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Carbonylated Proteins Are Detectable Only in a Degradation-Resistant Aggregate State in *Escherichia coli*[▽]

Etienne Maisonneuve,[1] Laetitia Fraysse,[1] Sabrina Lignon,[2] Laure Capron,[1] and Sam Dukan[1]*

Laboratoire de Chimie Bactérienne, Université de la Méditerranée, UPR 9043-CNRS, 31, Chemin Joseph Aiguier, 13402 Marseille, France,[1] and Service de spectrométrie de masse, 31, Chemin Joseph Aiguier, 13402 Marseille, France[2]

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Carbonylation is currently used as a marker for irreversible protein oxidative damage. Several studies indicate that carbonylated proteins are more prone to degradation than their nonoxidized counterparts. In this study, we observed that in *Escherichia coli*, more than 95% of the total carbonyl content consisted of insoluble protein and most were cytosolic proteins. We thereby demonstrate that, in vivo, carbonylated proteins are detectable mainly in an aggregate state. Finally, we show that detectable carbonylated proteins are not degraded in vivo. Here we propose that some carbonylated proteins escape degradation in vivo by forming carbonylated protein aggregates and thus becoming nondegradable. In light of these findings, we provide evidence that the accumulation of nondegradable carbonylated protein presented in an aggregate state contributes to the increases in carbonyl content observed during senescence.

Proteins can become modified by a large number of reactions involving reactive oxygen species. Among these modifications, carbonylation has attracted a great deal of attention due to its irreversible and irreparable nature. Carbonyl derivatives are formed by a direct metal-catalyzed oxidative attack on the amino acid side chains of proline, arginine, lysine, and threonine (2). With the development of sensitive immunochemical methods for the detection of protein carbonyls, the presence of such groups has been extensively used as a marker of reactive oxygen species-mediated protein oxidation (17) and associated with a large number of age-related disorders, including Parkinson's disease, Alzheimer's disease, and cancer (5, 17). While carbonylated proteins are considered soluble in healthy cells, a decrease in proteolysis has been suggested to provoke increases in levels of carbonylated protein which may form aggregates during aging or disease (5, 12–14). Interestingly, in starvation, aging, or disease states, only some proteins appear more prone to carbonylation (3, 11, 17, 24). Finally, in vivo studies using exponentially grown *Escherichia coli* cells or other organisms indicate that carbonylated proteins are more prone to degradation than their nonoxidized counterparts (10, 14–16, 18, 21). Moreover, several groups have postulated that carbonylation may act as a tag for degradation (10, 15, 21).

Here, contrary to observations made previously by Dukan et al. (10) and other groups, we show, using *E. coli* exponential- or stationary-phase cells, that carbonylated proteins are mainly cytosolic and that most of them are detectable in an aggregate state that does not degrade with time. As a consequence, we propose that increases in carbonyl content observed during bacterial senescence could be due at least in part to the accumulation of nondegradable carbonylated proteins presented in an aggregate state.

* Corresponding author. Mailing address: UPR 9043, 31, Chemin Joseph Aiguier, 13402 Marseille, France. Phone: 00 33 (4) 91 16 44 08. Fax: 00 33 (4) 91 16 89 14. E-mail: sdukan@ibsm.cnrs-mrs.fr.

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MATERIALS AND METHODS

Bacterial strain and medium. *E. coli* MG1655 was grown aerobically or anaerobically in liquid Luria-Bertani (LB) medium in a rotary shaker at 37°C and 200 rpm.

Protein preparation. Exponential (optical density at 600 nm [OD_{600}] = 0.5)- or stationary (24-h or 48-h)-phase-grown cells from, respectively, 10-liter or 1-liter cultures were harvested and then washed twice with phosphate buffer (pH 7, 0.05 M, 4°C) by centrifugation at 5,500 × *g* for 20 min at 4°C. Cells were resuspended in phosphate buffer and lysed by four cycles in a French press. Next, all samples were treated with 0.2 mg/ml DNase and 50 μg/ml RNase. Immediately afterwards and following various centrifugation times at 18,000 × *g*, we obtained SN_4 (limpid supernatant obtained after 4 min of centrifugation), SN_{30} (limpid supernatant obtained after 30 min of centrifugation), LP (large particles forming the large pellet obtained between 0 and 4 min of centrifugation), and SP (small particles forming a pellet obtained between 4 and 30 min of centrifugation). LP and SP were dried by speed vacuum and resolubilized in rehydration buffer before being subjected to isoelectric focusing. Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce). To evaluate the protein carbonyl content in each fraction, soluble or insoluble, we took two measurements. First, we measured the carbonyl content per mg of total protein in each fraction. Second, we measured the relative carbonyl content of each fraction, with SN_{30}, LP, and SP made up to the original volume of crude extract (CE) with phosphate buffer.

