Oxidation of thioanisole and *p*-methoxythioanisole by lignin peroxidase: kinetic evidence of a direct reaction between compound II and a radical cation

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The reaction of H₂O₂ with thioanisole and *p*-methoxythioanisole catalysed by lignin peroxidase from *Phanerochaete chrysosporium* has been studied spectrophotometrically under turnover and single turnover conditions with a stopped-flow apparatus. Pre-formed lignin peroxidase compounds I and II are each able to react with the sulphides to form a sulphide radical cation. The radical cation is then converted into the sulfoxide either by reaction with the medium or by reaction with compound II. This is the first report of a direct reaction between compound II and the substrate radical cation. With thioanisole, significant enantiomeric selectivity and high oxygen incorporation in the sulfoxide are obtained because compound II is preferentially reduced by the enzyme-bound thioanisole radical cation compared with the neutral substrate. By

contrast, with *p*-methoxythioanisole, the data imply formation of an intermediate ternary complex comprising compound II, radical cation and neutral substrate, such that a chain of electron transfer reactions starting from neutral molecule and progressing to oxidized haem via substrate radical cation is facilitated, yielding the native enzyme and two molecules of *p*-methoxythioanisole radical cation as products. The reactions of compounds I and II with sulphides imply flexing of the apoprotein moiety during catalysis.

Key words: electron transfer, lignin peroxidase, oxene process, oxygen rebound, peroxidase compound II, sulphide radical cation.

INTRODUCTION

LiP (lignin peroxidase), a haem (protoporphyrin IX)-containing enzyme secreted by the white-rot fungus *Phanerochaete chrysosporium*, oxidizes polymeric lignin and non-phenolic electron-rich aromatic compounds with redox potentials up to 1.4 V versus NHE, using H₂O₂ as the primary oxidant [1–6]. Initial two-electron oxidation of native (ferric) LiP by H₂O₂ yields compound I (LiP I), which is an iron (IV)-oxo porphyrin radical cation [P⁺•Fe(IV)=O]. Two subsequent one-electron reductions by electron donor substrates re-form the native (ferric) state via the intermediate compound II (LiP II) [PFe(IV)=O], which retains an oxyferryl haem [7–11].

Single-electron oxidation of methoxylated aromatic compounds yields radical cations and these have been identified as the products of LiP catalysis [12,13]. The X-ray crystal structure of LiP shows that the haem access is more restricted than in other peroxidases [14–17]; consequently it has been assumed that the LiP active site cannot directly interact with its substrates and that LiP oxidations proceed via a long-range ET (electron transfer) from substrate to the haem and that the intermediate substrate radical cations then undergo non-enzymatic reactions to yield the final products [13,18].

However, from recent research with alkyl aryl sulphides, *N,N*-dimethylanilines and benzyltrialkylsilanes, we found evidence suggesting that the pathway of degradation of the intermediate radical cation may be influenced by the apoprotein environment of one of the catalytic intermediates of LiP, namely PFe(IV)=O [19–21]. Furthermore, in the sulfoxidation of sulphides by LiP, we found that with increasing sulphide redox potential, the oxygen

incorporation from H₂O₂ in the sulfoxide increased together with the enantiomeric excess of S over R sulfoxide isomers. This led us to propose the mechanism reported in Scheme 1. In the first step, one electron is transferred from the sulphide (ArSR) to compound I [P⁺•Fe(IV)=O] (path *a*) to form a sulphide radical cation and compound II, PFe(IV)=O. The sulfoxide would then be formed either by transfer of the oxygen atom from PFe(IV)=O to the radical cation, the so-called 'oxygen rebound' step (path *b*), or by reaction with the medium (path *c*).

This mechanism is similar to that proposed for sulfoxidation by horseradish peroxidase and lactoperoxidase [22–27], but dissimilar to that proposed for chloroperoxidase, wherein an oxygen atom is transferred directly from compound I to the sulphide (oxene process) [28].

We now report on a detailed stopped-flow kinetic study of the LiP-catalysed oxidation of thioanisole and *p*-methoxythioanisole under single turnover and turnover conditions in order to shed further light on the role of LiP I and LiP II in such reactions, with the aim of obtaining kinetic evidence for the mechanism reported in Scheme 1.

