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Pyrroloquinoline quinone (PQQ) is the organic cofactor in soybean lipoxygenase-1

Robert A. van der Meer and Johannis A. Duine

Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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Treatment of soybean lipoxygenase-1 (SLO) with phenylhydrazine (PH) induced inactivation and a maximum (350 nm) in the absorption spectrum of the enzyme. To detach the product having this absorption maximum, proteolysis was required. The product appeared to be the C(5) phenylhydrazone of pyrroloquinoline quinone (PQQ). Quantification showed that one covalently bound PQQ is present per enzyme molecule. Inspection of published spectroscopic data leads to the conclusion that the established cofactor of SLO, Fe, present as one ion per enzyme molecule, interacts with PQQ, the latter functioning as a terdentate ligand for the Fe ion (the N atom and COOH group of the quinoline ring together with the hydrated C(5) carbonyl group). If it is assumed that the substrate has two sites of interaction with this two-cofactor complex, namely with the Fe ion and the C(5) carbonyl group of PQQ, the electron-relay system generated very well explains the reported mechanistic features as well as inhibition effects of substrates with extended conjugation systems. The finding of PQQ in SLO is an additional indication that this cofactor is very versatile with respect to involvement in different types of redox reaction. A further implication might be that PQQ has been overlooked in other well-known enzymes with an established cofactor but where the presence of this cofactor is unable to explain the mechanistic and spectroscopic features.

Lipoxygenase-1; Pyrroloquinoline quinone; Hydrazine; Cofactor; Quinoprotein; Iron-containing enzyme

1. INTRODUCTION

Enzymatic lipoxygenation is a process of considerable biological importance, since it is involved in the biosynthesis of regulators like leukotrienes, prostaglandins and lipoxins. The process is typified by regio- and stereo-specific conversion of arachidonic acid into the corresponding 15*S*-peroxide by means of soybean lipoxygenase (EC 1.13.11.12) (SLO). Although the enzyme was first crystallized 40 years ago and has meanwhile been investigated using several sophisticated techniques, a coherent picture, combining the structural and mechanistic information, is still lacking. The conclusion which can be derived from some recent reviews [1,2] is that the enzyme is an unelucidated, unique iron-protein.

It is generally accepted that non-haem iron is the only cofactor in SLO (present in a ratio of 1 Fe per enzyme molecule). Despite the fact that the nature of the residues involved in liganding is unknown, attempts have been made to interpret the (unusual) ESR and absorption spectroscopy parameters and from this to propose a mechanism [1,2]. For this purpose the assumption is made that Fe is involved in hydrogen abstraction from the substrate as well as in addition of dioxygen to the free radical substrate intermediate (fig.1A). Obviously, to perform this dualistic function, changes in the valency of Fe are required. From studies with mechanism-based inhibitors, however, another view has been proposed [3] in which this valency change is not required. Proton abstraction, catalyzed by an (very strong) unspecified, basic group in the active site,

Correspondence address: J.A. Duine, Department of Microbiology and Enzymology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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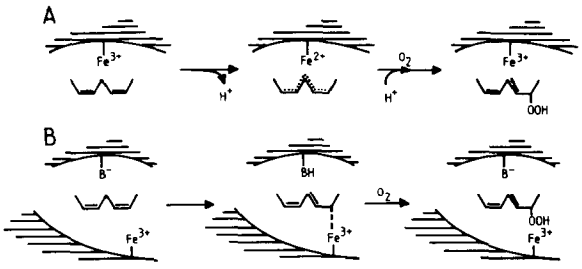

Fig.1. Proposed mechanisms for SLO: (A) the free radical mechanism [1,2]; (B) the organo-iron mechanism [3].

is supposed to occur with concurrent electrophilic attack of Fe on the substrate (fig.1B). It will be clear that these views are contradictory, but as long as real insight into the structure and functioning of the Fe centre is lacking, both mechanisms can be brought into agreement with the data available from the literature.