Two-dimensional (2D) SDS-polyacrylamide gel electrophoresis and carbonylation assays. Protein samples (100 μg) in rehydration buffer containing 7 M urea, 2 M thiourea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 100 mM dithiothreitol, 0.2% (vol/vol) ampholyte 3-10 (Bio-Rad), and 0.01% (wt/vol) bromophenol blue were adsorbed onto 17-cm immobilized pH gradient strips (pH 3 to 10, linear). After isoelectric focusing, the strips (i) were subjected to equilibration for 20 min in 60 mM Tris base containing 2.3% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.1% (wt/vol) bromophenol blue or (ii) were first 2,4-dinitrophenol derivatized (4). Molecular weight separation was achieved on 9% acrylamide gel by use of the Protean II XL Multi-Cells slab gel SDS-polyacrylamide gel electrophoresis system (Bio-Rad). Proteins were either stained by silver nitrate for mass spectrometry analysis (Amersham) or transferred onto a polyvinylidene difluoride membrane. Carbonylated proteins were detected as described previously (10).

Identification of proteins and carbonylated proteins by mass spectrometry. Excised silver-stained spots were destained using the ProteoSilver destainer kit (Sigma) and digested with trypsin (Promega, Madison, WI) as previously described (22). Proteomic analysis was performed by liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry as previously described (20) with one modification. Here, the oxidation modifications of turboSEQUEST search parameters were used on residues of amino acids methionine, proline,

threonine, arginine, and lysine (M-P-T-R-K), with mass variations of +16, +16, −2, −43, and −1 Da, respectively.

Anaerobiosis experiments. Exponentially (OD_{600} = 0.5) and aerobically grown cells were harvested by centrifugation at 5,500 × *g* for 10 min at 4°C and then resuspended in preincubated LB in a nitrogen gas chamber. These were then harvested after 0 h, 1 h, 2 h, and 3 h of anaerobiosis growth after the switch and washed twice with phosphate buffer (pH 7, 0.05 M, 4°C) by centrifugation at 5,500 × *g* for 20 min. Protein extraction was then performed as described above, though here, cell disruption was performed using a mini bead beater (Fisher Bioblock Scientific). Next, carbonyl detection was performed as described above, with all experiments performed in the nitrogen gas chamber.

Challenge conditions. As described previously (10), overnight cultures of *E. coli* were diluted 100-fold in LB with the addition of 10 μg of streptomycin/ml. Cells were then allowed to grow until the cell density reached an OD_{600} of 0.5. Protein synthesis was then stopped with spectinomycin (100 μg/ml) (T_0); carbonyl contents in the CE and supernatant after 30 min of centrifugation at 18,000 × *g* were measured at T_0 and after 2 hours of incubation (T_{120}).

RESULTS

Carbonylated proteins are mainly insoluble in exponentially grown cells. In vitro and depending on damage levels, oxidized proteins either remain soluble or coexist in an aggregate state (6, 14). Moreover, in vivo, no specifically carbonylated proteins are detected by use of a 2D gel electrophoresis approach on the supernatant extract from an exponentially grown *E. coli* culture (13). Taking into account these facts, we wondered if carbonylated proteins could be detected in the insoluble fraction. For this purpose, we split the CE obtained from an exponentially grown *E. coli* culture into several fractions, namely, SN_4, SN_{30}, LP, and SP, as previously described by Maisonneuve et al. (20). Next, as outlined in Materials and Methods, we quantified both the protein carbonyl content per mg of total protein within each fraction and also the relative amount of carbonyl content within each fraction. As depicted in Fig. 1A, we observed an important decrease in protein carbonyl content per mg of protein between CE and SN_4, and again between SN_4 and SN_{30}, suggesting that most carbonyl content sediments in pellets at between 0 and 30 min of centrifugation. We made a similar observation when CE was obtained via a mini bead beater and not a French press procedure, confirming that there was no influence of our experimental procedure on the observed results (data not shown). Finally, evaluations of the relative carbonyl content in each fraction confirmed that the carbonyl content in CE equaled the sum of SN_{30} (~3%), LP (~72%), and SP (~25%) (Fig. 1B). We also observed clearly that the majority of the protein carbonyl content present in the CE came from the LP fraction and to a lesser extent from the SP fraction, both representing, as described previously, just a small fraction of the total amount of protein present in the CE (20).

