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Free-radical-mediated fragmentation of monoamine oxidase in the mitochondrial membrane

Roles for lipid radicals

Roger T. DEAN,* Sian M. THOMAS* and Anthony GARNER†
*Cell Biology Research Group and †Department of Biochemistry, Brunel University, Uxbridge, Middlesex UB8 3PH, U.K.

A flux of hydroxyl radicals generated by γ-irradiation can fragment monoamine oxidase in the membrane of submitochondrial particles. This fragmentation can be inhibited by mannitol and in addition is more extensive in monoamine oxidase preparations that have been depleted of lipid. This latter observation is consistent with the higher yields of fragmentation induced by hydroxyl radicals in soluble proteins in the absence of added lipids. In the absence of oxygen, γ-irradiation of submitochondrial particles leads to cross-linking reactions. A flux of hydroperoxyl radicals also causes fragmentation, whereas one of superoxide is virtually inactive in this respect. The irradiation of submitochondrial particles leads in addition to the accumulation of products of lipid peroxidation. When these irradiated preparations are exposed to ferrous or cupric salts a further fragmentation of monoamine oxidase ensues, especially at acid pH. These transition-metal-catalysed reactions do not occur with irradiated preparations depleted of lipid, and the post-irradiation protein modifications are concomitant with further lipid peroxidation. The data indicate roles for lipid radicals in both fragmentation and cross-linking reactions of proteins in biological membranes. These reactions may have an important bearing on control of protein activity and of protein turnover in membranes.

INTRODUCTION

It is known that oxygen-centred free radicals can readily fragment proteins in solution and even in complex biological structures such as cartilage [1–6]. Amino acid modification and cross-linking reactions (the latter particularly in the absence of oxygen [3,5]) also occur. The products of these reactions are usually more susceptible to enzymic hydrolysis than the native molecule [6]. Consequently, free radical damage to proteins occurring within cells may positively influence the basal rate of protein breakdown therein. We have recently shown [7] for proteins synthesized in isolated mitochondria that this prediction is upheld: degradation is fastest when radical fluxes are greatest, in the case of mitochondria in State 4.

The majority of the endogenously synthesized mitochondrial polypeptides are membrane-bound [8] so the influence of radicals on protein breakdown may apply not only to proteins in aqueous environments but perhaps also within biological membranes. In the present study, we have therefore followed the reaction between free radicals and an intrinsic outer membrane protein of the mitochondria, monoamine oxidase. We have considered whether oxygen-centred radical attack may directly fragment the monoamine oxidase *in situ*. In biological membranes lipid peroxidation is frequently a consequence of radical attack [9] and so we have considered the possibility that radicals produced in lipid molecules may also interact with monoamine oxidase to cause degradation. Our results provide evidence both for direct oxygen-centred radical fragmentation of monoamine oxidase and for a role of some lipid-derived radicals in fragmentation of membrane-bound proteins. Such reactions are likely to influence the overall rate of protein turnover in membranes (consistent with our earlier evidence [7]), and may also relate to regulation of protein activity.

MATERIALS AND METHODS

Materials

Chelex-treated water and glow-cleaned glass were used for all experiments. [^{3}H]Pargyline was from New England Nuclear, and non-radioactive pargyline from Sigma. Desferrioxamine was a kind gift from Ciba–Geigy, and Trolox was from Aldrich. Other chemicals were the purest commercial grade.

Preparation of labelled monoamine oxidase within mitochondrial membranes

Rat liver mitochondria were isolated as described previously [7]; on each occasion, two rats were used. Monoamine oxidase was then labelled *in situ* using the active-site-directed, covalent-binding inhibitor pargyline. This provides a convenient tracer for the fate of molecules of the enzyme [7,10]. SDS/polyacrylamide-gel electrophoresis in reducing conditions showed that polypeptides of approx. 60 kDa were labelled, providing that pargyline was used at low concentrations (1 μM). This corresponds well to the reported polypeptides. Mitochondria were sonicated, to give submitochondrial particles, before use in experiments and were always washed (by sedimentation in an Eppendorf Microfuge for 5 min) immediately before use. Lipid-depleted mitochondria were made from portions of preparations of pargyline-labelled intact mitochondria, by extraction with aqueous methyl ethyl ketone by a modification of the procedure of Erkstedt & Oreland [11]. After

extraction, the residue was resuspended in 0.1 M-potassium phosphate buffer containing 1 mM-EDTA. The membranes were sedimented at 27000 *g* for 10 min, and then washed three times with distilled water, using the same sedimentation conditions. More than 80% of the lipid phosphorus was removed by this procedure.

