

# Identification of the Major Oxidatively Damaged Proteins in *Escherichia coli* Cells Exposed to Oxidative Stress\*

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In the present study we have analyzed protein oxidation on *Escherichia coli* when these cells were submitted to different stress conditions such as hydrogen peroxide, superoxide-generating compounds, and iron overloading. Carbonyl groups on oxidized cell proteins were examined by Western blot immunoassay. When anaerobically grown *E. coli* cells were exposed to hydrogen peroxide stress, alcohol dehydrogenase E, elongation factor G, the heat shock protein DNA K, oligopeptide-binding protein A, enolase, and the outer membrane protein A were identified as the major protein targets. A similar immunostained band pattern was found when cells were shifted from anaerobic to aerobic conditions in the presence of different concentrations of iron; it is relevant to note that oxidation of outer membrane protein C, not observed in peroxide stress conditions, was clearly detected as the concentration of iron was increased in the culture media. The hydrogen peroxide stress performed under aerobic conditions affected the  $\beta$ -subunit of  $F_0F_1$ -ATPase; the rest of the oxidized protein pattern was very similar to that found for anaerobic conditions, with the exception of alcohol dehydrogenase E, a protein not synthesized aerobically. Cells submitted to superoxide stress using menadione showed a more specific pattern in which elongation factor G and the  $\beta$ -subunit of  $F_0F_1$ -ATPase were affected significantly. When paraquat was used, although the degree of oxidative damage was lower, the same two modified proteins were detected, and DNA K was also clearly damaged. Cell viability was affected to different extents depending on the type of stress exerted. The results described in this paper provide data about the *in vivo* effects of oxidative stress on protein oxidation and give insights into understanding how such modifications can affect cellular functions.

Oxidative damage to DNA, RNA, proteins, and cell membrane occurs when the cellular concentration of reactive oxygen species exceeds the capacity of the cell to eliminate them. Aerobic prokaryotic and eukaryotic organisms have developed a set of cell defense systems to mitigate the damaging effects of reactive oxygen species (1). Genetic response systems have

been studied extensively in *Escherichia coli*, and they are grouped in two regulons, the *oxyR* and the *soxRS*. The *soxRS* locus controls the response to superoxide-generating agents; the *oxyR* gene controls the response to hydrogen peroxide stress (2). The response to oxidative stress conditions overlaps with other stress responses such as heat shock, starvation, and the SOS response.

Despite these defense systems, reactive oxygen species have been shown to be involved in oxidative cell damage in physiological and pathological processes such as aging (3–8), apoptosis (9, 10), neurodegenerative diseases (11, 12), and iron metabolism imbalance (13).

The highly reactive hydroxyl radicals are generated by the presence of hydrogen peroxide and iron (Fenton reaction) or by superoxide anion, hydrogen peroxide, and a metal catalyst (Haber-Weiss reaction). These species oxidize proteins, resulting in the formation of carbonyl groups in some amino acid residues (14). These proteins can be detected by Western blotting taking advantage of the fact that the carbonyl groups generated can react with 2,4-dinitrophenylhydrazine, and this group is recognized by anti-2,4-dinitrophenol (DNP)<sup>1</sup> antibodies; protein carbonyls are one of the modifications described in oxidized proteins and are used successfully to measure oxidative stress in tissue, cell, and protein samples (15). With the aim of obtaining a better comprehension of the *in vivo* effects of oxidative stress, we have analyzed the protein oxidative damage and the identity of the main oxidatively damaged proteins of *E. coli* when these cells are submitted to stressing conditions.

## EXPERIMENTAL PROCEDURES

**Materials**—Hydrogen peroxide 30% solution, menadione sodium bisulfite, paraquat (methyl viologen), 2,4-dinitrophenylhydrazine, protease from *Staphylococcus aureus* V8, and 3-(2-pyridil)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (Ferrozine®) were purchased from Sigma. Acrylamide/bisacrylamide solution was supplied by Bio-Rad. Polyvinylidene difluoride membranes (Immobilon Pseq) were from Millipore Corp. The chemiluminescent detection kit (Western Light) was from Tropix.

