Biochimica et Biophysica Acta, 291 (1973) 398–410

BBA 76122

PREPARATION OF MITOCHONDRIAL MEMBRANE PROTEINS FROM *NEUROSPORA CRASSA*: PREVENTION OF LIPID AUTOXIDATION DAMAGE BY AN ANTIOXIDANT

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(Received July 13th, 1972)

SUMMARY

Lipid autoxidation products, such as malonaldehyde, react with proteins, cross-linking them and decreasing their solubility. These reactions are of practical importance in experiments with membranes where lipids and proteins are closely associated.

When no precautions against lipid autoxidation were taken, both aged and freshly prepared mitochondrial membrane proteins from *Neurospora crassa* contained 1-amino-3-iminopropene groups formed by reaction of protein amino groups with malonaldehyde. This conclusion was derived by analysis of fluorescence emission, fluorescence polarization, the effect of borohydride reduction upon the fluorescence, and qualitative and quantitative determination of malonaldehyde with 4,4′-sulfonyldianiline after hydrolysis of the proteins.

The efficiency of antioxidants in the prevention of lipid autoxidation and consequent modification of protein was tested in a model system consisting of an aerated, aqueous solution of albumin, and methyl linolenate. The antioxidant, 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol, a sterically hindered phenol, appeared to be highly efficient in either the model system or in the isolation of membrane proteins. Mitochondrial membrane proteins, prepared in parallel procedure in the presence and absence of this antioxidant, differed in malonaldehyde concentration, iminopropene fluorescence and electrophoretic mobility.

Several unusual properties of aged membrane proteins, such as low solubility, resistance to trypsin hydrolysis, and changes in isoelectric points and electrophoretic mobilities can be explained as consequences of lipid autoxidation processes.

We suggest that antioxidants, such as sterically hindered phenols, be employed in the preparation and storage of proteins from membranes or other systems containing large amounts of lipids or unsaturated fatty acids in order to prevent artifactual modification of the proteins by lipid autoxidation products.

INTRODUCTION

Cellular membranes, such as those of microsomes, mitochondria, and chloroplasts, contain a mechanism for potential self-destruction; lipid autoxidation[1,2].

Destabilization of the structures of these membranes brings about the conditions necessary for lipid autoxidation; *i. e.* contact between lipids and molecular oxygen in the presence of catalysts which are also components of the membranes. Components of membranes with electron transport function, such as iron–sulfur proteins and cytochromes, serve as catalysts or initiators of lipid autoxidation, probably by generating free radicals which initiate the chain reaction.

Autoxidation products of unsaturated fatty acid acyl residues of membrane phospholipids attack the associated proteins, leading to covalent cross-linkage, the destruction of certain amino acids, a change in color, a decrease in solubility, and a loss of enzymatic activities[1–9]. Cellular damage by radiation and the formation of age pigments in living tissues is believed to follow the same class of reactions[6,10].

The self-destruction principle of these membranes, and perhaps others as well, dictates the prevention of lipid autoxidation in investigations of membrane structure and function and their genetic, pathological, and age-dependent changes. Control of lipid autoxidation *in vivo* in laboratory animals, with prolongation of their life span, appears to be feasible by diets that either contain antioxidants or are low in unsaturated fatty acids[11–14]. Non-toxic antioxidants, such as phenol derivatives, are often commercially included in human foods containing lipid and proteins to prevent oxidative spoilage[15]. Only a few studies of isolated cellular membranes have been concerned with the prevention of lipid autoxidation. According to Hanstein and Hatefi[1,2] and the present studies, lipid autoxidation may rapidly occur during the isolation of membranes and their proteins. Thus, it is more appropriate to include antioxidants to scavenge free radicals in the isolation media than to separate lipid from protein after the isolation.

We are investigating the structure, function, and biogenesis of mitochondrial membranes in the simple eucaryote, *Neurospora crassa*, because it has a simple diet and some mutations affect these membranes. We have frequently observed that the proteins of these membranes changed during storage[16], becoming indigestible by trypsin, decreasing in solubility, and isoelectric point, and changing from white to yellow. In these preparations, no attempt was made to prevent lipid autoxidation and the proteins were stored in air in either the aqueous or dry state at −20 °C. These observations can be now explained, in view of the present studies, as a consequence of lipid autoxidation damage.

In lipid autoxidation, reactions with free radicals are considered to be the main cause of protein damage, but the attack by a non-radical oxidation product, malonaldehyde, deserves special attention[4]. Malonaldehyde exists in solution and in solid phase predominantly in its enolic form, 3-hydroxyacrolein[17,18]. The latter reacts with both N-terminal and ε-amino groups of proteins to form azomethine or enamine bonds.

One molecule of malonaldehyde can form either an intra- or an inter-polypeptide cross-link:

$$2\ \text{protein–NH}_2 + \text{O=CH–CH=CHOH} \rightarrow \text{protein–N=CH–CH=CH–NH–protein}$$

In either case, the disubstituted 1-amino-3-iminopropene product has characteristic fluorescence properties[4]. Fluorimetric analysis of the reaction products of malonaldehyde with proteins and different reagents serves as a sensitive diagnosis of protein damage by lipid autoxidation.