All the preparations were stored at −20 °C for up to 4 weeks, in small portions so that repeated freeze–thawing was not needed.

Radical-generating systems

Oxygen-centred radicals were generated as in our previous work using steady state radiolysis at 50 Gy/min with a ^{60}Co source [4]. This permitted the selective generation of hydroxyl, superoxide and hydroperoxyl radicals, by the addition of N_2O/O_2 (4:1), 10 mM-formate (pH 7.2) gassed with air and 10 mM-formate (pH 4) gassed with air, respectively, and the investigation of their effects on the labelled enzyme *in situ*. Experiments were normally done in 10 mM-potassium phosphate buffer, pH 7.2; the only protein present was that of the mitochondria (1.0–2.5 mg/ml). Oxygen was not supplied for some irradiations.

Measurement of degradation of monoamine oxidase

Initially, we measured trichloroacetic acid-soluble fragments produced from the radioactively labelled enzyme. Carrier bovine serum albumin (0.5%, w/v) was added and mitochondria were then precipitated with a final concentration of trichloroacetic acid of 5% (w/v). The precipitate was sedimented on a Microfuge and the supernatant sampled for scintillation counting. The pellet was redissolved in formic acid and counted separately. Degradation is calculated as acid-soluble radioactivity as a percentage of the total in the system. This method only detects small fragments of monoamine oxidase. Larger fragments were detected by SDS/polyacrylamide gel electrophoresis (9% polyacrylamide) under reducing conditions [12]. Gels were silver stained or sectioned for radioactivity counting after dissolution in 100 vol. H_2O_2 (1 ml/gel slice) at 60 °C overnight.

For technical reasons (see results) it was later necessary to avoid the acid pH of the trichloroacetic acid method; instead, $ZnSO_4$ was added (2.5 g/100 ml final concentration), followed by 100 μl of saturated barium hydroxide/ml. The resulting suspension was centrifuged as above. The total radioactivity in each incubation was measured by counting an unprecipitated sample.

Two-phase degradation experiments (phase 1: oxygen-centred radical attack; phase 2: transition metal attack on the products)

Mitochondrial membranes containing labelled monoamine oxidase were exposed to 1000 Gy of irradiation in the system generating hydroxyl radicals in the presence of oxygen. Then they were transferred to a 37 °C incubator in 10 mM-potassium phosphate buffer, pH 7.2, in the presence of various additives such as ferrous or cupric ions. For addition to these experiments ferrous sulphate was made up as a fresh 10 mM stock in acetate buffer, pH 5.6 (100 mM). After incubation for chosen times at 37 °C in the presence of the transition metals, monoamine oxidase degradation was measured.

Characterization of products of radical attack on labelled monoamine oxidase

It was necessary to establish that the radioactive tracer used to label monoamine oxidase, pargyline, remained associated with peptides during radical attack. Using the trichloroacetic acid-soluble degradation products, two methods were applied. Firstly, gel filtration on Bio-Gel P-2; secondly, h.p.l.c. on Hypersil ODS (5 μm) with reverse-phase elution with a gradient of acetonitrile in 7 mM-ammonium acetate, pH 3.8. The column effluents were monitored at 257 nm (the absorption maximum of pargyline) and samples of fractions were also taken for scintillation counting.

Measurement of lipid peroxidation in mitochondria during radical attack

In various relevant conditions lipid peroxidation was measured as thiobarbituric-acid-reactive material [14].

Data presentation

Experiments are representative of several. They were performed routinely with duplicate samples and means are shown, except where otherwise indicated. Such duplicates differed from each other by less than 3%. In some other experiments greater statistical variation was obtained, and larger numbers of replicates were used: for these means are shown and degree of replication stated. In addition S.D. and/or statements of statistical significance are given. Although the results shown are all qualitatively reproducible, there were significant inter-experimental variations, and the data are selected to reveal the maximum extent of these variations: these mainly seem to originate in differences between successive mitochondrial preparations from pairs of animals. There are also differences due to the different mitochondrial preparations used (normal, lipid-depleted, and the control preparations obtained in parallel with the lipid depletion).