**Organisms and Growth of Cells**—The strain used was *E. coli* K12, strain ECL1 (kindly provided by E. C. C. Lin). The mineral medium used consisted of 34 mM  $\text{NaH}_2\text{PO}_4$ , 64 mM  $\text{K}_2\text{HPO}_4$ , 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 1  $\mu\text{M}$   $\text{FeSO}_4$ , 0.1 mM  $\text{MgSO}_4$ , and 10  $\mu\text{M}$   $\text{CaCl}_2$ . Glucose was added to a 20 mM final concentration. Growth of cells was carried out in bottles filled to the top with culture medium and stirred with a magnet (anaerobic cultures) or in 200-ml Erlenmeyer flasks, 1/10 filled and aerated in a rotary shaker at 250 rpm (aerobic cultures). All cultures were grown at 37 °C to an  $A_{420\text{ nm}}$  of 1 and 0.5 in anaerobic and aerobic conditions, respectively. To calculate cell viability, appropriate dilutions of the cultures were plated on LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar), incubated at 37 °C, and colony-forming units determined. The level of cell viability of stressed cultures was determined with reference to control cultures taken at the same absorbance.

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<sup>1</sup> The abbreviations used are: DNP, 2,4-dinitrophenol; HPLC, high performance liquid chromatography; EF, elongation factor; OMP, outer membrane protein.

**Stress Conditions**—Hydrogen peroxide was added to the cultures at final concentrations of 0.5, 1, and 2 mM. Menadione and paraquat were added to aerobic cultures to a final concentration of 20 mM and treated for 1 h. Ferrozine was added to the aerobic cultures at 0.2 and 0.4 mM final concentration. For shifts from anaerobic to aerobic conditions, cultures were taken at the end of the exponential phase, diluted 10-fold, and incubated as described before. Iron (as ammonium ferrous sulfate) was supplemented at 40 and 100  $\mu$ M. Samples were harvested at a  $A_{420 \text{ nm}}$  of 0.5.

**Preparation of Cell Extracts**—One-ml samples were taken from the cultures at the indicated times, centrifuged at  $13,000 \times g$  for 3 min, and resuspended in 50  $\mu$ l of 10 mM HEPES buffer, pH 7, containing 6% SDS and heated at 100 °C for 3 min. The protein content was measured using the Bio-Rad Dc protein assay for SDS-containing samples. Derivatization with dinitrophenylhydrazine was performed as published (15). These samples were ready to apply to SDS-polyacrylamide gel electrophoresis or to HPLC gel filtration chromatography.

**Enzyme Activities**—Alcohol dehydrogenase activity was determined according to the method described by McPhedran *et al.* (16). Enolase was assayed according to the method of Maitra and Lobo (17). ATPase activity was measured using the method of Pullman *et al.* (18). All of these activities were determined in crude extracts obtained by ballistic disintegration (19). Ultrasonic disintegration was discarded because unspecific protein oxidation was observed.

**Analytical and Preparative SDS-Polyacrylamide Gel Electrophoresis**—For analytical purposes cell extracts containing 10  $\mu$ g of protein were applied to 5% stacking gel and separated in a 9% resolving gel. Electrophoresis was performed according to the denaturing discontinuous buffer system of Laemmli (20). Proteins were stained with Coomassie Brilliant Blue (21).

Preparative electrophoresis was carried out in a model 491 PrepCell (Bio-Rad) using a gel tube with a 28-mm inner diameter. A volume of 6 ml of dinitrophenylhydrazine-derivatized cell extracts containing 20 mg of total protein was loaded into a 4% stacking gel (2 cm high) and separated in a resolving gel (8 cm high) in which the acrylamide concentration was varied according to the desired molecular weight interval resolution. Electrophoresis was also conducted according to the Laemmli system at a constant current of 75 mA until the dye front (unbound dinitrophenylhydrazine) reached the bottom of the gel. Subsequently, the elution chamber outlet was connected to a fraction collector by means of a peristaltic pump with a flow rate of 1 ml/min. Fractions of 3 ml were collected, and 15  $\mu$ l of each was analyzed by Western blotting to locate DNP-derivatized proteins. Fractions stored at -20 °C could be analyzed several times without loss of signal in the Western blot immunoassay.

**Immunodetection of Protein-bound 2,4-Dinitrophenylhydrazones**—Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to a polyvinylidene difluoride membrane using a semidry system. For immunodetection we followed the instructions outlined in the Tropix kit. The anti-DNP antibody was supplied by DAKO (V401) and used at a 1/2,000 dilution. The secondary antibody was a goat anti-rabbit antibody conjugated with alkaline phosphatase (Tropix) used at a 1/25,000 dilution.