In this report, we describe the results of fluorimetric and electrophoretic ana-

lysis of the effects of lipid autoxidation on mitochondrial membrane proteins of *N. crassa*. We prevented the modification of the proteins by including an antioxidant in the isolation media and during storage. A short summary of this work was presented elsewhere[19].

MATERIALS AND METHODS

Chemicals

Solvents in fluorescence studies were either reagent or spectrograde and were checked for light scattering and intrinsic fluorescence. Malonaldehyde bis(dimethylacetal) (99 %), 3,5-di-*tert*-butyl-4-hydroxybenzoic acid, 3,5-di-*tert*-butyl-2,6-dihydroxybenzoic acid, and 4,4'-sulfonyldianiline were obtained from Aldrich Chemical Co., Milwaukee, Wisc. The latter chemical was recrystallized twice from 95 % ethanol. Tetrapropylammonium hydroxide was from Eastman Organic Chemicals, Rochester, N.Y. Methyl linolenate was from Calbiochem, Los Angeles, Calif. The antioxidant, 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol (2,6 di-*tert*-butyl-4-hydroxymethylphenol) was obtained from K and K Laboratories, Plainview, N.Y. Bovine serum albumin (Fraction V) was obtained from Sigma Chemical Co., St. Louis, Mo.

Membrane protein preparations

The aged membrane proteins which were first analyzed for iminopropene fluorescence were prepared from mycelia of *Neurospora crassa* (wild-type 74A) to yield fraction P_0 as described by Swank *et al.*[16]. Fresh membrane protein preparations were isolated by the following procedure (Fig. 1). Conidia from 1-week-old cultures of wild-type *N. crassa* 74A (strain 5.5A Yale, FGSC No. 936) were grown on Fries' minimal medium[20] containing 2 % sucrose and 1.5 % agar. They were transferred to a 15-l carboy to a final concentration of 10^6 conidia per ml. After 20 h at 30 °C with aeration, the mycelia were collected by filtration through four layers of cheesecloth. The mycelial cake was pressed dry with cheesecloth. Mycelia (80–90 g fresh weight) were suspended in 300 ml of 50 mM Tris–HCl buffer (pH 7.4 at 4 °C) containing 0.5 M sucrose and 1 mM EDTA. Mycelia were collected by filtration, washed with 500 ml of the buffer, and pressed. The mycelial cake was disrupted into small lumps, soaked in an excess of glycerol, and blended in liquid nitrogen in a metal Waring blender for three periods of 30 s each. After the liquid nitrogen evaporated, the powder was suspended in the buffer. Mitochondria were collected from the homogenate by differential centrifugation as shown in Fig. 1. The mitochondria were purified by sedimentation in a step-gradient of 1.2 and 1.6 M sucrose (density 1.16 and 1.21, respectively), suspended in the buffer, and collected by centrifugation at 40000 × *g*. The mitochondria were swollen by suspension in 1 mM Tris–phosphate buffer (pH 8.0) and shrunk by the addition of 1/3 the original volume of a solution containing 0.5 M sucrose, 2 mM ATP, and 2 mM $MgSO_4$. The mitochondrial solution (about 15 mg protein/ml, 10 ml) in a 30-ml polypropylene tube in an ice-bath was probed with a Branson sonifier equipped with a tapered horn (3 mm final diameter) four times at 10-s intervals at a power setting of 4. The membranes were collected from the solution by centrifugation at 44000 × *g* and extracted three times with 10 ml of cold 99 % ethanol to remove lipids. The lipid-depleted membranes were suspended, with the aid of a glass–teflon homogenizer, in a solution containing 0.5 % sodium laurylsarcosine,

