation of xenobiotics. In our western society there is an ever-increasing demand on cytochrome P-450 to cope with xenobiotics. However, the involvement of cytochrome P-450 does not guarantee detoxification. It has been hypothesized that cytochrome P-450 played an early role in the detoxification of oxygen, during chemical evolution. It was probably present in living organisms

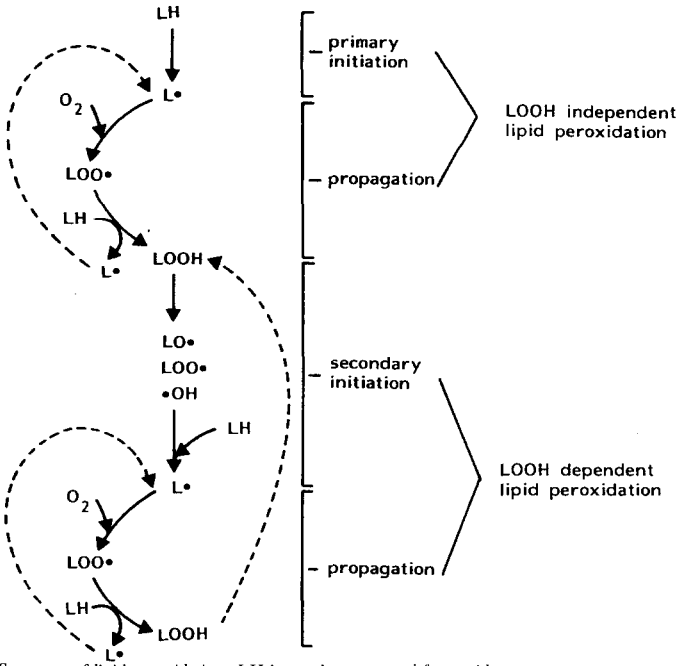

Fig. 1. Sequence of lipid peroxidation. LH is a polyunsaturated fatty acid.

before free oxygen appeared in the atmosphere. The assumed old age of cytochrome P-450 also helps to explain its ubiquity in organisms throughout the phylogenetic scale. In mammals, including man, cytochrome P-450 has been found in many organs, and particularly high concentrations are present in the liver. Cytochrome P-450 may function as a monooxygenase (a dioxygenase function has also been suggested[3], but has not been investigated extensively), as an oxidase and as a peroxidase (Fig. 2).

The *monooxygenase activity* of cytochrome P-450 depends on electron transfer via NADPH cytochrome P-450 reductase (first electron) and (still controversial) via cytochrome b_5 (second electron). The actual mechanism of insertion of the activated oxygen (oxene?) into the substrate is unknown. The monooxygenase function may be involved in primary initiation (Fig. 1) via the formation of metabolic intermediates of xenobiotics resulting in the production of lipid radical (L·) from LH.

A deviation from the expected stoichiometry of the overall NADPH, cytochrome P-450 catalysed oxygenation of a substrate (1:1:1) is often observed. This can be understood if the *oxidase activity* of cytochrome P-450 is taken into account, when the oxygen molecule is not incorporated into the substrate; rather, oxygen functions as a hydrogen acceptor. The H_2O_2 formation mainly results from dismutation of superoxide anion radicals ($O_2^{\cdot-}$) which are released from the oxycomplex of cytochrome P-450[4]. The contribution of NADPH cytochrome P-450 reductase, cytochrome b_5, fatty-acid desaturases, FAD monooxygenase or direct formation from peroxy-cytochrome P-450 to H_2O_2 production in microsomes is probably small[4,5]. It has frequently been suggested that a Fenton reaction (Fe^{2+} + H_2O_2) may give rise to the hydroxyl radical (·OH) which can be regarded as the initiating species. However the finding that microsomal H_2O_2 formation did not correlate with MDA formation[6] as well as the inability of catalase to inhibit MDA formation[7] are arguments against the involvement of free ·OH or H_2O_2 in microsomal lipid peroxidation. The actual species which causes primary initiation might be $O_2^{\cdot-}$ complexed in some way with chelated iron[8].