**Peptide Mapping**—Proteins of interest (10  $\mu$ g) were subjected to limited proteolysis for 30 min according to the method of Cleveland *et al.* (22) with either 0.5  $\mu$ g of *S. aureus* V8 protease or 0.1  $\mu$ g of subtilisin at 25 °C for 30 min. Peptides were separated by SDS-polyacrylamide gel electrophoresis in duplicate gels, blotted to polyvinylidene difluoride membranes, and analyzed either by Western blotting as described previously or stained with Coomassie Brilliant Blue. Selected anti-DNP immunostained peptide bands were excised from Coomassie Brilliant Blue-stained membrane and sequenced.

**Protein and Peptide Sequencing**—Proteins of interest were identified by Edman degradation (10–15 cycles) using a Beckman LF3000 sequencer equipped with a phenylthiohydantoin derivative analyzer (System Gold, Beckman). To eliminate any neighboring bands, samples of whole protein (obtained by preparative electrophoresis) or peptide (obtained after limited proteolysis) were electrophoresed and electroblotted to polyvinylidene difluoride membranes. In the case of oxidized proteins, the peptide bands that gave a clear signal after anti-DNP immunostain assay were chosen to assess the identity of these proteins further. The stained band was sliced out of the membrane and washed three times in Milli-Q water to reduce to background levels of Tris and glycine from electrophoresis and electroblotting steps.

**Quantification of Protein Carbonyls**—The protein carbonyl content of crude extracts and purified proteins was measured according to the dinitrophenylhydrazine derivatization method described by Levine *et al.* (23). Quantification was performed using a Shodex KW803 HPLC

TABLE I  
Carbonyl content of crude extracts and cell viability after exposure to oxidative stress conditions

Carbonyl content was determined by HPLC gel filtration chromatography. The results are given in nmol of carbonyl/mg of protein. The values summarized here are the mean value of three separate experiments with a variation of  $\pm 0.05$ . Cell viability is the percentage of the corresponding anaerobic and aerobic control conditions and is the mean value of three independent experiments with a variation of  $\pm 2\%$ .

Culture conditions	Carbonyl content Survival	
	nmol/mg	%
Anaerobic	1.9	100
Anaerobic + hydrogen peroxide (2 mM)	5.3	68
Aerobic	3.0	100
Aerobic + hydrogen peroxide (2 mM)	4.5	93
Aerobic + iron overloading (100 $\mu$ M)	4.3	90
Aerobic + menadione (20 mM)	5.3	60
Aerobic + paraquat (20 mM)	3.7	12

gel filtration column at flow rate of 0.6 ml/min at 25 °C. Absorbance at 276 and 370 nm was monitored using a Waters 996 diode array detector. Values of carbonyl content for crude extracts are given in nmol/mg of protein; those for purified proteins are given in mol/mol of subunit.

## RESULTS

**Hydrogen Peroxide-induced Oxidative Damage**—Anaerobic and aerobic cultures of *E. coli* were challenged with 2 mM hydrogen peroxide, and the resulting oxidative damage was analyzed by Western blotting. Carbonyl groups were measured as described under “Experimental Procedures.” Anaerobic cultures showed a transient growth arrest of 2 h (not shown), a loss of 30% of cell viability, and a near 3-fold increase in the protein carbonyl concentration in crude extracts (Table I). Fig. 1 shows the time course for increased protein damage resulting from the exposure of an anaerobic culture to hydrogen peroxide. Protein staining (panel A) and anti-DNP immunostaining (panel B) are shown. Time course experiments performed with 0.5 and 1 mM  $H_2O_2$  gave the same oxidative pattern after longer periods of time of exposure to hydrogen peroxide (not shown). Protein bands were sequenced as indicated under “Experimental Procedures,” and the results obtained are summarized in Table II. As can be observed, proteins involved in different cell processes such as glucose catabolism (alcohol dehydrogenase E and enolase), chaperone function (DNA K), protein synthesis (EF-G), and an outer membrane protein (OMP A) have been identified as major targets for oxidative stress. The values of carbonyl content given in this table for each protein showed a 5–10-fold increase with respect to control values for unoxidized proteins, whose values ranged between 0.05 and 0.1 mol of carbonyl/mol of protein. The enzymatic activities of alcohol dehydrogenase E and enolase decreased, respectively, 80% and 25% after a 1-h treatment. It is interesting to observe that major bands with apparent molecular masses of 38 kDa and 42 kDa, indicated in Fig. 1A, are clearly resistant to oxidation by hydrogen peroxide. These proteins were purified and identified by  $NH_2$ -terminal sequence as a major outer membrane protein, OMP C, and EF-Tu.