EXPERIMENTAL

Materials

All reagents were purchased from Sigma Chemical Co., apart from analytical grade acetic acid, ethanol, potassium mono-/dihydrogen orthophosphate and H₂O₂, which were from BDH. Q-Sepharose, Sephacryl-HR S-100, Ampholine PAG platesTM (pH = 3.5–9.5) and broad-range isoelectric focusing calibration

Abbreviations used: ET, electron transfer; LiP, lignin peroxidase; P⁺•Fe(IV)=O, iron (IV)-oxo porphyrin radical cation; LiP I, LiP compound I; LiP II, LiP compound II; SVD, singular value decomposition; VA, veratryl alcohol.

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Scheme 1 ET-oxygen rebound process

ArSR, sulphide.

kits (pH = 3–10) were purchased from Amersham Biosciences. The concentration of H_2O_2 in stock solutions was measured using a molar absorption coefficient (ϵ) of $39.4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [29].

Methods

Production and purification of LiP

LiP (EC 1.11.1.7) was produced in 2-litre flasks (with 600 ml of culture medium) using *Phanerochaete chrysosporium* ME-446 (A.T.C.C. 34541) according to Zacchi et al. [3]. Enzyme purification was achieved after initial concentration of the culture medium in an Amicon hollow-fibre concentrator (10 kDa filter), followed by 66% (v/v) cold acetone (-20°C) precipitation, ion exchange (Q-Sepharose) and size-exclusion chromatography (Sephacryl S-100) [10]. The resulting sample ($R_z = 4.8$) showed a single band on SDS/PAGE and isoelectric focusing/PAGE, corresponding to a protein with a molecular mass of 40 kDa and a pI of 4.59. Molar concentrations of native LiP were assessed at 408 nm ($\epsilon_{408} = 133 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) [7]. LiP activity was determined by the VA (veratryl alcohol) assay (1 mM VA, 0.4 mM H_2O_2 , in 50 mM phosphate buffer, pH 2.75, 20°C); the reaction was monitored at 310 nm ($\epsilon_{310} = 9.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) according to [1]. LiP samples were stored at -20°C .

Transient state stopped-flow spectroscopy

Transient spectroscopy was carried out at 20°C using a four syringe SX-18MV (Applied Photophysics, Leatherhead, U.K.) or a SF61-DX2 (Hi-Tech, Salisbury, U.K.) stopped-flow set-up equipped for both conventional and sequential stopped-flow measurements. The stopped-flow set-up was attached to a diode array linked to a computer. Multi-wavelength kinetic data were analysed by SVD (singular value decomposition) and global fitting procedures [30] using the commercial software packages Pro-K.2000 Global analysisTM (Applied Photophysics) and Specfit/32 (Spectrum Associates, Marlborough, MA, U.S.A.). Reacting peroxidase species were identified using wavelength-dependent spectral profiles and time-dependent concentration profiles generated by the software packages after SVD analysis and kinetic fitting of the data set. The advantage of SVD data analysis preceding kinetic analysis is a significant reduction in experimental noise compared with direct analysis of the raw spectro-kinetic data [30].

The fidelity of each fit was assessed from analysis of the total sum of squares and standard deviation of the fit. The Marquart factor was zero for all fitting procedures, and fitting convergence was typically reached after 3–6 iterations using a consecutive first-order kinetic model. Additional indication for the reliability of the global fitting procedure was visual examination of the three-dimensional residual matrix (plot of absorbance versus wavelength versus time residuals), which showed no retention of significant spectral or kinetic data after the fitting procedure [30].