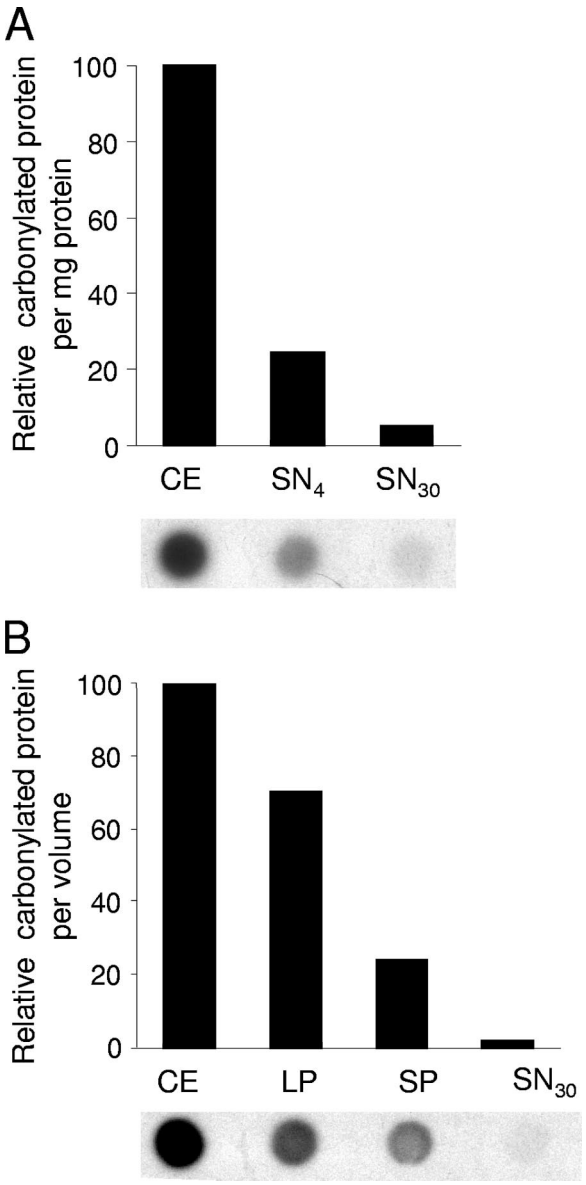

FIG. 1. Protein carbonyl content is within the insoluble fraction. MG1655 cells were grown in exponential phase (OD_{600} = 0.5), and extracts were obtained after 0 (CE), 4 (SN_4), and 30 (SN_{30}) minutes of centrifugation and processed for carbonylation assays. (A) Equal amounts of protein were loaded in each slot. (B) Similar volumes of LP, SP, SN_{30}, and CE were loaded in each slot. Carbonyl levels were quantified using Quantity One software (Bio-Rad), and analyses were repeated three times to confirm reproducibility.

Carbonylated proteins form protein aggregates in exponentially grown cells. To determine where the carbonyl content present in LP or SP came from, i.e., either from membrane-associated proteins and/or from aggregate proteins (20), we sought to identify the carbonylated proteins present in each of the fractions SN_{30}, SP, and LP. Using 2D gel electrophoresis and the same quantities of proteins as evaluated by use of Coomassie blue, we detected no carbonylated protein in SN_{30}, in agreement with previous observations (11) (Fig. 2). Interestingly, we detected carbonylated proteins in both pellet fractions, with the majority in LP and less in the SP. Using mass spectrometry analysis, we identified 23 different carbonylated proteins, which were found to be mostly cytosolic (Fig. 2 and Table 1). In addition, all proteins were localized at their apparently correct isoelectric points and molecular weights, indicating that there was no partial degradation. Altogether, these results show that in exponentially grown cells, carbonyl content comes mainly from cytosolic protein aggregates.