RESULTS

Direct radical attack on monoamine oxidase

As shown in Table 1, the hydroxyl radical was able to fragment monoamine oxidase only to a remarkably limited degree in the dose range studied. The superoxide radical was essentially ineffective, while an intermediate activity was observed with the hydroperoxyl radical generating system although this could be a result of conformational changes induced by acid pH. In the absence of oxygen, no fragmentation was detectable. The attack by hydroxyl and hydroperoxyl radicals could be inhibited by appropriate radical scavengers, such as mannitol in the case of the hydroxyl radical.

In Table 2, a comparison of the fragmentation of monoamine oxidase in normal and lipid-depleted submitochondrial preparations is shown. The hydroxyl radical could cleave the enzyme in the lipid-depleted membrane more readily than that in the replete membrane. The hydroperoxyl radical could cleave the enzyme similarly in both preparations, though it was much less effective than the hydroxyl radical against the lipid-depleted preparation. The data indicate that the

Table 1. Degradation of monoamine oxidase in mitochondrial membranes by oxygen-radical-generating systems

Degradation is measured as low-M_r fragments, and expressed as the increase over the no-irradiation blank, as % of the total. Degradation observed in these blanks, which were treated in parallel, was always less than 1%; generally those at pH 4 were slightly higher than those at pH 7.2. Similar results were obtained with either trichloroacetic acid or alkaline zinc processing methods.

Radical system	Irradiation dose (Gy)...	Degradation (%) 300	600	1200
$OH^{\cdot}(N_2O/O_2)$		0.06	0.23	0.68
+ 10 mM-Mannitol		0	0	0.06
$OH^{\cdot}(N_2O)$		0	0	0.01
$O_2^{\cdot -}$ (air)		0	0	0.02
$HO_2^{\cdot}$ (air, pH 4)		0.16	0.30	0.51
+ 10 mM-Mannitol		0.15	0.30	0.48

Table 2. Comparison of radical fragmentation of monoamine oxidase in lipid-depleted and normal submitochondrial particles

From a single preparation of labelled mitochondria, both a lipid-depleted and a normal preparation (treated in parallel throughout) were obtained. Degradation was expressed as in Table 1. Degradation in blanks treated in parallel was always less than 1%, and those for lipid-depleted preparations were much lower than those for the normal preparations. Each value represents the mean of four separate irradiations. *indicates a significant difference from the appropriate no-irradiation blank where $P < 0.01$ in a two-tailed t test.

Radical system	Irradiation dose (Gy)...	Degradation (%) 300	600	1200
$OH^{\cdot}(N_2O/O_2)$				
Normal		0.01	0.11	0.23*
Lipid-depleted		0.18*	0.25*	0.65*
$HO_2^{\cdot}$ (air, pH 4)				
Normal		0.06	0.12	0.35
Lipid-depleted		0.03	0.09	0.41*

action of the hydroxyl radical on the enzyme may be restricted by the presence of lipid, while that of the hydroperoxyl radical may depend on the presence of lipid, since this radical is inactive against bovine serum albumin in free solution [6].

There was also a marked inverse relationship between the protein concentration of the mitochondrial suspension during the irradiation and the extent of fragmentation observed. Similarly, mannitol was a more effective scavenger the lower the protein concentration (results not shown).

Characterization of the products of radical attack on monoamine oxidase

On Bio-Gel P-2 chromatography of the trichloroacetic acid-soluble material from radical attack on the monoamine oxidase several radioactive peaks were observed, mostly eluting earlier than free pargyline. About 25% in most cases was eluted at the position of free pargyline. Thus it seemed that a proportion of the degradation products might not result from proteolysis but from some other reaction producing free pargyline. However, the proteolytic enzyme trypsin could also generate a component eluting in the free pargyline position. Thus products other than free pargyline contaminate the peak corresponding to free pargyline. Thus the vast majority of the degradation products were other than free pargyline and were likely to be pargyline attached to peptides.

This was further supported by experiments with h.p.l.c. A variety of acetonitrile gradients were each able to separate more than 80% of the degradation products from the major peak of the radioactive free pargyline. Thus at a conservative estimate more than 80% of the acid-soluble degradation products in all the studied cases could be shown by the combination of these two separation methods to be other than free pargyline: the labelling method was thus sufficient to trace degradation of monoamine oxidase.

Two-phase experiments on degradation of monoamine oxidase

In view of the small degree of fragmentation achieved during irradiation, we hypothesized that many of the radicals generated react with lipid rather than with protein. Were this the case, the lipids in the irradiated membrane would be expected to contain a residue of hydroperoxides and other molecules which could be degraded during a subsequent transition metal attack, generating further radicals [15] which might be more able to fragment monoamine oxidase.