Aerobic metabolism generates reactive oxygen species and a set of antioxidant molecules to prevent oxidative damage (24, 25). Nevertheless, under oxidative stress conditions, proteins and other macromolecules become oxidized. The concentration of carbonyl content in aerobic cultures was 1.5 times higher than the values found in anaerobic conditions (Table I). When aerobic cultures were challenged with 2 mM hydrogen peroxide, a transient growth arrest of 2–3 h was also observed (not shown). Concentration of protein carbonyls showed an increase of 50% with respect to aerobic control conditions. Fig. 1D shows that as in the case of anaerobic culture, time-dependent protein oxidative damage was observed. Apart from alcohol dehydro-

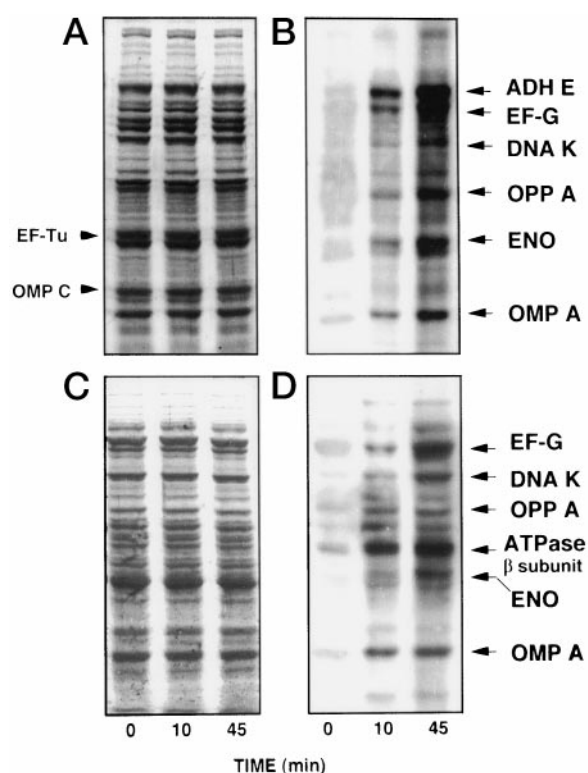


FIG. 1. Detection of oxidatively modified proteins of *E. coli* cells after 2 mM  $H_2O_2$  challenge. At the end of the exponential phase, *E. coli* cells grown anaerobically on glucose were challenged with 2 mM  $H_2O_2$ . Samples were taken at the indicated times, and cell extracts were prepared as described under "Experimental Procedures." Protein stain (panel A) and anti-DNP immunoassay (panel B) are shown. Identified proteins (corresponding to those summarized in Table II) are shown in panel B. In panel A, two major unoxidized protein bands identified as EF-Tu and OMP C are indicated. The effect of 2 mM  $H_2O_2$  on aerobically grown cells is shown in panels C (protein stain) and D (anti-DNP immunostain). Note the presence of the oxidized ATPase  $\beta$ -subunit.

TABLE II  
Identification and carbonyl content of proteins oxidized by hydrogen peroxide stress under anaerobic conditions

Oxidized proteins detected by anti-DNP immunoassay were purified and identified as described under "Experimental Procedures." Carbonyl content was determined by HPLC gel filtration chromatography. The results are given in mol of carbonyl/mol of subunit of each protein purified from control and stressed cultures after a 1-h treatment. ADH-E, alcohol dehydrogenase E; OPP A, oligopeptide-binding periplasmic protein.

Molecular mass kDa	Identified protein	Carbonyl content	
		Control	Oxidized
		nmol/mol	
96	ADH E	0.05	0.52
78	EF-G	0.04	0.61
70	DNA K	0.03	0.63
55	OPP A	0.03	0.43
46	Enolase	0.04	0.92
35	OMP A	0.07	0.35

genase E, a protein not synthesized when oxygen is present, one prominent band of 50 kDa was detected and identified as the  $\beta$ -subunit of  $F_0F_1$ -ATPase. This protein showed a decrease in enzymatic activity of 30% and 4.1 mol of carbonyl/mol of subunit, after stressing conditions. The rest of immunodetected protein bands were the same as those presented in Fig. 1B.