Detectable carbonylated proteins do not degrade over time. Upon finding carbonylated proteins mostly in an aggregate state, considered a state less prone to degradation (7, 8, 21), we wondered if these carbonylated proteins were degraded over

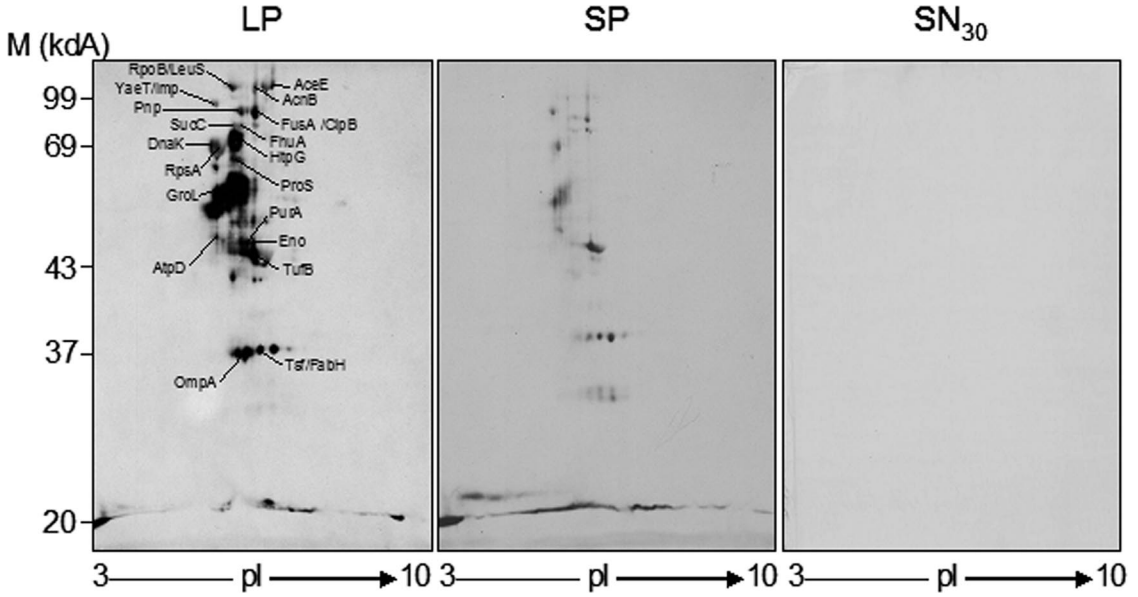

FIG. 2. Specific protein carbonylation of SN_{30}, LP, and SP from an exponentially grown *E. coli* culture. Extracts from a French press CE of an exponentially grown culture of *E. coli* (OD_{600} = 0.5) obtained after various centrifugation times were processed for resolution on 2D polyacrylamide. Autoradiograms were obtained after carbonyl immunoassay of proteins. The analysis was repeated three times to confirm reproducibility. Molecular masses (M) in kDa are indicated on the left.

time. For this purpose, aerobically grown exponential *E. coli* cells were switched to anaerobiosis, thereby preventing the generation of new carbonylated proteins, and carbonyl content was followed during growth (3 h). As depicted in Fig. 3A, no variation in carbonyl content per volume unit of culture could be detected in fractions CE, SN_4, and SN_{30}. As expected, however, when protein carbonyl content per mg protein was quantified, a decrease was observed during growth as a result of the dilution of carbonylated protein by de novo protein synthesis (data not shown). Moreover, in order to exclude the partial degradation of carbonylated protein over time, we followed the carbonylated proteins by use of 1D gel analysis. As depicted in Fig. 3B, we observed no variation in the level of carbonylated proteins over time. Altogether, these results indicate that the carbonylated proteins detected were not degraded over time.