Until recently, a similar situation existed with respect to dopamine β-hydroxylase (EC 1.14.17.1). This enzyme has long been considered as a colourless copper-protein, the Cu being involved as the only cofactor operating in the hydroxylation process. It now appears that this picture is incorrect, the enzyme also containing covalently bound pyrroloquinoline quinone (PQQ), probably operating as an additional cofactor in the catalytic cycle [4]. The fact that the presence of PQQ has been overlooked for so long in this well-known enzyme might indicate that the same has occurred in other cases where a cofactor is known but its presence is unable to explain the essential structural and mechanistic features of the enzyme in an adequate way. As may be clear from the foregoing, SLO seemed a good candidate to check the validity of this hypothesis.

All eucaryotic quinoproteins (PQQ-containing) discovered so far contain PQQ in covalently bound form. A proven, reliable way to demonstrate the presence of the cofactor in such a case is to apply the so-called 'hydrazine method' [5], derivatizing PQQ in situ to a reasonably stable adduct before protein hydrolysis is carried out. Since SLO becomes irreversibly inhibited with phenylhydrazine (PH) (but the product is unknown) [6], this could be interpreted in favour of the hypothesis and thus of the feasibility of the hydrazine method for this enzyme.

2. MATERIALS AND METHODS

2.1. *Purification of SLO*

Lipoxygenase from Sigma (type I) was further purified on DEAE-Sepharose (Pharmacia) followed by HPLC gel filtration on an SI 300 polyol column (Serva). The amount of purified SLO was determined using the specific absorption coefficient determined by Petersson et al. [7] ($A^{0.1\%}_{280\,nm} = 1.6$).

2.2. *Inhibition with PH and characterization of the product*

Derivatization of the cofactor in SLO with PH and isolation of the adduct were essentially as described [5], under the following conditions: to a solution (14 ml) of SLO (31 mg) in 0.1 M borate buffer, pH 10.0, 16 μl of a 0.1 M solution of PH in concentrated H_3PO_4/ethanol (1 : 1, v/v) [8] was added; the mixture was incubated at 40°C for 16 h under air; proteolysis was performed by addition of 16 mg pronase E (Boehringer, Mannheim) and incubation at 40°C for 6 h. Separation on a Seppak cartridge, HPLC (on a 4 μm C_{18} RCM cartridge) of the isolated adduct, its transformation into PQQ, the preparation of the model hydrazone from PQQ and PH, and the determination of PQQ concentrations were all performed as in [5].

2.3. *Enzyme assay*

SLO activity was determined by measuring the increase in absorbance at 234 nm of the hydroperoxide product, formed from linoleic acid (Sigma, ~99% free acid) according to Galey et al. [9]. Activities were calculated as μmol hydroperoxide product formed/min using a molar extinction coefficient for the hydroperoxide product of 25 000 $M^{-1} \cdot cm^{-1}$ [9].

2.4. *Iron determination*

Quantitative determination of Fe^{2+} in SLO was performed spectrophotometrically according to Massey [10]. Fe^{2+} concentrations were calculated using a molar extinction coefficient of 11 000 $M^{-1} \cdot cm^{-1}$ [10].

3. RESULTS

The indicated purification steps provided an SLO preparation with a specific activity of 188 μmol hydroperoxide product formed $\cdot$ $min^{-1} \cdot mg^{-1}$ (protein), a value comparable with that found by others for a preparation considered to be homogeneous [11]. Similarity and homogeneity were also apparent from the Fe content found (0.91 Fe^{2+} per enzyme molecule) and that reported [11]. No heterogeneity in activity was observed during the chromatographic steps so that the enzyme preparation only contains lipoxygenase-1 [7].

The SLO preparation had a structureless absorption spectrum (fig.2). Inhibition with PH induced a maximum at 350 nm (fig.2), a phenomenon which has been observed for several copper-quinoproteins [4,5]. Acidification of inhibited enzyme to

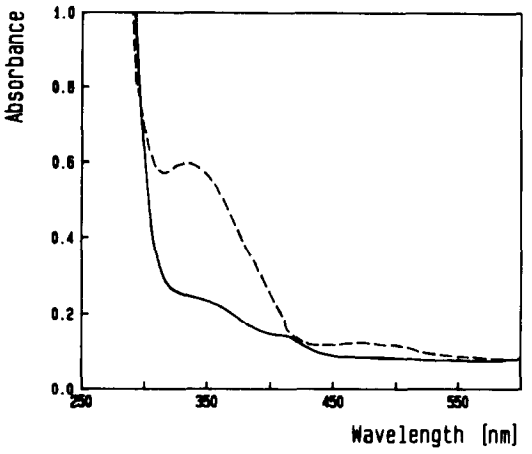

Fig.2. Absorption spectra of SLO (2.2 mg/ml in 0.1 M borate buffer, pH 10.0) before (——) and after (– – –) derivatization with PH.