Thus, when irradiated mitochondria were subsequently

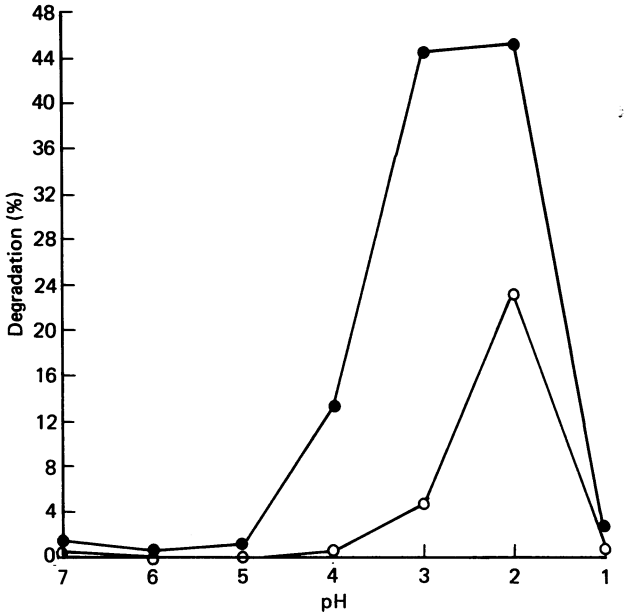

Fig. 1. Fe^{2+}-catalysed degradation of monoamine oxidase *in situ* at low pH

A large volume of submitochondrial particles (10 mg of protein/ml) was irradiated in glass-distilled water at pH 7 (under $OH^{\cdot}/O_2$ conditions; 1000 Gy). The particles were then diluted into 10 vol. of water titrated to each desired pH, with (●) or without (○) 100 μM-Fe^{2+}. The second-phase incubation was then at 37 °C for 30 min, and the measurement of degradation used the alkaline zinc method. Degradation is expressed as the increment from 0 min to 30 min of incubation in the second phase.

exposed to ferrous iron at 100 μM much larger quantities of trichloroacetic acid-soluble material (up to 6% of the total) were observed, and then the amount of acid-soluble products declined, giving a biphasic reaction. The decline is presumably due to cross-linking reactions. However, it was notable that the zero-time values, determined as soon as possible after addition of the transition metal, were much above those of controls which did not receive transition metal. Generation of acid-soluble radioactivity occurred after a lag in non-irradiated samples also. The effects of Fe^{2+} in both irradiated and non-irradiated samples could be drastically reduced by a 1.5-fold molar excess of desferrioxamine. These data suggested that degradation might be continuing even during the brief processing in trichloroacetic acid.

Thus we found, as predicted, that very rapid production of acid-soluble material occurs when irradiated mitochondria with labelled monoamine oxidase are incubated in the presence of transition metal in 5% (w/v) trichloroacetic acid, which had a pH of approx. 1.5. Up to 15% of the label can be made soluble in 5% trichloroacetic acid within 90 min in these conditions. This reaction can be inhibited only 40% by the iron chelator desferrioxamine at a 1.5-fold molar excess added after the trichloroacetic acid. Greater but still incomplete inhibition was obtained with a 10-fold molar ratio of desferrioxamine to transition metal. Better inhibition is obtained when desferrioxamine is present with iron before the trichloroacetic acid is added. Fig. 1 shows that this catalysis of fragmentation of the monoamine oxidase is a feature of a low pH and not of the presence of trichloracetic acid. Strikingly, fragmentation of lipid hydroperoxides also occurs best with transition metals at low pH [16]. It is also notable that Fe^{2+}/trichloroacetic acid fragments non-irradiated monoamine oxidase only slightly (result not shown). The data indicate a substantial difference in the action of Fe^{2+} at low and neutral pH, and also that the fragmentation observed in irradiated samples in the two-phase experiments above occurred mainly during the acid phase.