**Shift from Anaerobic to Aerobic Conditions: Effect of Iron Overload**—Changing cultures from anaerobic to aerobic conditions requires an induction of antioxidant molecules to protect the cell from superoxide and hydrogen peroxide generated un-

der this new condition (24). Superoxide can be converted to hydrogen peroxide, and this, in turn, can react with transition metals such as iron to form hydroxyl radical-promoting cell damage (14, 26, 27). A deregulation in iron metabolism in *E. coli fur* mutants has been demonstrated to produce an oxidative stress resulting from iron overload (28). We have tested the oxidative damage promoted by shifting cultures from anaerobic to aerobic conditions in minimal medium supplemented with iron at a 40–100  $\mu$ M final concentration once in aerobic conditions. No addition was made in the aerobic control. Fig. 2 shows the results obtained with samples tested at 20 and 60 min after the shift. The initial anaerobic conditions are shown in 0 time lane 0. It is clear (Fig. 2B) that a slight oxidative damage is observed after 60 min in aerobic control conditions and that the oxidative damage was greater as the iron concentration increased. It is evident that there is a great similarity with the pattern obtained for the hydrogen peroxide stress. The identified proteins are shown in the figure. It is worth noting that OMP C, which was not oxidized under hydrogen peroxide stress, was clearly damaged under iron overloading conditions.

The carbonyl content obtained after supplementing the culture with 100  $\mu$ M iron for 60 min is 1.4 times higher than aerobic control conditions. A decrease of 10% of cell viability was observed (Table I).

**Superoxide Stress Conditions**—Electrophilic quinone compounds can be reduced *in vivo* to semiquinones, which in turn, are able to reduce oxygen to superoxide anion radical, regenerating the oxidized quinone (29). To study the oxidative damage promoted by redox cycling agents, 20 mM menadione or paraquat was added to aerobically growing *E. coli* cells. In contrast to hydrogen peroxide stress conditions, a definite growth arrest was observed in these experiments (not shown). Table I shows protein oxidative damage after challenging the cells for 60 min. This treatment increased the value of the aerobic control conditions by 2.3 mol of carbonyl/mg of protein. The electrophoretic pattern of specific oxidative damage found was time- and dose-dependent (not shown). Fig. 3B shows that two of the protein bands became highly oxidized; purification of these proteins allowed their identification as EF-G and  $\beta$ -subunit of  $F_0F_1$ -ATPase. The results of the same experiment performed with paraquat showed a slight increase in total protein carbonyl content and a dramatic decrease in cell viability (Table I). The Western blot pattern obtained (Fig. 3B), indicated that in addition to the above mentioned proteins, oxidized DNA K is clearly detected. Table III indicates the carbonyl content of these three proteins after menadione or paraquat treatment. The values obtained for EF-G with menadione treatment were several times higher than those observed with paraquat or  $H_2O_2$  treatment. The  $\beta$ -subunit of  $F_0F_1$ -ATPase exhibited the highest value observed among all of the data presented (8.1 mol of carbonyl/mol of protein) with a concomitant decrease of 65% in enzymatic activity.

To study whether oxidation of these proteins depends on the iron present in the cell, cultures were grown in the presence of an iron chelator, Ferrozine, at 0.2 and 0.4 mM final concentration, and a menadione-induced oxidative stress was performed. Fig. 4 shows a clear decrease in the oxidation level of EF-G and  $\beta$ -subunit of  $F_0F_1$ -ATPase compared with control conditions where no Ferrozine was added.

## DISCUSSION

Exposure of *E. coli* cells to oxidative stress conditions resulted in selective protein oxidative damage. This was evident in superoxide stress conditions where three proteins were clearly oxidized. Protein oxidation observed with anti-DNP immunoassay did not depend on the amount of a particular protein because major protein bands such as EF-Tu or OMP C



FIG. 2. Effect of aerobic metabolism and iron overload on protein oxidation. Detection of oxidatively modified proteins after a shift from anaerobic to aerobic conditions is presented in panel B. Increasing concentrations of iron (as ammonium ferrous sulfate) are indicated. In each case, samples were taken at the indicated times, and cell extracts were prepared as described under "Experimental Procedures." Protein stain is shown in panel A.