pH 2.0 did not release a product with this absorption maximum, but proteolysis, followed by acidification, did (as evident from adsorption to and elution from the Seppak cartridge). Since all chromatographic and spectral properties of the product (not shown) were identical to those of the C(5)-hydrazone of PQQ and PH [5], and the product could be converted into PQQ, it is concluded that the 350 nm product is the C(5)-hydrazone of PQQ and PH. Quantification indicated that 0.88 PQQ is present per enzyme molecule.

Although immediate inhibition occurred, formation of the so-called azo compound [5] was not observed. Formation of the hydrazone in the enzyme required the usual (long) incubation time, but in contrast to the copper-quinoproteins [4,5] proceeded efficiently under air and did not require high oxygen tensions.

4. DISCUSSION

4.1. *Product identification: evidence for PQQ*

The spectroscopic data on the inhibited enzyme (fig.2) and the spectroscopic and chromatographic data of the isolated product indicate that PH inhibition leads to the formation of the C(5)-hydrazone of PQQ and PH in SLO. Since product release required protein hydrolysis, PQQ is covalently bound to the protein, probably via an amide bond. From the fact that the enzyme preparation was homogeneous and PH inhibition has been observed by others [6], it is concluded that the finding is not adventitious but that PQQ is a real constituent of SLO. A ratio of 0.88 PQQ per enzyme molecule was determined, indicating that the monomeric enzyme contains 1 PQQ and 1 Fe. PH inhibition of SLO has been intensively studied [6]. Although product analysis was not carried out, it was demonstrated that phenyldiazene, formed from PH under oxidative conditions, is the genuine inhibitor. Furthermore, it was suggested that phenyldiazene is degraded into a phenyl radical which attacks SLO [6]. Although it now appears that the suggestion is incorrect, the diazene could indeed be the inhibiting species, since no azo compound was formed and hydrazone formation required aerobic conditions, in contrast with amine oxidoreductases where the azo compound is formed under anaerobic conditions, suggesting that the hydrazine itself is the inhibiting species [4,5]. Since the latter enzymes occur in their oxidized form but (as discussed below) native SLO seems to be in a reduced form, the difference is comprehensible.

4.2. *Accordance with spectroscopic data*

After ascertaining that PQQ is a constituent of the enzyme, the question can be raised as to whether spectroscopic data are in accordance with this, and more intriguing, whether there is any indication for changes in its redox state, pointing to a function as cofactor. To answer this, the spectroscopic properties of the enzyme forms of SLO should be inspected and compared with those of PQQ, its biologically relevant redox forms and derivatives (see fig.3 for structures), and established quinoproteins.

Before discussing the particular enzyme forms, it is necessary to comment on the overall spectroscopic properties. A remarkable feature of the absorption spectra is the structureless, broad band belonging to the non-protein chromophores, extending from 300 to 700 nm and having low molecular absorbances. ESR spectra suggest that the enzyme forms are mostly homogeneous with respect to the redox state of the Fe. Since the redox forms of PQQ as well as of Fe have well-defined absorption bands, it must be assumed that more than two chromophores exist in the preparation of the enzyme form, this phenomenon being related to the occurrence of either several redox forms of

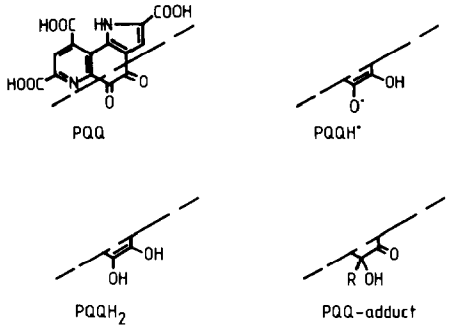

Fig.3. Redox forms and adducts of PQQ. The following adducts are mentioned in the text: R, CN, cyanide adduct; R, O-CH_2-CH_3, ethanol adduct; R, OH, hydrated PQQ.