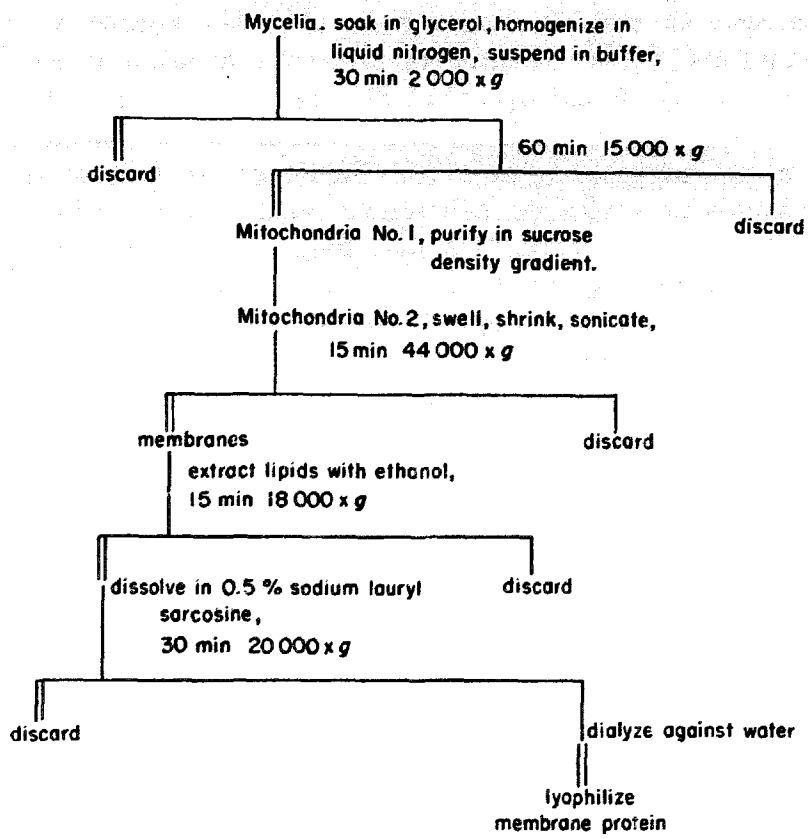


Fig. 1. Flow chart of the preparation of mitochondrial membrane proteins from *N. crassa*.

0.1 % mercaptoethanol and 10 mM Tris–HCl buffer (pH 6.8). The solution was heated for 3 min at 100 °C and centrifuged at 20000 × *g*. The supernatant was dialyzed against five changes of water at 4 °C for 3 days. The precipitated protein was collected by centrifugation and lyophilized. Protein concentrations were determined by the procedure of Lowry *et al.*[21].

Fluorescence analysis

Fluorescence measurements were made with an Aminco–Bowman spectrofluorimeter, Model SP-100, equipped with an IP-28 photomultiplier, ellipsoidal off-axis condensing sytem, Glan prism polarizers, and temperature-controlled cuvette chamber.

Spectra were recorded with either an Aminco X-Y or a Beckman 10-inch recorder. The reported spectra are uncorrected[22], but adjustments were made as follows. The emission monochromator was calibrated by using the first-order light scattering of an aqueous solution of Ludox[22]. When fluorescence intensities were to be compared, they were normalized relative to a quinine sulfate standard to correct for changes in lamp intensity and photomultiplier sensitivity. In fluorescence polarization experiments, the stability of the lamp and the detection system was checked by repeating the first spectrum of a series at the conclusion of the experiment. Corrections for grating anomalies were calculated by the method of Chen and Bowman[23]. All fluorescence measurements were made with samples at 21 °C.

Malonaldehyde test

Qualitative and quantitative analysis of malonaldehyde by reaction with 4,4′-

sulfonyldianiline was based upon the method of Sawicki *et al.*[24]. The samples were freed of sucrose (buffer component) by centrifuging them from aqueous suspension and lyophilized. The powder was hydrolyzed overnight in 0.03 M HCl in dimethylformamide to obtain malonaldehyde from iminopropene cross-linkages. (The HCl/dimethylformamide solution was prepared immediately before use to prevent the formation of chlorine which interferes with the subsequent reaction with sulfonyldianiline.) Insoluble material was removed from the hydrolyzate by centrifugation. The supernatant (100 µl) was added to 1.45 ml of a dimethylformamide solution containing 0.03 M HCl and 0.25 % 4,4'-sulfonyldianiline. The mixture was heated at 80 °C for 45 min in a water bath, cooled, mixed with 0.25 ml of a 10 % aqueous solution of tetrapropylammonium hydroxide, and incubated for 10 min at 21 °C. The fluorescence of the reaction product at 545 nm was measured after excitation at 485 nm. Malonaldehyde bis(dimethylacetal) was used as a standard and was treated in the same way as the sample.

Electrophoresis

Polyacrylamide gel electrophoresis of membrane proteins was performed according to Swank and Munkres[25] with gels that contained 0.1 % sodium dodecyl sulfate, 8 M urea, and monomer concentrations of either 8 % and 0.27 % acrylamide and bisacrylamide, respectively (System 1) or 12.5 % and 1.25 % (System 2). Gels were 13 cm in length and 0.6 cm in diameter.

Freshly prepared membranes were dissolved by heating them in a solution of 2 % sodium dodecyl sulfate and 1 % mercaptoethanol for 3 min at 100 °C. The solution was cooled to room temperature. Then solid urea was added to the solution to a final concentration of 8 M. Electrophoresis was performed with a Büchler Polyanalyst apparatus, with potentials of 4 to 7.5 V/cm. Bromophenol blue, cytochrome *c*, sperm whale myoglobin and bovine pancreatic carboxypeptidase *A* were used as standards. The gels were stained with Coomassie blue and scanned with a Gilford recording spectrophotometer equipped with a linear transport device.

RESULTS

Proteins cross-linked by malonaldehyde are soluble in chloroform–methanol (2:1, v/v)[24]. Samples of mitochondrial membrane proteins which had been stored at −20 °C in the lyophilized state for 6 to 24 months were extracted with chloroform–methanol and filtered through a Millipore apparatus. The extracts exhibited fluorescence with excitation and emission maxima at 375–385 and 450–470 nm, respectively (Fig. 2). These observations agree with published values for iminopropene bonds in proteins as found with the same instrument and solvent. The same fluorescence characteristics were exhibited by a model compound synthesized from glycine and malonaldehyde by the method of Chio and Tappel[5].