Ageing times for the transient species LiP I and II were determined by conventional dual syringe stopped-flow, where syringe one contained LiP stock solution ($350 \mu\text{l}$) and syringe two was filled with H_2O_2 ($350 \mu\text{l}$). After mixing, the formation and decay of peroxidase transient species was followed by diode array detection. Exact ageing times for peroxidase intermediates were determined after kinetic fitting of the collected traces and calculation of time-dependent concentration profiles. All reactions were carried out in 50 mM phosphate buffer at pH 4.6 (20°C), which increased the stability of LiP I and II. LiP I [$\text{P}^{+}\text{Fe(IV)=O}$; $\lambda_{\text{max}} = 408 \text{ nm}$; $\epsilon_{408} = 73 \text{ mM}^{-1} \cdot \text{cm}^{-1}$], characterized by its decreased absorbance at 408 nm compared with the native (ferric) state [8], was fully formed after 340 ms by mixing equimolar concentrations of native LiP ($1.5 \mu\text{M}$) and H_2O_2 . From the analysis of the time-dependent concentration profiles, LiP I lifetime was 7.51 s. Mixing of $1.5 \mu\text{M}$ native LiP with $3 \mu\text{M}$ H_2O_2 formed LiP II [PFe(IV)=O ; $\lambda_{\text{max}} = 420 \text{ nm}$; $\epsilon_{420} = 81 \text{ mM}^{-1} \cdot \text{cm}^{-1}$] after 3 s [8,9]. The lifetime of LiP II in the absence of substrate was $>560 \text{ s}$ under these conditions. Reactions of LiP catalytic intermediates were studied by sequential stopped-flow mixing. LiP I or II was pre-formed in an ageing loop, and then mixed with thioanisole or *p*-methoxythioanisole ($220 \mu\text{l}$, syringe 4) in the reaction chamber. Any peroxidase intermediates that formed were detected by diode array spectroscopy and identified after kinetic fitting of the acquired traces and calculation of wavelength-dependent absorbance profiles.

Determination of catalytic constants

Reactions of LiP I and II ($0.75 \mu\text{M}$) with thioanisole or *p*-methoxythioanisole (0 – $650 \mu\text{M}$) were performed by sequential stopped-flow spectrometry using diode array detection (dead time: 1.25 ms). All reactions were carried out in 50 mM phosphate buffer (pH 4.6, 20°C) containing 1% (v/v) CH_3CN as the co-solvent. Reduction of LiP I and II with the substrate was monitored between 350–750 nm. Each measurement contained at least 1000 spectra. The pseudo-first-order rate constants (k_{obs}) were calculated as the mean of 5–8 repeat rate measurements by (global) fitting of the whole spectro-kinetic data set using the Specfit/32 software package [30]. Second-order rate constants for each reaction were calculated from the slope of the plots of the pseudo first-order rate constants (k_{obs}) against substrate concentrations. In control experiments, the reaction of LiP I either in the absence of an electron donor or with the one-electron reductant VA [8] was examined. Both reactions yielded a single species that accumulated at 420 nm, with a single isosbestic point at 411 nm. These absorbance features are diagnostic for formation of LiP II [8,9].

RESULTS AND DISCUSSION

Single-step turnover kinetics of pre-formed LiP I reduction by thioanisole or *p*-methoxythioanisole

The reaction between pre-formed LiP I ($0.75 \mu\text{M}$) and one molar equivalent of thioanisole (ArSMe) was examined over the wavelength range 350–750 nm and on a time-scale of 100 s. Initially (0–4.7 s), a single species accumulated with an absorbance maximum at 420 nm. This reaction was characterized by a single isosbestic point at 411 nm (Figure 1A, traces a–c). These absorbance features are diagnostic for formation of LiP II [8,9] and indicate that LiP I was reduced to LiP II by a single electron transfer from thioanisole, with the quantitative formation of the thioanisole radical cation ($k_{\text{obs}} = 1.23 \pm 0.05 \text{ s}^{-1}$). In Figure 1(B) the time-dependent profiles for the formation of LiP II in the

Figure 1 Single turnover reactions of pre-formed LiP compound I with thioanisoles

(A) Single-turnover reduction of LiP I with thioanisole. Rapid scan spectrophotometric measurements of the reaction of LiP I ($0.75 \mu\text{M}$) with thioanisole ($0.75 \mu\text{M}$) in 50 mM phosphate buffer containing 1% (v/v) CH_3CN (pH 4.6, 20°C). Traces a–g were recorded at 0.002, 0.85, 1.32, 4.65, 30.7, 41.63, 54.32 s after thioanisole addition. Isosbestic points ($\uparrow$): 411, 418 nm. (B) Calculated time-dependent concentration profile of LiP II formation in the absence (solid trace, autocatalytic decay) and presence of thioanisole (dashed trace) or *p*-methoxythioanisole (dashed dotted trace).

absence (solid trace) and the presence of an electron donor substrate (thioanisole, dashed trace; *p*-methoxythioanisole, dashed dotted line) are compared. The rate of transformation of LiP I was accelerated by the presence of an electron donor compared with its absence and was faster for *p*-methoxythioanisole [$E^0(\text{S}^{+\bullet}/\text{S}) = 1.36 \text{ V}$ versus NHE in H_2O] [31] compared with that of the parent compound thioanisole [$E^0(\text{S}^{+\bullet}/\text{S}) = 1.49 \text{ V}$ versus NHE in H_2O] [31]. These data accord with an ET process for the reduction of LiP I to LiP II by thioanisoles.