PQQ or close interaction of the two cofactors so that new species are formed, leading to overlap of absorption bands.

4.3. *Native SLO*

This is an ESR-silent enzyme form showing the characteristic broad absorption band but with slightly visible shoulders at 330 and 400 nm [12] and a weak circular dichroism (CD) band around 330 nm [13]. From mechanistic and ESR spectroscopic features it is generally assumed that Fe is in an ESR-silent Fe^{2+} form [14,15]. Treatment with NO leads to the characteristic ESR spectrum of an Fe^{2+}-NO complex, the signal showing changes in the range pH 9–10 [16]. Also, addition of primary alcohols has a strong effect on the signal and these compounds have a remarkably high affinity for the enzyme, clearly not by direct binding to Fe^{2+}. It is known [17], however, that alcohols, cyanide, and H_2O easily form reversible adducts with PQQ at the C(5) position (fig.3). Moreover, the pyridine carboxylic acid moiety of PQQ is well suited for binding of an Fe ion. In fact, pyridine 2,4-dicarboxylic acid is an excellent Fe chelator and antagonist of the iron-protein, prolyl 4-hydroxylase (EC 1.14.11.2) [18]. An even better (terdentate) coordination is possible if the C(5) carbonyl group of PQQ is incorporated as ligand, in agreement with the strong binding of the Fe ion to SLO. The spectroscopic data can, therefore, be reconciled with a complex of Fe^{2+} and PQQ in the configuration given in fig.4 [additional evidence for this structure derives from model studies of PQQ and metal ions (Jongejan, J.A., unpublished) and from arguments provided in the discussion of the other

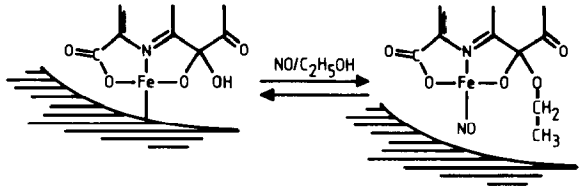

Fig.4. The complex of PQQ and the Fe ion in SLO and the mode of binding of NO and ethanol to the complex.

enzyme forms]. The OH group at the C(5) position of hydrated PQQ (having a pK_a value between 9 and 10, unpublished) could explain the pH effect on the ESR spectrum of the NO-treated enzyme. Indeed, this phenomenon is not observed if the OH group is replaced by adding a small amount of ethanol to the enzyme [16].

Although the complex is proposed to consist of Fe^{2+} and PQQ, in fact the redox state of the constituent species cannot be indicated and formally the system could also be described as a diamagnetic complex of Fe^{3+} and $PQQH^{\cdot}$. This explains why the characteristic absorption band of one of the PQQ redox species is not observed and the requirements of inhibition with PH, suggesting that PQQ is in a reduced state. It could also be possible that the preparation is not homogeneous with respect to either conformation (leading to differences in or absence of interactions of PQQ and Fe^{2+}) or the redox state of PQQ. In view of the weak CD band at 330 nm, a small amount of the fully reduced form, $PQQH_2$ (λ_{max} $PQQH_2$ is at 310 nm, leading to an absorption band in some quinoproteins at 335–340 nm [19]) could indeed be present. The same applies to the semiquinone form, $PQQH^{\cdot}$, since a weak ESR signal at $g = 2$ has frequently been reported (this has been ascribed to metal ion contamination, but in view of the fact that several quinoproteins contain $PQQH^{\cdot}$, the $g = 2$ signal of SLO should be carefully inspected in the future). The slight heterogeneity, as suggested here, is in accordance with the broad absorption band and the ESR data, indicating some variability in ligand configuration of the Fe ion.