Since the emission fluorescence of the extracts of the proteins was polarized (observed polarization = 0.10–0.14), the fluorophores were bound to macromolecules, (The emission polarization at 450 nm of quinine sulfate in 0.1 M sulfuric acid was 0.009, as expected for a low molecular weight compound.) Dialysis of the protein against water for several days could not remove the fluorophore.

Iminopropene cross-linkages in proteins contain reducible, azomethine bonds;

so hydrogenation can be expected to quench the fluorescence. Borohydride reduction in the chloroform–methanol extracts of membrane proteins was carried out in the presence of tetrapropylammonium hydroxide to prevent decomposition of the borohydride by methanol. (The hydroxide increases the first-order light scattering, but does not influence the fluorescence pattern). As shown in Fig. 3, 80 % of the fluorescence at 470 nm was ultimately quenched by borohydride reduction.

The extracts of the membrane proteins, after excitation at 280 nm, exhibited

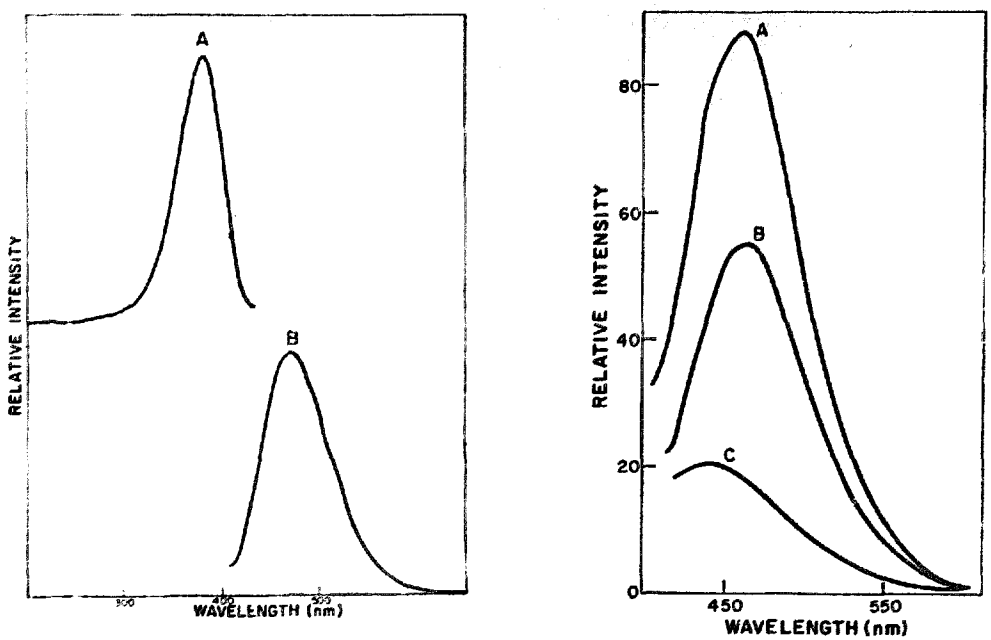


Fig. 2. Fluorescence emission and excitation spectra of chloroform–methanol extracts of aged mitochondrial membrane proteins of *N. crassa* (Fraction P_0). A, excitation spectrum (emission 465 nm); B, emission spectrum (excitation 380 nm). Aged mitochondrial membrane protein (stored for 22 months at −20 °C in the lyophilized state) was dialyzed against water and extracted with chloroform–methanol at 4 mg protein per ml.

Fig. 3. Quenching of iminopropene fluorescence in membrane proteins by borohydride reduction. 1.5 ml of fresh sodium borohydride was added to 1.5 ml of protein extract (prepared as in Fig. 2). Excitation at 380 nm. A, before borohydride reduction; B, 30 min after borohydride was added; C, 136 min after borohydride was added. Normalized relative intensities of A:B:C = 88:63:18.

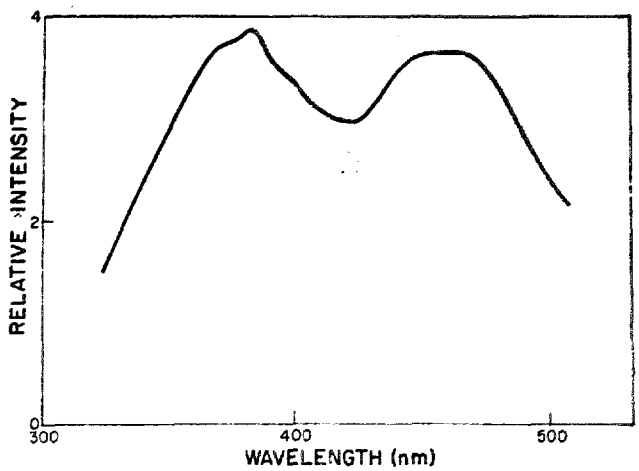


Fig. 4. Fluorescence emission spectrum of chloroform–methanol extract of aged mitochondrial membrane protein after excitation at 280 nm. The same sample as in Fig. 2.

two emission maxima; 380 and 465 nm (Fig. 4). We attribute the first emission maximum to protein fluorescence, although in aqueous solutions, tryptophan residues of proteins fluoresce at 340–360 nm. However, the shift to a longer wavelength is due to the effects of the solvent. An emission maximum of 375 nm for tryptophan in the chloroform–methanol solvent was observed. Since the protein emission coincides with the main excitation maximum of the iminopropene fluorescence, energy transfer from tryptophan residues to iminopropene bonds is the most likely explanation for the 465-nm fluorescence. However, with the glycine–malonaldehyde model compound, we also found a minor second excitation maximum at 280 nm. Therefore, the 465-nm fluorescence of the modified proteins may be due to a combination of both energy transfer and a second excitation maximum at 280 nm.