However, analysis of the reaction with thioanisole on a longer timescale (4.7–53 s) revealed evidence for a second reaction, in which the Soret maximum shifted from 420 nm back to 408 nm and increased in intensity, and a single isosbestic point at 418 nm was identified. These features are characteristic for reduction of LiP II to the native state (Figure 1A, traces c–e). The reduction of LiP II showed first-order kinetics, suggesting that

the decay of LiP II took place within a LiP II/ $\text{ArS}^{+\bullet}\text{Me}$ complex. The first-order rate constant (k_{obs}) for the reduction of LiP II to the native state was $(2.4 \pm 0.3) \times 10^{-2} \text{ s}^{-1}$ under these conditions, approx. 20 times faster than the autocatalytic decay of LiP II in the absence of substrate [$k_{\text{obs}} = (1.2 \pm 0.6) \times 10^{-3} \text{ s}^{-1}$]. Similar results were obtained with *p*-methoxythioanisole, except that the rate of the reaction of LiP II with the *p*-methoxythioanisole radical cation was $(6 \pm 1) \times 10^{-2} \text{ s}^{-1}$, approx. 2.5 times faster compared with the corresponding reaction with the thioanisole radical cation.

Thus these single-step turnover experiments not only indicate an ET mechanism for the reaction between LiP I and the sulphides, but also they provide for the first time kinetic proof that LiP II is able to react with the product of the reduction of LiP I, a sulphide radical cation, very probably by the donation of an oxygen atom. They are in line with our previous hypothesis [19], regarding both the formation of an intermediate radical cation in the LiP-catalysed oxidation of sulphides by an ET mechanism and the capability of LiP II to donate an oxygen atom to the radical cation (oxygen rebound mechanism). The latter conclusion implies that the radical cation should be able to come quite close to the iron-oxo complex, which, as already noted [19], is significant in view of the fact that the X-ray analysis of the crystal structure of LiP [14–17] indicates that in this enzyme the haem is more recessed than in other similar peroxidases. Hitherto, a direct interaction between a substrate and LiP active site has been considered unlikely.

A probable explanation is that in solution the protein is flexible, as recently demonstrated from molecular dynamics simulations [32] in which large fluctuations of the residues defining the access channel to the haem have been observed. These movements relax the crystallographic side chain conformations, thus opening the rim of the access channel and facilitating the access of small substrates toward the active site.

A surprising result is, however, the faster reduction of LiP II by *p*-methoxythioanisole radical cation compared with the thioanisole radical cation; the reverse would have been expected [19], in view of the higher stability of the former radical cation. A greater reactivity of LiP II with thioanisole compared with *p*-methoxythioanisole might have been anticipated by the larger enantioselectivity and O-incorporation in the sulfoxide from the iron-oxo complex observed for the former substrate. A possible explanation is that LiP II behaves as an electrophilic species and its reactivity can be more subject to polar than enthalpic effects [33]. Thus LiP II might react faster with *p*-methoxythioanisole radical cation than with thioanisole radical cation, because in the former, the positive charge is partially delocalized on the *p*-methoxy group.

Turnover kinetics of pre-formed LiP I reduction by thioanisole or *p*-methoxythioanisole

Under turnover conditions, reduction of pre-formed LiP I by each of the two substrates led to the formation of LiP II with a single exponential behaviour. The data were fitted to a sequential first-order mechanism ($\text{LiP I} \rightarrow \text{LiP II}$) to obtain the pseudo first-order rate constants (k_{obs}) for the reaction of LiP I with the substrates. The plots of k_{obs} for the reduction of LiP I against either thioanisole or *p*-methoxythioanisole concentrations showed evidence of saturation kinetics, indicating that a LiP I/ ArSMe complex is formed en route to the oxidation products.