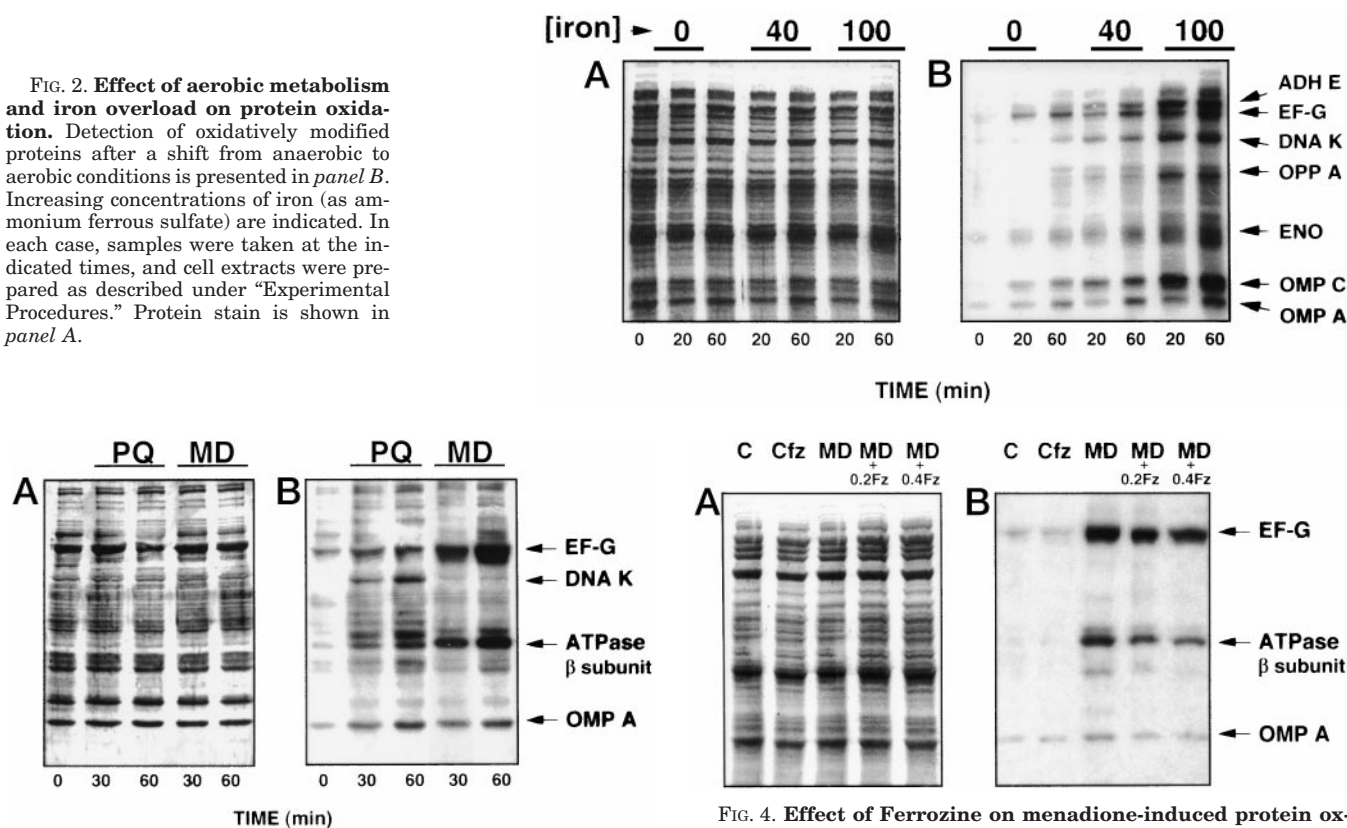


FIG. 3. Protein oxidative damage promoted by menadione and paraquat. Cultures of aerobically growing *E. coli* cells were challenged with menadione (MD) or paraquat (PQ) (20 mM each), and protein carbonyl detection was performed after 30 and 60 min of exposure to these superoxide generating compounds (panel B). Protein stain is shown in panel A.

TABLE III

Identification and carbonyl content of proteins oxidized by menadione and paraquat stress under aerobic conditions

Oxidized proteins detected by anti-DNP immunoassay were purified and identified as described under "Experimental Procedures." Carbonyl content was determined by HPLC gel filtration chromatography. The results are given in mol of carbonyl/mol of subunit of each protein purified from stressed cultures.