TABLE 1. Data analysis of carbonylated protein identified by liquid chromatography-nanoelectrospray ionization-tandem mass spectrometry

Gene name	Protein	Accession no.[a]	% Sequence coverage
aceE	Pyruvate dehydrogenase (decarboxylase)	2506964	59
acnB	Aconitate hydrase B	2506132	51
atpD	Membrane-bound ATP synthase, F1 sector	114546	91
clpB	Heat shock protein-dependent protease, Hsp 100	54036848	34
dnaK	Chaperone Hsp70, DnaK	12512692	66
eno	Enolase (2-phosphoglycerate dehydratase)	68566315	62
fabH	3-Oxoacyl-[acyl carrier protein] synthase III	68566320	34
fhuA	Outer membrane pore protein	2507464	40
fusA	GTP-binding protein chain elongation factor EF-G	62288080	63
groL	Chaperone Hsp60, GroEL	30065518	80
htpG	Chaperone Hsp90, heat shock protein	16128457	58
imp	Organic solvent tolerance protein precursor	2507089	49
leuS	Leucine tRNA synthetase	2507435	30
ompA	Outer membrane protein A precursor	12514142	73
sucC	Succinyl-coenzyme A synthetase beta chain	67473117	56
pnp	Polyribonucleotide nucleotidyltransferase	1172545	51
proS	Proline tRNA synthetase	34395980	48
rpoB	RNA polymerase, beta subunit	67472412	31
purA	Adenylosuccinate synthetase (IMP-aspartate ligase)	67471590	45
tsf	Protein chain elongation factor EF-Ts	67462328	66
tufB	Protein chain elongation factor EF-Tu	30064737	78
rpsA	30S ribosomal subunit protein S1	30062446	66
yaeT	Hypothetical protein SF0167	71164818	50

[a] Protein accession numbers are from the NCBI website.

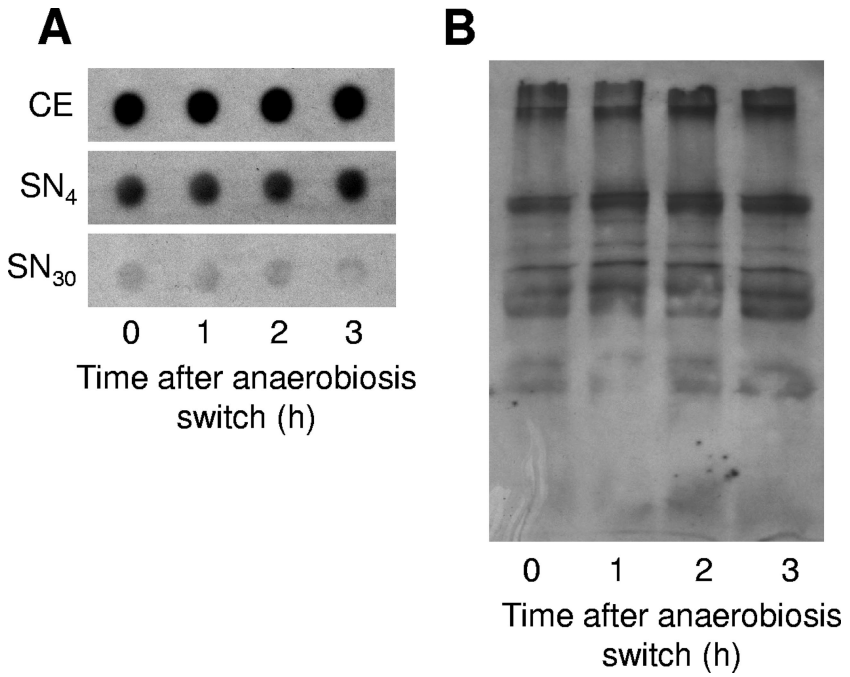

FIG. 3. Detectable carbonylated proteins were not degraded over time. Exponentially (OD_{600} = 0.5) aerobically grown cells were switched to anaerobiosis growth and harvested 0, 1, 2, and 3 h later. Similar volumes of culture were used to prepare French press CEs. (A) CEs were obtained after 0 (CE), 4 (SN_4), and 30 (SN_{30}) minutes of centrifugation and processed for carbonylation assays by directly loading equal volumes of sample in each slot. (B) Specific carbonylation pattern after 1D protein electrophoresis from CE (equal volumes of sample loaded) after the anaerobiosis switch.