LOOH-dependent peroxidation (secondary initiation, Fig. 1) is initiated by chelated iron[1] or haem iron[9]. Cytochrome P-450 has been proposed as an important endogenous initiating species in LOOH-dependent lipid peroxidation via its *peroxidase activity*[1]. The peroxidase function may proceed using organic hydroperoxides as oxygen donors. A homolytic cleavage of the peroxide bond is probably involved[10] (Fig. 3). Hydrogen donors other than LH or LOOH such as NADPH or oxidizable substrates, RH[2], may depress the secondary initiation catalysed by cytochrome P-450 in lipid peroxidation, because in that way a non-toxic decomposition of LOOH is achieved (Fig. 3). Also, competition between substrates and LOOH for binding loci at cytochrome P-450 may prevent its functioning as peroxidase.

The remarkably varied utilization of oxygen by cytochrome P-450 is not astonishing in view of the assumed primordial role of cytochrome P-450 in defence against oxygen toxicity.

Glutathione

Like cytochrome P-450, the tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) is also involved in the metabolism of xenobiotics. It serves as a conjugating factor for many electrophilic xenobiotics, before irreversible alkylation occurs. Conjugation with GSH occurs much more readily in the presence of GSH S-transferases (GSH-tr). Conjugation with GSH does not always lead to detoxification, but may cause bioactivation. Metabolism and some physiological functions of GSH have been reviewed by Meister[11].

GSH is often present in millimolar concentrations, and acts as an intracellular reductant, when converted to glutathione disulfide (GSSG). High concentrations of GSH are maintained by the reduction of GSSG to GSH catalysed by the NADPH-dependent enzyme GSH reductase (Fig. 4).

In the late 1950s, Mills[12] discovered that a GSH-dependent peroxidase (GSH-px) was able to protect against effects of H_2O_2. Some years ago, two GSH-px activities were described: a selenium-dependent GSH-px which utilizes H_2O_2 and organic hydroperoxides as substrates, and a selenium-independent GSH-px which preferentially utilizes organic hydroperoxides. The selenium-independent GSH-px is similar to GSH-tr[13]. It is generally accepted that GSH-px reduces lipid hydroperoxides to less reactive lipid alcohols. It would therefore be expected to affect LOOH-

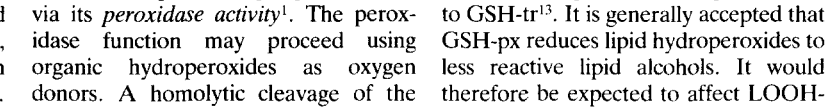

Fig. 2 Proposed stoichiometry of various functions of cytochrome P-450: RH, ROH and XOOH are the substrate, the product and a peroxy compound, respectively.

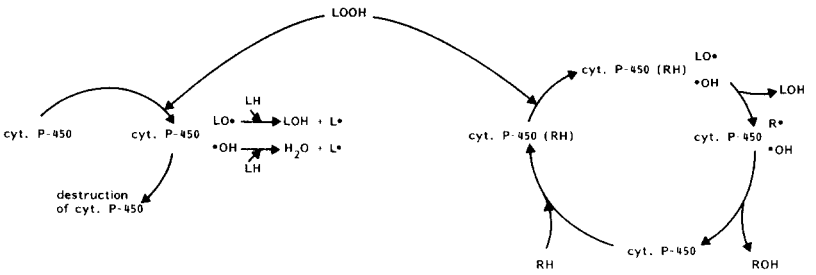

Fig. 3. Cytochrome P-450 as peroxidase. Left, *without a suitable hydrogen donor, leading to lipid peroxidation.* Right, *with a hydrogen donor leading to innoxious decomposition of the peroxide.*

dependent lipid peroxidation. As expected free fatty acid hydroperoxide salts in solution or as micellar suspensions are good substrates for GSH-px. However, the actual significance of this reaction for microsomal membranes has been questioned by McCay *et al.*[13] since their investigations on GSH-dependent inhibition of microsomal lipid peroxidation by cytosol showed that:

(1) Polyunsaturated fatty acids of microsomes are not consumed in systems containing GSH and cytosol. This indicates that primary initiation is inhibited rather than the reduction of lipid hydroperoxide to lipid alcohol.
(2) Micellar peroxidized microsomal lipids (almost entirely phospholipids) precludes access of GSH-px.

Burk *et al.*[14] suggested that this GSH-dependent cytosolic inhibitory activity could be attributed to GSH-tr. However, further purification of GSH-px and GSH-tr showed that the rat cytosolic GSH-dependent lipid peroxidation inhibiting factor is neither GSH-px, nor a GSH-tr[15]. The recently described inhibiting protein of phospholipid peroxidation[16] shows peroxidase activity and is therefore probably not identical to the cytosolic factor described by McCay *et al.*[15]. This has yet to be verified.