To avoid this acid-catalysed degradation of the monoamine oxidase we precipitated intact protein by the alkaline zinc method. In control experiments we showed that incubation of pre-irradiated mitochondria in the presence of zinc and barium hydroxide with or without the presence of transition metal resulted in fragmentation indistinguishable from that in comparable controls not incubated in the presence of alkaline zinc. This precipitation method was therefore acceptable. Fig. 2 shows the kinetics of transition-metal-catalysed degradation of pre-irradiated monoamine oxidase using this alkaline zinc precipitation method. A lipid-dependent transition-metal-catalysed reaction in pre-irradiated samples was thus observed, which could be prevented by a 1.5-fold molar excess of desferrioxamine. Non-irradiated samples treated in parallel showed no degradation until 90–120 min of second-phase incubation, after which it accelerates. Under these experimental conditions, iron may be cycling between its two oxidation states, by virtue of several different reactions. Ca^{2+} (100 μM) can also cause fragmentation. Its effects are largely inhibited by 150 μM-EDTA (result not

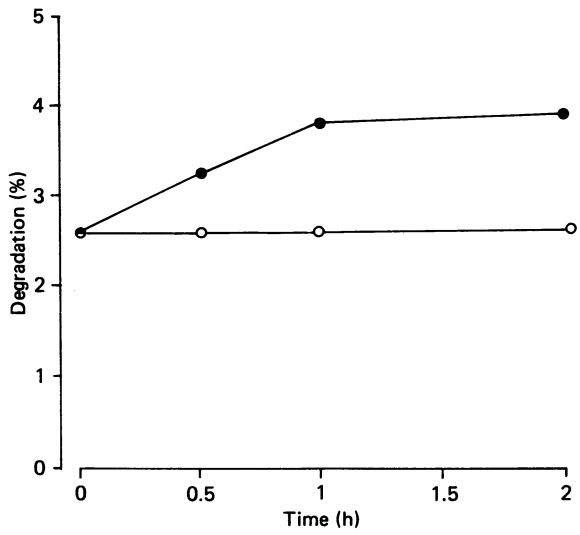

Fig. 2. Lipid-dependent degradation of pre-irradiated monoamine oxidase by Fe^{2+}

The two-phase experiment was conducted as for Fig. 1, except that in the second phase samples were diluted into 10 mM-potassium phosphate buffer, pH 7.2, either with no further addition (○) or in the presence of 100 μM-Fe^{2+} (●) in the second phase. Results from samples incubated in the second phase with iron plus a 1.5-fold molar excess of desferrioxamine were very similar to those of the controls shown in the Figure.

Table 3. Lack of iron-dependent degradation in pre-irradiated lipid depleted monoamine oxidase

The two-phase experiment was conducted as described in Fig. 1. Each value above represents the mean of four separate irradiations treated separately in the second phase, and S.D. values are given in parentheses. **indicates a significant difference between iron-containing and control samples at $P < 0.05$, and *similarly at $P < 0.01$.

Sample	Time...	Degradation (%) 1.5 h	6 h	22.5 h
Normal				
Control		1.14 (0.12)	1.22 (0.23)	1.21 (0.18)
$+Fe^{2+}$		1.03 (0.09)	1.28 (0.14)**	1.60 (0.16)*
Lipid-depleted				
Control		1.20 (0.50)	1.16 (0.59)	1.33 (0.53)
$+Fe^{2+}$		1.05 (0.49)	1.35 (0.45)	1.66 (0.43)

shown). In parallel experiments on bovine serum albumin in solution in the absence of lipids no such post-irradiation effect of transition metals on polypeptide fragmentation was observed either at neutral pH, or in the presence of 5% trichloroacetic acid or zinc/barium hydroxide (results not shown.) Nor can transition metals alone fragment bovine serum albumin [6], though they can eventually lead to fragmentation of monoamine oxidase in the membrane.

Thus two lines of evidence indicate that some of the fragmentation of monoamine oxidase we observed depends on the presence of lipid: firstly the reaction of the hydroperoxyl radical, and secondly the lack of effect of transition metals on pre-irradiated soluble proteins in comparison with their considerable effectiveness on pre-irradiated protein within lipid membranes. This interpretation was supported by further experiments comparing lipid-depleted with normal monoamine oxidase preparations. Table 3 shows that in pre-irradiated lipid-depleted preparations, there is very little subsequent fragmentation during incubation in the presence or absence of iron. In contrast, iron can induce a detectable fragmentation of the enzyme in the pre-irradiated normal mitochondrial preparations. The corresponding controls (without iron) showed no significant degradation.

Gel electrophoresis of products of radical attack on monoamine oxidase

During attack by $OH^{\cdot}/O_2$ (up to 1000 Gy) only a slight reduction in the radioactivity associated with the 60 kDa labelled band on the gels could be detected, but no clear fragments. The $HO_2^{\cdot}$ and $O_2^{\cdot -}$ radical-generating systems had no detectable effect on the electrophoretic distribution of radioactivity. The $OH^{\cdot}$ radical in the absence of oxygen gave significant amounts of radioactivity in larger molecules, presumably due to cross-linking. The products of the two-phase experiments contained many labelled components, some larger and some smaller than the enzyme polypeptide. Radioactivity was also detected at the dye front in amounts correlated with those observed by zinc/barium hydroxide or trichloroacetic acid precipitation (as appropriate).