Transfer of carbonylated proteins from supernatant to pellet explains the apparent degradation of carbonylated proteins. In light of the surprising finding that the carbonylated proteins detected were not degraded over time, we wondered why Dukan et al. (10) and others concluded that carbonylated proteins were more susceptible to degradation in vivo. One explanation could be that the apparent degradation of carbonylated proteins seen by Dukan et al. (10) in fact resulted from the transfer of these carbonylated proteins from the supernatant fraction to the pellet. In order to test this hypothesis, we reproduced these earlier experiments (10) with the modification of measuring protein carbonyl content present not only in SN_{30} as previously described but also in the CE. Cells were grown in the presence of streptomycin (10 μg/ml), which induced mistranslation until the cell density reached an OD_{600} of 0.5. Protein synthesis was then stopped with spectinomycin (100 μg/ml) (T_0). As depicted in Fig. 4 and as expected, we observed the disappearance of protein carbonyl content between T_0 and T_{120} in SN_{30} supernatants. In addition and as previously described (10), cells grown in the presence of streptomycin resulted in an increased protein carbonyl content in SN_{30} (T_0) supernatant (Fig. 4) compared to what was seen for untreated culture (Fig. 1). However, we observed no decrease in protein carbonyl content between T_0 and T_{120} in the CE, indicating that the apparent degradation of carbonylated proteins reported in earlier studies resulted from the transfer of these carbonylated proteins from the supernatant fraction to the pellet.

Accumulation of carbonylated protein aggregates during growth arrest. Next, we wondered if a previously observed increase in carbonyl content during growth arrest (11) came from soluble or insoluble proteins. Indeed, the carbonyl content in these earlier experiments was measured without taking into account the possible presence of protein aggregates in cell extracts (10). With this in mind, we analyzed the carbonyl content in each of the fractions CE, LP, SP, SN_4, and SN_{30} in exponentially grown *E. coli* and during the stationary phase (24

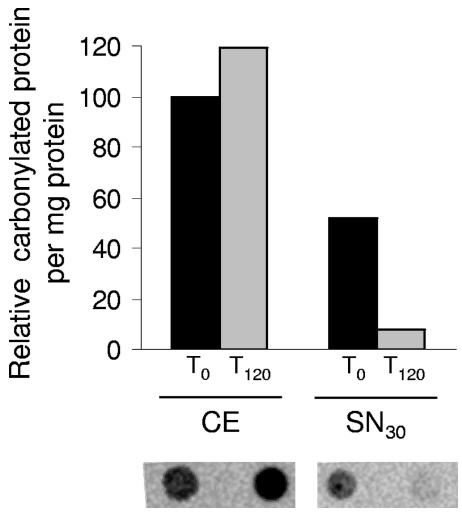

FIG. 4. Protein carbonyl content transfer between supernatant and pellets after streptomycin/spectinomycin treatment. *E. coli* cells were grown in LB with the addition of 10 μg of streptomycin/ml until exponential phase (OD_{600} = 0.5). Protein synthesis was then stopped with spectinomycin (100 μg/ml) at T_0. We measured the protein carbonyl content in the CE and supernatant after 30 min of centrifugation at T_0 and T_{120}. Equal amounts of protein were loaded in each slot, and quantification of carbonyl levels was obtained using Quantity One software (Bio-Rad). The analysis was repeated three times to confirm reproducibility.