All of these observations are consistent with what one could expect to find in aged protein preparations cross-linked by malonaldehyde. However, we questioned whether lipid autoxidation might not also occur early during the isolation procedure after the cells are broken and natural antioxidants are removed or diluted.

The first step in our approach to this question was to survey the properties of synthetic antioxidants to choose a suitable one to add to the membrane isolation medium to prevent lipid autoxidation. Antioxidants should be relatively stable to autoxidation, at least sparingly soluble in water, and chemically inert except for their ability to scavenge free radicals. By these criteria, some sterically hindered phenols with additional hydrophylic groups appear to be most suitable. The efficiency of some antioxidants of this class was determined with a model system which consisted of a mixture of bovine serum albumin and methyl linolenate in water. The mixtures were shaken in air and the change in absorbance at 420 nm from colorless to yellow was measured as an index of lipid autoxidation. The observed difference in efficiency between the structurally related compounds, 3,5-di-*tert*-butyl-2,6-dihydroxybenzoic acid and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid is noteworthy (Fig. 5). The most effective antioxidant, 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol was chosen for the following experiments.

Mitochondrial membrane proteins were isolated in parallel procedure in the absence and presence of the antioxidant, as outlined in Fig. 1. The antioxidant was dissolved in 1,2-propanediol (50 mg/ml), and added as a suspension of 100 μg/ml in the buffer until the crude mitochondria were obtained. A concentration of 10 μg/ml (approximately the aqueous solubility limit) was employed in all subsequent operations, except for the lipid extraction with an ethanolic solution of 100 μg/ml.

The preparations started with the same harvest of mycelia. The mycelial pad was divided in half. Membrane proteins were prepared simultaneously from the two portions with and without 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol in the solutions. Freshly isolated membranes, prepared in the absence of the antioxidant, yielded at least a 10-fold greater concentration of malonaldehyde than those prepared in the presence of the antioxidant (Table I). (Malonaldehyde concentration in the protected samples was at or below the limit of detection.) The date in the last row of Table I indicate that the difference between the two preparations is not due to interference of the antioxidant with the malonaldehyde assay.

In correlation with the observations on malonaldehyde concentrations in membranes, the proteins isolated from them showed marked difference in iminopropene fluorescence depending on whether they were isolated in the absence or presence of the

antioxidant. When freshly prepared lyophilized proteins were extracted with chloroform–methanol, the fluorescence emission intensities of the extracts were 5-fold greater in the unprotected than in the protected preparations (Fig. 6). (The antioxidant did not affect the fluorescence *per se*; when the antioxidant was added to

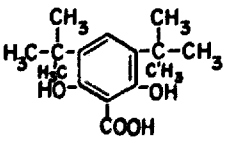
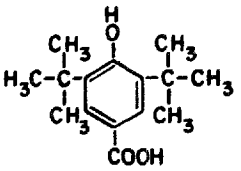
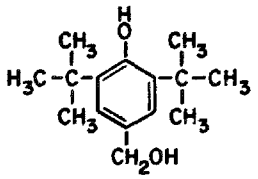
Antioxidant added	Absorbance of bovine serum albumin–methyl linolenate mixture at 420 nm
none	0.127
3,5-di-tert-butyl-2,6-dihydroxybenzoic acid 	0.121
3,5-di-tert-butyl-4-hydroxybenzoic acid 	0.042
3.5-di-tert-butyl-4-hydroxybenzylalcohol 	0.023

Fig. 5. Comparison of the efficiency of some antioxidants in the prevention of lipid autoxidation. Bovine serum albumin (20 mg) was mixed with 20 mg of methyl linolenate in 4 ml of isolation buffer. Antioxidants were 10 μg/ml. Duplicate mixtures were shaken in air at room temperature for 2 weeks.

TABLE I

CONCENTRATION OF MALONALDEHYDE IN MITOCHONDRIAL MEMBRANES OF *N. crassa* AFTER PREPARATION OF THE MEMBRANES WITH AND WITHOUT ANTIOXIDANT

Membranes were isolated in parallel simultaneous procedure with and without the antioxidant, 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol, in the isolation media. Malonaldehyde was obtained from the proteins by acid hydrolysis of iminopropene bonds and determined fluorimetrically after reaction with sulfonyldianiline.