Kinetic constants for the reduction of LiP I by thioanisole or *p*-methoxythioanisole are listed in Table 1. Comparison of the second-order rate constants showed that the reaction with *p*-methoxythioanisole ($k_2/K_D = 9.9 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) is about 5.4 times faster than with thioanisole ($k_2/K_D = 1.8 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$). This

Table 1 Kinetic parameters for pre-formed LiP I reduction by thioanisole and *p*-methoxythioanisole under turnover conditions

	ArSMe	<i>p</i> -Methoxythioanisole
R ²	0.991	0.990
Standard error of fit	0.24	0.17
K _D (M)	$(139 \pm 8) \times 10^{-6}$	$(80 \pm 3) \times 10^{-6}$
k ₂ (s ⁻¹)	25.6 ± 0.6	79.3 ± 0.7
k ₂ /K _D (M ⁻¹ · s ⁻¹)	$(1.8 \pm 0.2) \times 10^5$	$(9.9 \pm 0.5) \times 10^5$

Figure 2 Pre-formed LiP II reduction by thioanisole

Rapid scan spectrophotometric measurements of the reaction of LiP II (0.75 μM) with thioanisole (50 μM) in 50 mM phosphate buffer containing 1% (v/v) CH₃CN (pH 4.6, 20 °C). Traces a–e were recorded at 0.010, 4.2, 36, 48.4 and 112 s. Isosbestic point (↑): 418 nm.

trend is also consistent with an ET mechanism: the faster reaction occurs with the substrate (*p*-methoxythioanisole) with the lower redox potential [31]. In the same experiments the decay of LiP II formed from LiP I was followed. In the case of thioanisole, reduction of LiP II showed only marginal variations ($k_{\text{obs}} = 0.024\text{--}0.035\text{ s}^{-1}$) with increasing thioanisole concentration (0.75–500 μM), which indicates that LiP II in the presence of both the neutral substrate and its radical cation is preferentially reduced by the enzyme-bound thioanisole radical cation. This finding rationalizes the significant enantioselectivity and the high O-incorporation in the sulfoxide from the iron–oxo complex that we observed in the LiP-promoted oxidation of thioanisole [19].

In contrast, the reduction of LiP II in the presence of both *p*-methoxythioanisole and its radical cation showed a dependence of the rate on the concentration of the sulphide and evidence of saturation kinetics. From a non-linear least-squares fit of the data, $k_2 = 5.1 \pm 0.1\text{ s}^{-1}$, $K_D = (108 \pm 6) \times 10^{-6}\text{ M}$, and $k_2/K_D = (4.7 \pm 0.3) \times 10^4\text{ M}^{-1} \cdot \text{s}^{-1}$. This leads us to suggest that, in this case, the LiP II/ArS⁺•Me complex reacts with the neutral sulphide molecule to form a ternary complex, a situation very similar to that envisaged for the LiP-catalysed oxidation of VA [10,34], where a ternary complex LiP II/VA⁺•/VA formed by reaction of the complex LiP II/VA⁺• with VA was suggested. Inside the complex the neutral sulphide molecule may be oxidized to a radical cation, leading to the formation of the native enzyme and the release of two molecules of sulphide radical cation, which then react with the medium to produce the sulfoxide. Such a route might be in competition with the oxygen rebound path in the LiP II/ArS⁺•Me complex and so explain the

Figure 3 Kinetics of the reduction of pre-formed LiP II by aromatic sulphides

(A) Plot of the pseudo-first-order rate constants (k_{obs}) for the reduction of LiP II (0.75 μM) against thioanisole (+, solid line) concentrations (0–650 μM). (B) Plot of the pseudo-first-order rate constants (k_{obs}) for the reduction of LiP II (0.75 μM) against *p*-methoxythioanisole (▲, dashed line) concentrations (0–650 μM) in 50 mM phosphate buffer containing 1% (v/v) CH₃CN (pH 4.6, 20 °C). The plot of k_{obs} versus [sulphide] represents a non-linear least-squares fit of the data using eqn (1). Each value of k_{obs} is the mean of at least eight measurements.

relatively low O-incorporation in the sulfoxide from the iron–oxo complex observed for the LiP-promoted sulfoxidation of *p*-methoxythioanisole [19].

The different behaviour of thioanisole and *p*-methoxythioanisole with respect to the fate of the LiP II/ArS⁺•Me complex (the reaction with the neutral sulphide occurring only in the second case) warrants further investigation.