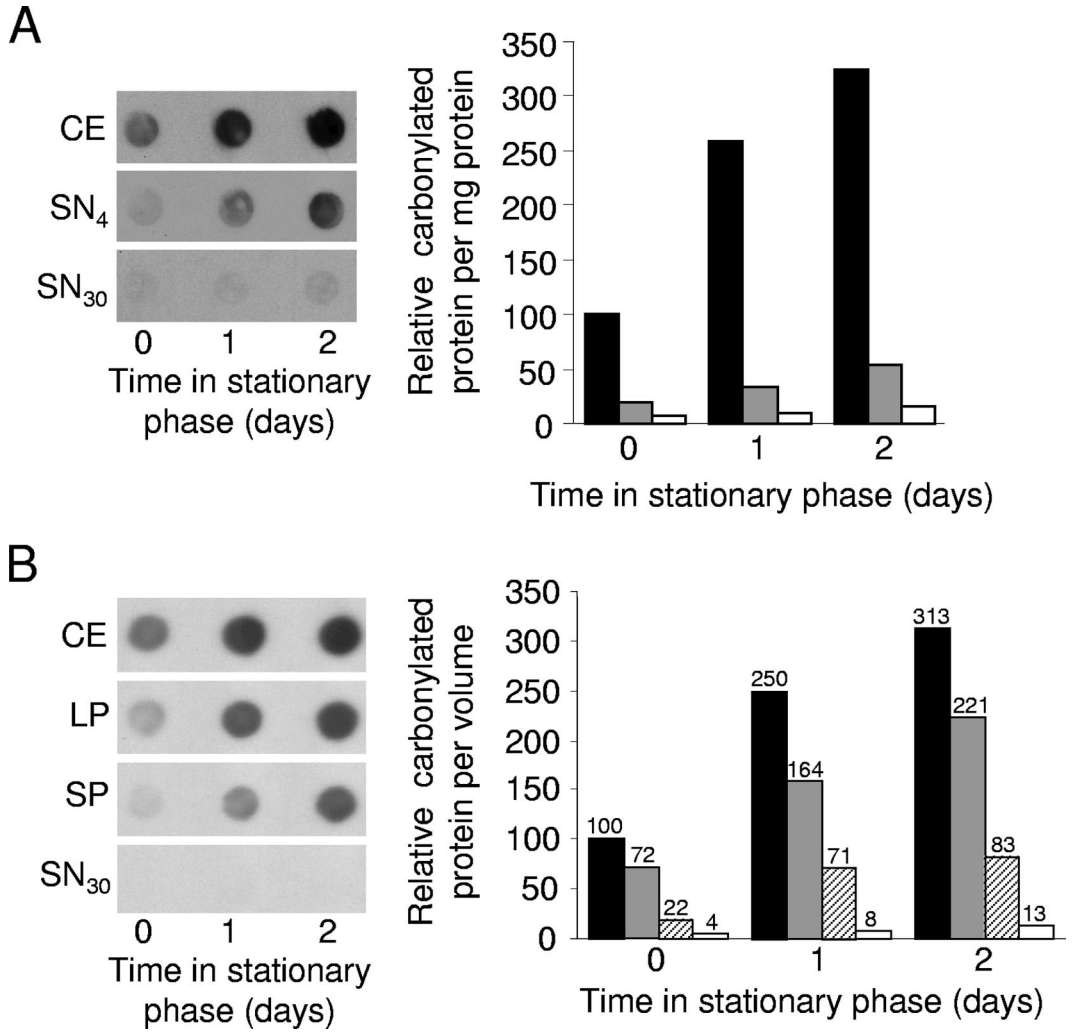

FIG. 5. Accumulation of carbonylated protein aggregates during growth arrest. *E. coli* cells were grown in LB until exponential phase (time zero; OD_{600} = 0.5) and stationary phase (1 to 2 days). (A) Extracts were obtained after 0 (CE [black bars]), 4 (SN_4 [gray bars]), and 30 (SN_{30} [white bars]) minutes of centrifugation and processed for carbonylation assays. We loaded equal amounts of protein in each slot. (B) Similar volumes of CE (black bars), LP (gray bars), SP (hatched bars), and SN_{30} (white bars) were loaded in each slot.

h and 48 h). As depicted in Fig. 5A, the greatest increase in carbonyl content per mg of protein occurred in the CE and, to a lesser extent, in the SN_4 fraction during growth arrest. As previously described, we observed a fivefold increase in carbonylated proteins in the SN_4 fraction in 2-day-old stationary-phase cultures (11). Interestingly, no significant increase in carbonyl content could be detected in the SN_{30} fraction. Evaluation of the relative amount of carbonyl content confirmed that the carbonyl content of CE approximately equaled the sum of those for SN_{30}, LP, and SP and that SN_4 equaled approximately the sum of SN_{30} and SP at each time of culture, suggesting a transfer between each fraction which depended on the centrifugation time (Fig. 5A and B). Finally, at each point in time, evaluations of the relative amount of carbonyl content revealed that the majority of carbonyl content consisted of insoluble protein (Fig. 5B). Taken together, these results suggest that the increase in carbonyl content observed during growth arrest came from an accumulation of carbonylated protein aggregates over time.