Origin of sample	*ng malonaldehyde per mg protein*		
Replicate No:	*1*	*2*	*3*
Preparation + antioxidant	+2	−2	−1
Preparation − antioxidant	26	25	22
Preparation − antioxidant (antioxidant was added to the preparation just before malonaldehyde analysis)	—	25	25

the unprotected sample before the test, no change in fluorescence was observed.) These observations correlate reasonably well with the 10-fold difference in malonaldehyde concentration in the original membranes, if it is taken into account that cross linked proteins might be less extractable from the membranes with sodium lauryl sarcosine and that not all of the iminopropene cross-linked proteins might be extracted by the chloroform–methanol solvent.

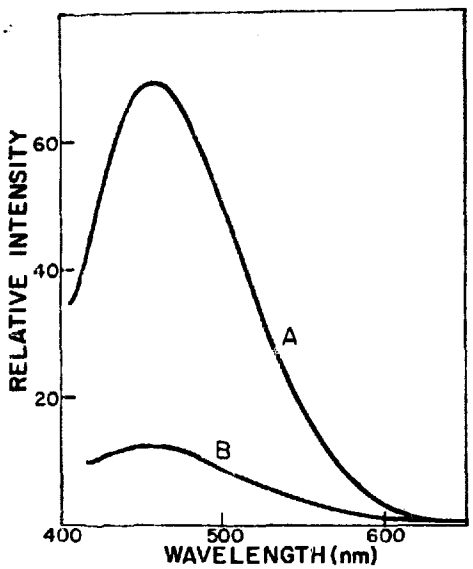


Fig. 6. Comparison of iminopropene fluorescence in chloroform–methanol extract of mitochondrial membrane proteins which were freshly prepared, either in the presence or absence of antioxidant. A, unprotected preparation; B, preparation in presence of 3,5-di-*tert*-butyl-4-hydroxybenzylalcohol. Excitation 377 nm. Extraction was done with 1 mg of protein per ml of chloroform–methanol.

If an average molecular weight of the proteins of 25000 is assumed, one molecule of malonaldehyde would be present among 100 of the protein molecules in the membranes isolated in the absence of antioxidant (Table I). This is a minimum estimate of the total damage to the proteins because malonaldehyde is considered to be a minor component among the various products of lipid autoxidation which modify proteins[7].

The electrophoretic mobilities of the membrane proteins in polyacrylamide gels containing sodium dodecyl sulfate were determined to assess the total autoxidation damage (Table II). The results indicate that over half of the proteins bands were modified in the absence of antioxidant, with a slight but reproducible increase in mobility. The antioxidant itself does not interfere with the mobilities; they were the same in the unprotected preparation when antioxidant was either added just before electrophoresis or omitted (Expt 4, Table II). The estimate of the degree of modification is probably low in these experiments because optimum resolution of the entire range of molecular weight is not achieved in any one electrophoretic system (Table II)[25].

Griffith[26] observed that disulfide and glutaraldehyde intrapolypeptide cross-linkages cause proteins to migrate faster in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Intrachain cross-linked polypeptides may not fully assume the rod-like conformation that the detergent normally forces upon them[27] so that their average Stokes radius is less and their mobility in gel electrophoresis is consequently

greater than that of proteins that are not cross-linked. This model is also consistent with the observation that the mobilities of the higher molecular weight polypeptides were altered more than those of the lower ones (Table II). Detailed interpretation of the molecular basis of the modifications from the electrophoretic data is not possible

TABLE II

ELECTROPHORETIC MOBILITIES OF MITOCHONDRIAL MEMBRANE PROTEIN AFTER ISOLATION OF THE MEMBRANES WITH AND WITHOUT ADDED ANTIOXIDANT

Proteins were stained with Coomassie blue in polyacrylamide gels after electrophoresis and scanned with a recording device. The positions of the peaks from the origin were recorded to the nearest 0.1 mm. The numbers in the table are the average of 3 or 4 replicates of each sample. Three preparations of membrane proteins, prepared in the absence (U) or presence (AO) of the antioxidant were analyzed in the 4 experiments. Two sodium dodecyl sulfate-polyacrylamide gel systems were employed (see Methods). In Expts 2, 3, and 4 electrophoretic System I was used, but in Expt 1, electrophoretic System 2 was used. In Expts 2 and 3, the same preparations were analyzed, but the effective time of electrophoresis was less in 3 than in 2. U (+AO) indicates that the antioxidant at 10 μg/ml was added to the unprotected sample (U) just before electrophoresis. In Expts 1–3, *D* is the percentage deviation of the mobility of U from AO. In Expt 4, *D* is the percentage deviation of the average of U and U (+AO) from AO.