Molecular mass <i>kDa</i>	Identified protein	Carbonyl content	
		Menadione	Paraquat
		<i>mol/mol</i>	
78	EF G	2.20	0.53
70	DNA K		0.42
50	F <sub>0</sub> F <sub>1</sub> -ATPase (β-subunit)	8.12	2.21

remained undamaged after hydrogen peroxide, menadione, or paraquat treatments. Furthermore, OMP C was only oxidized in the iron overload experiments, indicating once again the selective nature of the protein oxidation observed. It should be noted that oxidized proteins present in very low quantities are not detected easily unless they were highly oxidized. The detection limits are discussed by Shacter *et al.* (15).

The results presented in Table I show that the total protein oxidative damage, assessed by quantification of protein carbonyl content, depends on the type of stress produced. Even in the case of shifting an anaerobic culture to aerobic conditions there was, once in equilibrated aerobic growth conditions, an increase in carbonyl content from 1.9 to 3. It is interesting to note that the increase in the protein carbonyl content of crude extracts of anaerobic cultures challenged with 2 mM hydrogen peroxide is greater than that obtained from aerobic cultures. One explanation for this could be that aerobic cultures have

FIG. 4. Effect of Ferrozine on menadione-induced protein oxidation. *E. coli* cells grown aerobically on glucose in the presence of 0.2 or 0.4 mM Ferrozine were challenged with 20 mM menadione. After a 30-min treatment, crude extracts were analyzed to detect protein oxidative damage. Panel C, aerobic control conditions; Cfz, aerobic control conditions in the presence of 0.4 mM Ferrozine. MD, cells treated with 20 mM menadione; MD+0.2 Fz, cells grown in the presence of 0.2 mM Ferrozine and treated with 20 mM menadione; MD+0.4 Fz, the same treatment on cells grown in the presence of 0.4 mM Ferrozine.

antioxidant enzymes (such as catalase) already induced, so the formation of hydroxyl radicals by Fenton reaction will decrease, thus reducing the extent of protein and other macromolecule oxidation. In this situation cell viability will be better preserved than in anaerobic cultures (Table I).

It is clear that loss of cell viability depends not only on the protein damage but also, to a great extent, on DNA damage (28). However, loss of cell viability is higher as the carbonyl content increases except for the case of paraquat where there is a moderate increase in carbonyl content and a high loss of cell viability. One possible explanation for this result could be the poor induction of catalase by paraquat in contrast to the high induction exerted by menadione. In both situations similar values of SOD activity have been reported (29, 30).

Major immunostained protein bands observed with anti-DNP immunoassay have been selected to be identified by NH<sub>2</sub>-terminal sequencing; these proteins were purified by preparative electrophoresis. Although during electrophoresis, the pH in the resolving gel is unfavorable for stability of hydrazone derivatives, we have not observed a significant loss of signal compared with immunoassays of whole crude extracts.

Oxidatively damaged proteins tend to be eliminated more rapidly by proteases than their normal counterparts (31); this increased susceptibility to proteolytic degradation might make detection of highly modified proteins more difficult. Nevertheless, this does not seem to be the case in our study for two reasons. First, our observations in time course experiments indicated that the signal for each oxidized protein band increases constantly soon (5 min) after challenging cultures with hydrogen peroxide or redox cycling agents. Second, normal rat

liver epithelia cells challenged with hydrogen peroxide have been shown to cause a transient depression in protein degradation with respect to control cells; thereafter proteolysis was increased significantly (32).

The results obtained indicated that EF-G became oxidized in all tested stress conditions, including aerobic growth. Recent data published by Ayala *et al.* (33) showed the oxidative damage of EF-2, the eukaryotic counterpart of EF-G, by exposure of hepatocytes to cumene hydroperoxide. This treatment caused a doubling in carbonyl content (from 0.3 to 0.6 mol of carbonyl/mol) and a concomitant inhibition of EF-2 activity. In our case, the concentration of carbonyl groups detected for EF-G after peroxide (0.6 mol of carbonyl/mol) and superoxide stress conditions (2.2 mol carbonyl/mol with menadione and 0.5 mol of carbonyl/mol with paraquat) clearly account for the inactivation of this translocase.

Sequence data from a 36-kDa peptide obtained after limited proteolysis of EF-G with V8 protease indicated major oxidative damage to the carboxyl-terminal region (domains 4–5). Domain 4 would have the residue where the ADP-ribosylation of EF-2 occurs (34, 35). If such a reaction takes place in EF-G, this could promote the observed oxidative damage to this protein because (i) ADP-ribosylation increases under oxidative stress (36, 37); (ii) this nucleotide binds easily to divalent metal ions (38); and (iii) we proved that the oxidation of EF-G depends on the availability of iron.