Kinetics of pre-formed LiP II reduction by thioanisole or *p*-methoxythioanisole

To investigate further the mechanism of LiP II reduction, pre-formed LiP II was reacted with either thioanisole or *p*-methoxythioanisole. Under these conditions LiP II can only react with the neutral sulphide molecule.

Figure 2 shows the reduction of LiP II to the native (ferric) state by thioanisole. The kinetic profiles for the reduction of pre-formed compound II by either thioanisole or *p*-methoxythioanisole

Scheme 2 Reaction of LiP II with thioanisolesArSMe, thioanisole, *p*-methoxythioanisole.**Table 2** Kinetic parameters for pre-formed LiP II reduction by thioanisole and *p*-methoxythioanisole under turnover conditions

	ArSMe	<i>p</i> -Methoxythioanisole
R ²	0.996	0.998
Standard error of fit	0.002	0.031
K _D (M)	(8 ± 1) × 10 ⁻⁶	(2.8 ± 0.2) × 10 ⁻⁶
k ₁ (s ⁻¹)	0.052 ± 0.003	0.13 ± 0.03
k ₁ /K _D (M ⁻¹ · s ⁻¹)	(6.5 ± 1.2) × 10 ³	(46 ± 14) × 10 ³
k ₂ (M ⁻¹ · s ⁻¹)	62.0 ± 0.6	2342 ± 1

showed single exponential behaviour. Therefore, pseudo-first-order rate constants were determined by fitting the data to a sequential first-order model (LiP II → native LiP). The pseudo first-order rate constant (k_{obs}) for the reduction of pre-formed LiP II depends on sulphide concentration (Figures 3A and 3B). The plots of k_{obs} for the reduction of LiP II versus thioanisole (Figure 3A) or *p*-methoxythioanisole (Figure 3B) concentrations show non-linear behaviour, with a concave curvature toward the abscissa, which failed to approach a maximum at high substrate concentrations. The kinetic behaviour of LiP II with sulphides can formally be described by an extended Michaelis–Menten scheme (Scheme 2), involving the initial formation of an enzyme substrate complex between LiP II and the sulphide, which can

oxidize a second sulphide molecule. Similar kinetic behaviour has previously been reported for the reaction of horseradish peroxidase compound II with *p*-cresol [35].

Table 2 lists the kinetic constants obtained by non-linear least-squares fitting of the kinetic data to eqn (1) [35]:

$$k_{\text{obs}} = k_1[\text{S}] + k_2[\text{S}]^2 / (K_D + [\text{S}]) \quad (1)$$

Comparison of the productive second-order rate constants (Table 2) revealed that LiP II reduction by *p*-methoxythioanisole ($k_1/K_D = 46 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) is approx. 7.1-fold faster than the corresponding reaction with thioanisole ($k_1/K_D = 6.5 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$). The trend in the second-order rate constants is similar to that previously observed for the reduction of LiP I by these substrates and suggests that the reaction between LiP II and a neutral sulphide leads to a sulphide radical cation. Presumably, the latter should be converted into a sulfoxide by reaction with the medium.

Conclusion

On the basis of the results reported in the present study, the mechanism illustrated in Scheme 3 can be proposed for the LiP-catalysed oxidation of thioanisole and *p*-methoxythioanisole.

LiP I (formed by oxidation of the native enzyme with H₂O₂, path *a*) reacts with the aromatic sulphide ArSMe, forming first a complex between the enzyme and the sulphide (path *b*), which is then converted, by an ET step, into a LiP II/ArS⁺⁺Me complex (path *c*). This complex decays to form the native enzyme and the sulfoxide (path *d*).

However, the decay of the LiP II/ArS⁺⁺Me complex was found to depend on the concentration of the neutral sulphide in the case of *p*-methoxythioanisole, but not for thioanisole. These observations suggest that in the second case the LiP II/ArS⁺⁺Me complex is directly converted into the native enzyme and the sulfoxide (oxygen rebound, path *d*), whereas with *p*-methoxythioanisole at least a part of the LiP II/ArS⁺⁺Me complex may react with the neutral *p*-methoxythioanisole to form a ternary complex (path *e*), which presumably decays producing native LiP and two molecules of ArS⁺⁺Me (path *f*).

Scheme 3 LiP-catalysed oxidation of thioanisolesArSMe, thioanisole, *p*-methoxythioanisole.

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