4.4. *Yellow SLO*

This enzyme form is obtained by mixing native enzyme with a stoichiometric amount of product, e.g. the hydroperoxide of linoleic acid. This treat-

ment produces active enzyme and a general increase in absorbance in the 330–450 nm region, most significantly around 350 nm [12]. Two bands are induced in the CD spectrum, namely at 345 and 425 nm [13]. A maximum around 425 nm has been reported for several (ferric) iron-proteins and is in accordance with the observed $g = 6$ ESR signal [20], the intensity accounting for about 75% of the iron present in the protein (perhaps the remaining part still existing in the diamagnetic complex of the native form). The band at 345–350 nm could originate from (uncomplexed) PQQH$^{\cdot}$, since an identical band has been found for the semiquinone form of methanol dehydrogenase [21]. Another indication that some PQQH$^{\cdot}$ occurs in uncomplexed form, might be the weak $g = 2$ ESR signal in this enzyme form [22] (although this has been implicitly ascribed to decomposition products of the hydroperoxide, it is rather narrow in accordance with a top-top value of 0.6 mT for PQQH$^{\cdot}$ [23]).

Also, for this enzyme form, the effects of alcohol addition were investigated [20]. From the line broadening due to binding, as measured by ^{1}H-NMR spectroscopy, it was calculated that the protons at the C1 and C2 of ethanol are rather remote from the Fe^{3+} centre (at 5 and 6 Å, respectively). Cyanide also has strong effects on the ESR spectrum but experiments [20] with ^{13}CN showed that cyanide is definitely not bound to the Fe^{3+}. All these data support the idea that the main yellow enzyme species consists of a complex of Fe^{3+} and PQQ with a structure as depicted in fig.4.

4.5. *Purple enzyme form*

On treating native enzyme with an excess of hydroperoxide product [25], a new maximum is generated at 578 nm, concomitant with a signal at $g = 4.2$ in the ESR spectrum [since there is also an increase in absorbance in the 350–400 nm region and the amount of ESR-visible Fe increases to 90% (consisting of 80% $g = 6$ and 10% $g = 4.2$ signal), this is another indication that the yellow enzyme form is not completely in the Fe^{3+}/PQQ redox state]. A band at 580 nm and a double band at 390/410 nm are seen in the CD spectrum [13]. An identical double band has been observed in the absorption spectrum of fully oxidized methanol dehydrogenase and this has been ascribed to PQQ occurring as a C(5) adduct in the enzyme [21,27]. In view of the unusually high maximum and molar absorption coefficient of the 580 nm band, the Fe must be in a special environment, achieved by direct binding of the hydroperoxide to either the Fe^{3+} or PQQ at the C(5) position (the latter possibility could be responsible for the 390/410 nm double band). Since only 10% of the Fe exists in this state [25], this suggests that the major part of the purple enzyme form contains the Fe^{3+}/PQQ couple as described for the yellow enzyme form.

Although an adequate interpretation of the spectroscopic data is now possible from the proposed complex, this forms only the beginning of their assignment. Quantitative studies on the Fe/PQQ species in the enzyme and model studies should shed more light on the exact structure of the complexes and on heterogeneity.

4.6. *Mechanistic considerations*

Taking the proposed complex (fig.4) and the properties of the enzyme forms into account, the next question to arise is whether a mechanism can be derived with a role of PQQ in the catalytic cycle. Mechanistic studies strongly suggest that O_2 insertion is preceded by hydrogen abstraction, these reactions taking place at different sites of the substrate molecule in a regio- and stereo-specific way [1,2]. Hydrogen abstraction leads to a substrate radical from which the hydroperoxide product can be formed or to aberrant reactions (e.g. dimerization) if the O_2 concentration is too low. Current mechanisms [1,2] propose the involvement of Fe in hydrogen abstraction as well as dioxygenation, although it is difficult to see how this dualistic role can be performed and high selectivity be achieved with only one Fe ion in the enzyme molecule. This problem is solved, however, if it is assumed that the substrate remains bound to the enzyme molecule during the catalytic cycle and that both PQQ and Fe are involved in the overall process, so that the intermediate is shielded from unspecific O_2 attack and the stereo- and regio-specific reactions can proceed in the appropriate way. Since NO binds to the Fe ion and substrate competes for the same binding site, but O_2 does not [16], it is reasonable to assume that the oxygen insertion site of the substrate molecule is a ligand for Fe but that O_2 binding occurs via another Fe ligand. With this in mind, it is easy to see that the other interaction of the substrate could occur with the C(5) carbonyl

group of PQQ, a site where hydrogen abstraction from the substrate is possible. The resulting electron-relay system (fig.5A) allows abstraction of hydrogen from the substrate with concomitant conversion of Fe^{3+} into Fe^{2+}, the latter being well suited to perform O_2 insertion (see fig.5B for a tentative scheme of the steps).