Electrophoretic mobility (mm)

Experiment number

1			2			3			4			
AO	*U*	*D*	*AO*	*U*	*D*	*AO*	*U*	*D*	*AO*	*U*	*U(+AO)*	*D*
22.2	22.8	+2.7	6.7	6.7	0	5.6	5.6	0	5.3	6.4	6.3	+21.0
25.0	25.4	+1.6	9.8	10.0	+2.0	7.6	7.6	0	7.5	8.5	8.5	+13.3
29.5	30.2	+2.4	10.9	11.2	+2.8	9.6	9.9	+3.1	8.6	9.5	9.4	+10.5
31.4	32.7	+4.1	13.3	13.8	+3.8	13.2	14.0	+6.1	10.6	11.6	11.4	+ 8.5
32.8	33.6	+2.4		15.8		16.4	17.3	+4.9	12.3			
35.0	37.3	+6.6		17.5		18.8	19.7	+4.8	13.6	14.5	14.4	+ 6.6
39.7	41.0	+3.2	19.3	20.0	+3.6	26.4	27.0	+2.3	15.6	16.2	16.1	+ 3.8
42.2	43.2	+2.4	20.7	21.2	+2.4	27.8	29.1	+4.7	19.5	19.9	19.9	+ 2.1
45.7	48.5	+6.1	23.5	24.0	+2.1	29.6	30.8	+4.1	20.7			
49.0	51.5	+5.1		25.7		32.8	33.6	+2.4	22.4	22.7	22.7	+ 1.3
52.2	55.0	+5.4	26.4	27.0	+2.3	36.7	36.7	0	26.8	26.9	27.0	+ 0.8
61.7	60.7	−1.6	31.8	32.2	+1.3	40.0	40.8	+2.0	30.5	30.7	30.7	+ 0.7
65.7	65.7	0	35.8	37.2	+3.9	44.7	44.7	0	33.8	33.8	33.5	− 0.3
73.2	75.5	+3.1	39.3	40.7	+3.6	50.8	50.8	0	36.7	36.5	36.5	− 0.5
79.0	81.5	+3.1		42.7		57.5	58.0	+2.0	39.1	39.1	38.9	− 0.3
			41.8	43.5	+4.1	71.7	72.5	+1.1	42.7			
			45.0	45.5	+1.1				44.7	43.4	44.7	− 1.5
			48.0	49.8	+3.8				49.8	49.3	49.3	− 1.0
			50.5	52.0	+3.0				55.3	55.1	54.3	− 1.1
			56.2	57.0	+1.4				62.0	60.1	59.8	− 3.3
			61.0	62.5	+2.4				66.7	65.2	65.2	− 2.2
			66.0	67.0	+1.5				68.6			
			69.5	71.0	+2.2				73.5	72.0	72.6	− 1.6
			75.0	76.5	+2.0				78.0	79.1	78.0	+ 0.7
			77.0	78.0	+1.3				84.8	82.7	82.5	− 2.6
			84.0	84.0	0				90.1	87.4	86.5	− 3.5
			87.5	88.0	+0.6				96.8	92.1	93.4	− 4.2
			91.5	92.5	+1.1				103	100	100	− 2.4
			96.5	97.5	+1.0							
			103	103	0							
			108	111	+2.8							
			115	116	+0.9							

because some cross-linkage may be both inter- and intrachain and the modifications may also interfere with sodium dodecyl sulfate binding and lead to amino acid destruction[3,4]. Both of the latter two factors would change the net charge and mobility. Moreover, the exact history of the preparation may influence the kind and extent of modification. Thus, in Expt 4, the higher molecular weight proteins exhibited relatively greater mobilities in the unprotected samples, as in the other two preparations, but the low molecular weight ones exhibited a tendency to be slower in mobility. Perhaps in this preparation the autoxidation damage caused interchain cross-linkage and interfered with sodium dodecyl sulfate binding and outweighed the effects of intrachain linkage of the lower molecular weight proteins.

DISCUSSION

The present results may serve to explain and overcome certain difficulties which we have previously encountered in studies of mitochondrial membrane proteins of *N. crassa*. These difficulties are related to the solubility of the intrinsic membrane proteins. Unlike other cellular proteins, intrinsic membrane proteins are often insoluble in aqueous solutions containing detergents or other protein denaturing substances. Such insolubility is generally regarded as an expression of the hydrophobic character of these proteins. It is questionable, however, to what extent the insolubility is correlated with innate hydrophobicity as determined by the composition and sequence of hydrophobic amino acids in the proteins. We have frequently observed that fresh preparations of mitochondrial membrane proteins (Fraction P_0) were readily soluble in detergent solutions, but that they became insoluble after storage for several months at -20 °C (ref. 16). Denaturing solutes, such as long chain alkyl sulfates or hexafluoroacetone, failed to dissolve the proteins. The insolubility was unrelated to the formation of disulfide bridges since mercaptans failed to increase the solubility. Furthermore, an irreversible conformational change of the proteins appeared to be highly unlikely. The conformation of a protein is basically determined by its amino acid sequence and the surrounding solvent. Lyophilized proteins which could be dissolved once in a certain detergent solution should always be soluble in that solution unless they are altered chemically by processes such as lipid autoxidation.