DISCUSSION

Surprisingly, our results with *E. coli* demonstrate that in exponentially growing cells the majority of the carbonyl content comes from insoluble cytosolic proteins detectable in an aggregate state. Moreover, these detectable carbonylated proteins were not degradable over time. Interestingly, several in vitro studies suggest that depending on the level of oxidative damage, oxidized protein either remains soluble or coexists in an aggregate state (6, 14, 21). Like most partially denatured proteins, modestly oxidized proteins are usually more sensitive to proteolytic attack by most proteases (7, 18, 21, 26), whereas heavily oxidized proteins generally show decreased susceptibility in vitro (7, 8, 21). If the results obtained in vitro showing preferential degradation of weakly oxidized carbonylated proteins are transferable to an in vivo setting, our results indicate that the majority of the carbonylated proteins we detected were aggregated and thus in the state that is the least susceptible to degradation, whereas the weakly oxidized carbonylated proteins were rapidly degraded, thus preventing detection.

The fact that detectable carbonylated proteins appear in a state less prone to degradation could apparently contradict a earlier study from Fredriksson et al. (13). Indeed, using CE, Fredriksson et al. (13) showed that mutants defective in proteolysis accumulated more carbonylated proteins in starvation, suggesting that carbonylated proteins are more prone to degradation. However, when the carbonyl contents in the SN_{30} fractions of all mutants were evaluated, no further differences could be observed (data not shown). A simple way to explain this discrepancy is that a reduced degradation of soluble carbonylated protein in mutants makes them more prone to aggregation, thus explaining the observed augmentation of carbonyl content in the CE. In our study, we claim that even if soluble proteins are more prone to degradation in vivo, detectable carbonylated proteins are found predominantly in a degradation-resistant aggregate state.

The idea that we detected mainly nondegradable carbonylated protein in an aggregate state led us to believe that the demonstration of carbonylated protein as more prone to degradation in vivo by the experiments previously performed by Dukan et al. (10) was in fact due to a misinterpretation of results. Indeed, when the same experimental procedure was repeated and when carbonyl contents were obtained not only for SN_{30} but also for CE, we observed that whereas protein carbonyl content decreased in the SN_{30} extract as previously described, no decrease was found in the newly measured CE between T_0 and T_{120}. Considering CE, our results suggest that at T_0, carbonylated proteins present in SN_{30} are either soluble or in an aggregate form too small to sediment during the 30 min of centrifugation. These then become bigger inside the cells and sediment during the 30 min of centrifugation at the end of the experiment at T_{120}. In light of our results, we propose increasing the scope of our demonstration and suggest that the apparent specific degradation of carbonylated proteins observed in previous studies (10, 14–16, 18, 21) was in fact due to the aggregation of the protein carbonyl content over time during the experimental procedure. Indeed, none of the previous studies demonstrating in vivo carbonylated proteins as more prone to degradation in eukaryotic or prokaryotic cells (10, 23, 25) evaluated the carbonyl content in CE.

Because protein carbonyl content was always evaluated only in the supernatant fraction, current opinion states that carbonylated proteins are considered soluble in healthy cells (3, 4, 9–11). It was also speculated that a decrease in proteolysis provokes increases in carbonylated protein levels, which may in turn form aggregates during aging or disease (5, 13, 14). The unexpected results in the present study lead us to propose that, in fact, even in healthy cells, carbonylated proteins are detectable mainly in an aggregate state which is able to pass from one generation to the next. These carbonylated protein aggregates would thus accumulate over time and could therefore be associated with bacterial cell death during senescence. Interestingly, this assumption is in good agreement with previous observations for (i) *Saccharomyces cerevisiae*, where carbonylated proteins segregated in an aggregate state in the mother cell (1, 12), and (ii) *E. coli*, where dead cells formed during stasis showed higher carbonyl and aggregate protein contents (9, 19).

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