An essential feature of lipoxygenation is migration of the unpaired electron through the substrate molecule to the carbon atom where O_2 addition occurs. Using mechanism-based inhibitors with extra double bonds, upstream of and in conjugation with the double bond where O_2 insertion takes place, after hydrogen abstraction the unpaired electron in the substrate molecule has other possibilities to reside. Thus, the dioxygenation step becomes more difficult but the probability of wrong O_2 insertions and reaction of the unpaired electron with residues of the enzyme increase. This is indeed what has been found [3], O_2 insertion occurring at high oxygen tensions (but mainly at the wrong sites) and irreversible inactivation at low tensions. Unfortunately, product analysis in inhibited enzyme has been scarcely performed. Using an acetylenic fatty acid analogue, it was found that

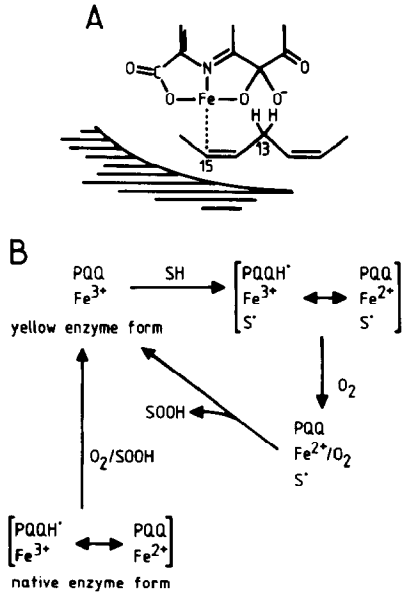

Fig.5. Hydroperoxidation mechanism of SLO with arachidonic acid. The enzyme-substrate complex is depicted in (A) and a tentative catalytic cycle in (B).

inhibition was related to conversion of a methionine residue into methionine sulfoxide [26]. However, the fact that arachidonic acid conversion under anaerobic conditions also leads to irreversible inactivation suggests that free radical derangement might also occur in the substrate moiety interacting with the Fe^{3+}/PQQ system so that other modes of inactivation could exist. In this context, it is interesting to mention the inactivation of methanol dehydrogenase by cyclopropanol where the free radical, due to ring opening, adds to PQQ [27]. Since a similar reaction might occur under anaerobic conditions with arachidonic acid as a substrate, product analysis might be worthwhile to substantiate further the involvement of PQQ and the mechanism proposed.

Although the Fe^{3+}/PQQ system is in principle also suited for concerted proton abstraction (with the deprotonated OH group at the C(5) position of PQQ) and electrophilic attack of Fe^{3+} on the substrate, as required in the organo-iron mechanism [3] (fig.1B), the free radical mechanism seems more attractive, since it provides the best explanations for all features presently known. To substantiate it further, in view of the participation of $PQQH^{\cdot}$ and substrate radical, extensive ESR studies of SLO in the $g = 2$ region should be performed.

4.7. *Further implications*

The finding that SLO is a quinoprotein strongly supports the notion that the cofactor PQQ is versatile and ubiquitous as it occurs in dehydrogenases, oxidases, hydroxylases and dioxygenases and has meanwhile been detected in enzymes present in species from microbes to man. In view of their properties, lipoxygenases of mammalian organisms, being involved in the route of biosynthesis of leukotrienes and prostaglandins, might also be quinoproteins. Curiously, the already established mammalian copper-quinoproteins are also involved in biosynthesis or degradation of bioregulators (histamine, polyamines, dopamine). The discovery of PQQ in these enzymes might, therefore, have an important impact in the field of pharmacology. A further consequence of the finding is that other well-known enzymes for which a cofactor has already been indicated might eventually appear to be quinoproteins as well. Since PQQ is a versatile chelator (at least 3 sites

available) and several precedents exist now already, metallo-enzymes should be considered in particular.

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