The aged proteins were virtually indigestible by trypsin (K.D. Munkres, unpublished). Andrews *et al.*[3] reasoned that since denatured proteins are generally more easily hydrolyzed by proteolytic enzymes than are their native counterparts, whereas cross-linked proteins resist that proteolysis, the extent and rate of proteolytic hydrolysis would serve as an index of the occurrence of cross-linkage. In model experiments with pure proteins and an auto-oxidizing unsaturated fatty acid, the cross-linked proteins were indeed more resistant to hydrolysis than were the unmodified proteins. Moreover, since the lipid oxidative modification of proteins involves the ε-amino groups of lysine[3,4], trypsin may fail to hydrolyze such proteins not only because of their higher state of aggregation but because of the specificity of trypsin for lysine.

The color of the membrane proteins, unprotected by antioxidant, changes from white to yellow during the isolation and upon storage. The yellow pigment was not removed from the proteins by dialysis, extraction with organic solvents, or isoelectric focusing. It has been frequently suggested that yellow, fluorescent age pig-

ments occurring *in vivo* are derived from modified proteins caused by lipid oxidation in damaged membranes[10]. Chio and Tappel[4] noted the similarity of such age pigments with the yellow, fluorescent product obtained from the reaction of malonaldehyde and *N*-acetyl-L-lysine. Roubal[7], in a study of protein damage in a dry lipid–protein system, noted that the change in color of the protein during lipid oxidation from white through tan to yellow was correlated with the appearance of 3-iminopropene fluorescence and free radicals.

Freshly isolated membrane proteins exhibited relatively greater electrophoretic mobilities when unprotected by antioxidant. Furthermore, upon aging in the absence of antioxidant, the isoelectric points of these proteins decreased (M. Minssen, unpublished). These modifications may reflect, in part, the known modifications of terminal and ϵ-amino groups of the proteins by lipid autoxidation. Extensive damage[3,4] or long storage[16] leads to the formation of aggregates of higher molecular weight which are not dissociable by treatments which disrupt noncovalently bound aggregates.

The results of the present studies indicate that lipid autoxidation commences during the isolation of the membranes and their proteins after the cells are broken and natural antioxidants are removed or diluted. To the extent that various membranes differ in their content of phospholipids, natural antioxidants such as α-tocopherol, and catalysts of lipid autoxidation such as proteins containing iron and heme, the potential of the self-destruction process of lipid autoxidation may also differ. Membranes with electron transfer functions such as in microsomes, mitochondria, and chloroplasts may be especially susceptible to lipid autoxidation damage because components of their enzymatic complexes catalyze the autoxidative process.

In the analysis of malonaldehyde as an indicator of lipid autoxidation, we preferred the reaction with 4,4′-sulfonyldianiline rather than a generally employed method in which thiobarbituric acid is the reagent. The former method is far more sensitive and specific than the latter[24]. We found, however, that the time of heating during the reaction must be longer than that specified by Sawacki *et al.*[24] to insure reproducibility.

Small but significant and reproducible differences in the electrophoretic mobilities of most of the mitochondrial membrane proteins were observed when they were protected by the addition of a synthetic antioxidant during the isolation procedure. Since the mobility of proteins in the sodium dodecyl sulfate–polyacrylamide gel system is a function of a protein's molecular weight, charge, and shape, it is not possible to deduce precisely the molecular details of lipid autoxidation modification of the proteins. Isoelectric focusing in polyacrylamide gel might appear to be a useful approach to analysis of the change in charge of proteins after lipid autoxidation damage. However, antioxidant prevents the free radical polymerization of the gel when the protected proteins are mixed with the acrylamide monomer solutions. Application of the proteins to the gel after polymerization also proved to be unsatisfactory because many of the proteins failed to enter the gel after an electric field was applied.

3,5-Di-*tert*-butyl-4-hydroxybenzylalcohol proved to be a useful antioxidant. In addition to its effectivity, it has other favorable features. Due to the sterical hinderance of the phenolic hydroxylic groups, it is chemically relatively inert, and unless heated, is stable to autoxidation. In addition, the dissociation constant of the phenol appears to be extremely small. Only at about pH 12 is a pink color formed in the formation of the phenolate. On the other hand, the compound has a very limited solubility in water. One chemical manufacturer lists its solubility at 35 μg/ml. We found

that 5–10 μg/ml was about the limit of water solubility. However, because of the good solubility of this antioxidant in non-polar solvents, its local concentration in the hydrophobic phase of a membrane may be greater than that in the surrounding aqueous phase.

Historically, antioxidants, especially those of the sterically hindered phenol group, have been designed for hydrophobic systems such as rubbers, plastics, fats and oil. However, for biochemical purposes and because of the possible biological importance of antioxidants in the prevention of aging process[11–13], perhaps new sterically hindered phenols with greater water solubility should be synthesized. Introduction of hydroxylic groups in the aliphatic side chains may serve this purpose without affecting the antioxidant effectivity.

ACKNOWLEDGEMENTS

Contribution No. 1557 of the Laboratory of Molecular Biology and Genetics of the University of Wisconsin. This investigation was supported by the College of Agriculture and Life Sciences, the Graduate School, the American Cancer Society (E-640), the National Institute of Health (GM-15751) and by the Deutsche Forschungsgemeinschaft. The technical assistance of L. Kort, M. Munkres, and B. Sauer is acknowledged. Valuable suggestions by Dr W. Behr on the topic of antioxidants are acknowledged.

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