A recent study showed that selenium-dependent GSH-px can engender its protective function with membrane-bound lipid hydroperoxides, but only after liberation of the lipid hydroperoxides from their membrane-bound status by phospholipase action[17]. An analogous dependence on phospholipase has been reported for the action of epoxide hydrolases on membrane-bound phospholipid epoxides[18]. This is all the more interesting if one realizes that intramolecular rearrangements during propagation can yield epoxides. In this way also, cyclic dialkyl lipid peroxides are formed which are not substrates for GSH-px.

Morgenstern *et al.*[19] demonstrated GSH-tr activity in rat liver microsomes which differs from cytosolic GSH-trs. The microsomal GSH-tr also has GSH-px activity. Based on the substrate utilization, it has been concluded that microsomal GSH-tr/px is not dependent on selenium[20]. The peroxides which originate in the hydrophobic matrix of the membrane might be substrates for the bound GSH-px, if indeed the objections formulated for cytosolic GSH-px[13] do not apply for membrane GSH-px. This has still to be determined.

GSH is able to protect liver microsomes against lipid peroxidation[21–23]. This microsomal GSH-dependent factor is of utmost importance because it probably protects against effects of reactive (oxygen-) intermediates which are formed by the cytochrome P-450 system. Lipid peroxidation may also destroy the GSH-dependent protection in microsomes. It has been suggested that GSH protects the microsomes via inhibition of free-radical formation, in which vitamin E might be involved[22]. The microsomal protective factor is not a GSH-px, since a substantial GSH consumption during the protection by GSH was not observed[22,23]. Moreover, the microsomal factor seems different from the GSH-dependent cytosolic factors[22,23]. This has to be verified. The microsomal factor provides local protection against and restriction of microsomal lipid peroxidation by inhibiting primary initiation. The precise mechanism of this GSH action still remains unclear.

Obviously, an array of GSH-dependent protective mechanisms exist, of which the significance *in vivo* still has to be established.

Interrelationship of cytochrome P-450 and glutathione

Cytochrome P-450 and GSH may influence both LOOH-independent and LOOH-dependent lipid peroxidation.

Primary initiation (Fig. 1) might be stimulated via the monooxygenase function of cytochrome P-450 (xenobiotic reactive intermediate formation) or its oxidase activity ($O_2^{\cdot-}$; ·OH formation), or even its peroxidase activity (LH as H-donor leading to L·). However, it is generally assumed that NADPH cytochrome P-450 reductase is the main causative or primary initiation agent in NADPH-dependent lipid peroxidation, via reduction of iron or chelated iron. Also, GSH may act as a reducing agent. In the presence of iron and H_2O_2, the thiol may generate ·OH radicals[24] which probably can cause primary initiation. On the other hand, prominent inhibition of primary initiation by both GSH-dependent-cytosolic and GSH-dependent-microsomal factors has been observed. Clearly, mediation of primary initiation in LOOH-independent lipid peroxidation by the cytochrome P-450 system and GSH should be considered; the significance of this *in vivo* is unclear.

Secondary initiation (Fig. 1) can be stimulated via the peroxidase function of cytochrome P-450 (homolytic scission of LOOH leading to LO· and ·OH, or LOOH as H-donor leading to LOO· – see Fig. 3). GSH (cytosolic and microsomal) might reduce LOOH to LOH and inhibit secondary initiation. However, the possibility that GSH-px utilizes peroxidized microsomal lipids (LOOH) as substrates is currently under debate[13,17]. Most studies in which the peroxidase function of cytochrome P-450 has been investigated in relation to lipid peroxidation have been conducted either in model systems or by using simple organic hydroperoxides. It is important to note that the question of whether membrane-bound LOOH can

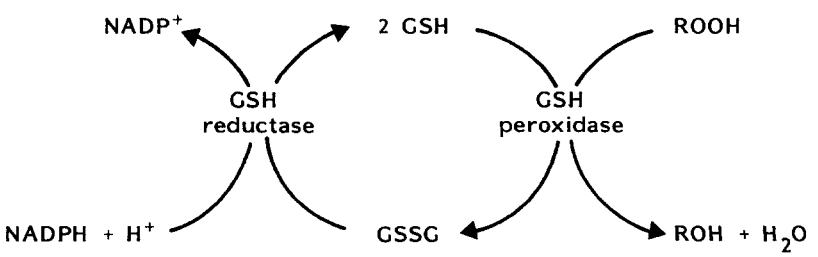

Fig. 4. Outline of GSH peroxidase and GSH reductase action.

be a substrate for cytochrome P-450 has not yet been studied.