Comparison of fragmentation of monoamine oxidase with lipid peroxidation

We have measured thiobarbituric acid-reactive products of lipid peroxidation in conditions corresponding to those described above. In agreement with the literature on mitochondrial peroxidation [17,18], little peroxidation was observed *in vitro* up to 3 h at 37 °C unless 100 μM-Fe^{2+} was added, when thiobarbituric acid-reactive materials were rapidly generated, reaching plateau values corresponding to approx. 0.7 nmol of malondialdehyde/mg of protein within 60–120 min. Ascorbate (0.5 mM) together with Fe^{2+} (5–100 μM) did not enhance peroxidation over that with iron alone, and also had no effect on proteolysis (not shown). 1000 Gy of irradiation in the $OH^{\cdot}/O_2$ system gave larger quantities of malondialdehyde/mg of protein. When pre-irradiated mitochondria are incubated with the transition metals rather little alteration in the thiobarbituric acid-reactive material present is observed. Only extremely small quantities of thiobarbituric acid-reactive materials could be generated from the lipid-depleted preparations. The kinetics of lipid peroxidation and protein fragmentation were compared in many experiments. It was observed that the two processes were concomitant. The lag before peroxidation and fragmentation were detected in unirradiated samples exposed to iron could be substantially compressed by repeated thawing and brief storage at 4 °C of the labelled submitochondrial particles, perhaps due to progressive depletion of antioxidants [17]. In such samples peroxidized by storage, reactions causing cross-linking, such as to decrease the quantity of acid-soluble pargyline-peptide radioactivity, could also be detected.

DISCUSSION

The evidence above indicates that free radicals can fragment and otherwise damage monoamine oxidase when it is in the outer mitochondrial membrane. However, it implies also that lipids protect monoamine oxidase from fragmentation in comparison with protein in lipid-free solution. Thus for a given dose of hydroxyl radical attack, for instance, a smaller degree of fragmentation of monoamine oxidase is observed in the normal mitochondrial membrane than in the lipid-depleted membranes, and a much smaller degree than is observed with bovine serum albumin in solution [6]. One reason for this is presumably that lipid consumes, directly or indirectly in peroxidative reactions, many of the primary radicals, leaving far fewer reacting directly with the protein. The two-phase experiments, however, show that

the consequence of lipid peroxidation, the presence of lipid hydroperoxides and other materials, may allow another route for protein fragmentation. Thus, when these products react with transition metals more radical production is initiated and some (unknown) components of this reaction are relatively effective in protein fragmentation.

It seems likely therefore that some lipid radicals can fragment proteins and that this effect is occurring during both phases of the monoamine oxidase experiments described above, in conjunction with other cross-linking reactions. Previous literature [2,17,19] has primarily emphasized the possibility of lipid-induced cross-linking of protein, rather than fragmentation. However, a limited amount of data indicate fragmentation due to lipid radicals, in particular, fragmentation of cytochrome *c* [20]. Lipid-dependent degradation of gelatin has also been observed [21]; this may have involved peptide-bond scission, but this was not conclusively shown. A further formal possibility exists for the mechanism of fragmentation of monoamine oxidase in the two-phase experiments: that other oxidized proteins generated during the first phase give rise to further radical fluxes which fragment. This possibility is perhaps unlikely in view of the lack of a second-phase fragmentation in the lipid-depleted enzyme, but requires further investigation with simpler model systems containing membrane proteins.

The relevance of lipid-dependent polypeptide fragmentation to intact biological membranes rather than to artificial mixtures of proteins and lipids has not previously been described. We suggest that this interaction is probably of considerable biological significance. We imagine that lipid alkoxy and peroxyl radicals may be involved: the reactive moieties require identification. In mitochondria and chloroplasts, whose radical fluxes are metabolically controlled (see [7] for discussion), such reactions may modify protein activities and influence overall protein turnover; but such regulatory effects may be much more widespread [24]. For example, the accentuated degradation at low pH may magnify the role of this lipid-dependent process in low-pH environments such as lysosomes [22], and the sequestered eroding surface of inflammatory sites [23].

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