*In vivo* oxidation of the  $\beta$ -subunit of  $F_0F_1$ -ATPase was detected in aerobically grown cells under menadione, paraquat, and hydrogen peroxide stress conditions. The addition of Ferrozine, an iron chelator, to the cultures stressed with menadione diminished the degree of oxidative damage to this subunit (and also to the EF-G protein), thus indicating that a Haber-Weiss reaction is involved in the oxidation mechanism of both proteins (39). This finding agrees with previous data reporting *in vitro* metal-catalyzed oxidation of mitochondrial ATP synthase (40, 41) or inactivation by exposure of bovine heart sub-mitochondrial particles to continuous flux of hydroxyl radical or superoxide anion radical or a mixture of the two (42). Our finding also supports the early depletion of ATP levels observed *in vivo* under oxidative stress conditions (43, 44) because the high carbonyl content of the catalytic  $\beta$ -subunit is consistent with the enzyme inactivation observed.

Enzymes involved in the glycolytic flux are also oxidized; alcohol dehydrogenase E in anaerobic cultures and enolase in both aerobic and anaerobic conditions were identified as targets for the oxidative process. These data support the observation that hydrogen peroxide causes a decrease in the activity of glycolytic enzymes in transformed eukaryotic cells (37).

Metal-catalyzed oxidation, a site-specific protein oxidation mechanism (14), would be responsible for the oxidation of alcohol dehydrogenase E, an iron-containing enzyme. The sequence of the immunostained peptide band (35 kDa) obtained after limited proteolysis, which was used to confirm the identity of the target protein, corresponds to the carboxyl terminus of the enzyme. This domain, which includes an iron binding motif, has sequence homology with the microbial iron-activated group III of dehydrogenases (45) such as propanediol oxidoreductase of *E. coli* and alcohol dehydrogenase II of *Zymomonas mobilis*. We proved that these two enzymes were inactivated by metal-catalyzed oxidation resulting from modification of histidine 277, which lies 10 residues apart from the iron-chelating motif His-X-X-His (46). The specificity of this mechanism explains that the oxidative damage measured in alcohol dehydrogenase E must affect the active center directly; however, the high oxidation observed in ATPase probably affects amino acid residues not directly involved in the catalytic

mechanism. The same explanation could also apply to enolase.

Enolase was described as a heat shock protein in yeast because it shares some degree of homology with DNA K (47). Recently, enolase and DNA K have been found associated to RNase E in a multicomponent ribonucleolytic complex (RNA degradosome) essential for RNA processing and degradation (48, 49). Increased degradation of 16 S ribosomal RNA has been described to occur in mitochondria in response to hydrogen peroxide exposure (50). In this situation, proteins involved in the RNA degradosome would be oxidized preferentially as a consequence of the reactive oxygen species generated by metal ions associated with RNA (51).

DNA K oxidation may also be explained on the basis that as a molecular chaperone it may interact with proteins damaged (unfolded) by stress conditions. In fact, it has been suggested (52) that oxidized proteins contain reactive species that can damage other proteins. The oxidation of this chaperone due to interaction with nascent polypeptides with oxidized amino acid side chains was ruled out as no changes in anti-DNP immunostained pattern were observed in cultures treated previously with chloramphenicol to stop protein synthesis.

The peptide used to identify further OMP A protein indicated that oxidative damage was located in the periplasmic COOH-terminal domain. Interestingly, oligopeptide-binding periplasmic protein A protein is also oxidized in peroxide and iron overload experiments. Even though the mechanisms that cause this oxidative damage are unclear, it seems that the membrane could play a role in protecting the protein domains embedded in it. Experiments carried out with monoamine oxidase seem to indicate this (53). This would also apply to undamaged OMP C protein under hydrogen peroxide stress conditions. The oxidative damage to OMP C observed in iron overload conditions suggests that this protein is involved in iron uptake in such overdose conditions; the acidic internal loop of this porin (54) may be responsible for binding metal ions and promoting the formation of reactive oxygen species, which would account for the oxidation detected.

It is known that oxidative stress induces GADD (growth arrest and DNA damage) genes (55, 56) to stop growth, which, in turn, minimizes cell damage. The oxidative modification of EF-G and the  $\beta$ -subunit of ATPase clearly plays an important role in the above mentioned mechanism because ATP and protein synthesis decrease in this situation.

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