Oxidizable substrates of cytochrome P-450 inhibit NADPH-dependent lipid peroxidation, possibly by providing H-atoms for the peroxidase function of cytochrome P-450 (Fig. 3). When the peroxidase activity of cytochrome P-450 is insignificant for lipid peroxidation, because membrane peroxidized microsomal lipids are not substrates, the observed inhibition of cytochrome P-450 substrates on NADPH-dependent lipid peroxidation might be the result of diverting electrons from the NADPH cytochrome P-450 reductase. The real issue in determining the importance *in vivo* of many data obtained *in vitro* is whether LOOH-dependent lipid peroxidation forms a major part of overall lipid peroxidation – a question which has not been fully answered yet.

Obviously GSH and cytochrome P-450 may affect the sequence of lipid peroxidation at the same steps. Moreover cytochrome P-450 activities are coupled to the GSH/GSSG system via the $NADPH/NADP^+$ ratio. Cytochrome P-450 activity and reduction of GSSG to GSH result in consumption of NADPH. Moreover, cytochrome P-450-catalysed oxidation of xenobiotics results in GSH consumption. This is not necessarily caused only by formation of electrophilic intermediates, but may also be attributable to an increased flux through GSH-px. However, it seems that GSH consumption is only partly the result of enhanced H_2O_2 and other hydroperoxide formation[25]. In this respect the recently assured interaction of O_2^- with GSH deserves further attention[26]; we found that H_2O_2 originating from the oxidase activity of cytochrome P-450 is not a rate-limiting step in microsomal lipid peroxidation. However, indirectly via a decrease in the GSH/GSSG ratio, H_2O_2 may effect lipid peroxidation, as it is possible that GSH reduction *per se* may lead to lipid peroxidation[27].

In considering the reciprocal effects, i.e. the effects of lipid peroxidation on cytochrome P-450 and GSH, it was found that cytochrome P-450 is destroyed during lipid peroxidation. Interestingly, it has also been hypothesized that LOOH plays a key role in the induction of cytochrome P-450[28]. GSH concentrations also decrease during lipid peroxidation, and GSH may protect cytochrome P-450 from destruction by lipid peroxidation[29].

Initial work on the interrelationship between cytochrome P-450 and GSH makes it clear that, in order to understand the complex process of lipid peroxidation, functional competition and cooperation of various cell components (which may reside in different cell compartments) should not be overlooked. Adequate and ingenious models *in vitro* of the situation *in vivo* are required.

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Letters to the Editor

Is dicyclohexylcarbodiimide a probe for proton-translocating enzymes?

Marc Solioz[1] recommends *N,N'*-dicyclohexylcarbodiimide (DCCD) as a tool for investigating the mechanism of proton translocation and for identifying the structures involved in this process. He places particular emphasis upon the fact that two systems, F_1F_o-ATPase and cytochrome *c* oxidase have been shown to be inhibited in their proton-pumping activity by the interaction of DCCD with a single carboxylic group located in a hydrophobic domain in one of the subunits[2,3]. From these two observations Solioz draws the broad conclusion that all enzymes involved in proton translocation and inhibited by DCCD possess DCCD-reactive stuctural domains essential for proton translocation and that DCCD is thus a probe for them. Unfortunately, we cannot share his optimism.

There is no space to argue about some marginal aspects of Solioz's article[1], such as the interpretation of the data concerning subunit III of cytochrome *c* oxidase[4]. It was never proposed that this subunit forms a proton channel similar to that of the F_1F_o-ATPase, and actually DCCD-labelling data cannot show whether the labelled polypeptide is a pump, a channel or a regulatory subunit. There are, however, more important discrepancies between our view and that of Solioz on the use of DCCD:

(1) Solioz presents DCCD as a reagent which binds specifically to carboxylic groups located in hydrophobic domains of proteins[1]. In fact, this has only been demonstrated for the two systems mentioned above and for the Ca^{2+}-ATPase of sarcoplasmic reticulum[5]. These systems may well be exceptional. High reactivity of carbodiimides towards many functional groups[6] gives little chance for specific interactions, unless the system is carefully controlled and