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Analysis of Protein Carbonyls with 2,4-Dinitrophenyl Hydrazine and Its Antibodies by Immunoblot in Two-Dimensional Gel Electrophoresis

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Protein carbonyls are reported to increase in aging and in pathologies such as Alzheimer's disease and ischemic injury. Detailed study of this important issue has, however, been hampered by lack of an appropriate method to identify individual carbonylated proteins. We describe here an immunoblot method to investigate protein carbonyls reactive to 2,4-dinitrophenyl hydrazine. Rabbit polyclonal antibodies against 2,4-dinitrophenyl hydrazine were used to study the proteins derivatized by the reagent in one- or two-dimensional polyacrylamide gel electrophoresis followed by immunoblotting. More than 25 proteins with high carbonyl contents were clearly demonstrated in two-dimensional immunoblot of rat tissue soluble proteins. The method could detect concentrations as low as 1 pmol of carbonyls. The signals were mostly abolished by prior treatment of tissue proteins with sodium borohydride to reduce carbonyls. Fragments generated by V8 protease digestion of a single protein exhibited signal intensities of varying degrees, indicating that carbonylation is not uniform in different amino acid sequences. Proteins treated with glucose or aldehydes gave rise to positive signals, suggesting that the finding of carbonyls in tissue proteins is not necessarily an indication of direct oxidation of side chains of amino acid residues.

Key words: aging, immunoblot, protein oxidation, protein carbonyl, rat.

Oxidative modifications of proteins have recently attracted considerable interest in aging (*1-3*) and in age-associated diseases such as Alzheimer's disease (*4*), arteriosclerosis (*5*), cataract (*6*), and rheumatoid arthritis (*7*). Reactive oxygen species give rise to a variety of modifications in amino acid residues like cysteine, methionine, tryptophan, arginine, lysine, proline, and histidine (*8-11*). Among these, carbonyls formed by oxidation of arginine, lysine, threonine or proline residues are often employed as a marker of major forms of the modification because they are readily measured by the reaction with 2,4-dinitrophenyl hydrazine (DNPH) to form hydrazones (*12, 13*) or labeling *via* reduction with tritiated sodium borohydride (*14, 15*). In most investigations so far reported, however, carbonyls are determined as a mixture of tissue proteins and hence little information is available on what protein species are carbonylated *in vivo*. In view of possible changes in the extent of oxidation of tissue proteins in aging and pathology, it is of great interest to know the specificity of proteins that might be more susceptible or resistant to oxidation than others.

In this communication we report an indirect immunological method to detect protein carbonyls by immunoblot using monospecific antibodies against 2,4-dinitrophenyl hydrazones of oxidized bovine serum albumin (BSA). While it is generally believed that carbonyls are generated by direct oxidation of amino acid residues, they can also be generated by either nonenzymatic glycation (*16*) or reaction with aldehydes derived from lipid peroxides (*17*). We therefore evaluated protein carbonyls generated by reactions with glucose or aldehydes *in vitro* by the present method. During preparation of this manuscript, Levine and his collaborators reported an approach similar to the one described here (*18, 19*). Preliminary results of our investigations have been reported elsewhere (*20-22*).

MATERIALS AND METHODS

Chemicals—2,4-Dinitrophenyl hydrazine (DNPH), Staphylococcal Protease V8, urea, glucose, acetaldehyde, and sodium borohydride were purchased from Wako Pure Chemicals, Tokyo. Chicken lysozyme, BSA, ovalbumin, and catalase were from Sigma Chemical, USA. Endoglycosidase F was from New England Bio Labs. ^{125}I-Protein A was from ICN Biomedicals, USA. Cyanogen bromide activated Sepharose 4B was from Pharmacia LKB. PVDF and nitrocellulose membranes were obtained from Millipore, USA, and Schleicher and Schull, Germany, respectively. Other reagents used were of analytical grade.

Animals and Preparation of Tissue Extracts—Male Fischer 344 rats were maintained under specific pathogen-free conditions in our animal facility and used for experiments. Tissue samples were homogenized in 10 to 20 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA using a Polytron-type Nition homogenizer at speed control 100 for 2×15 s. The homogenates were

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Abbreviations: DNPH, 2,4-dinitrophenyl hydrazine; TCA, trichloro acetic acid; SDS-PAGE, SDS polyacrylamide gel electrophoresis.

centrifuged at 15,000×*g* for 10 min at 4°C. The supernatants were saved for analysis of protein carbonyls.

Determination of Carbonyl Contents—Carbonyl content of proteins was determined by the DNPH method as described by Ref. *12* with some modifications to improve reproducibility in our laboratory (Nakamoto, H. and S.G, unpublished results). Briefly, proteins in two equal portions of the supernatant were precipitated with 10% TCA. The precipitates were treated with either 2 N HCl alone (control) or 2 N HCl containing 10 mM DNPH at 15°C for 1 h. After the reaction, the mixture was centrifuged, the precipitates were washed with an ethanol-ethyl acetate (1 : 1) mixture three times and the final precipitates were dissolved in 6 M guanidine chloride or 8 M urea. The absorbancy was measured at 360 nm and the carbonyl content was obtained as nmole per mg protein using a molar extinction coefficient of $22{,}000^{-1}$. Samples processed similarly but without DNPH treatment were used as controls. Concentrations of protein were measured with a BioRad assay kit using bovine serum albumin as a standard.

Preparation of Antibodies—BSA (1 mg/ml) was oxidized by incubation in 50 mM sodium phosphate buffer (pH 7.4) containing 0.155% (v/v) H_2O_2, 1.2 mM $FeSO_4$, and 1.2 mM EDTA 2Na for 5 min at 37°C. The oxidized BSA was precipitated with 10% TCA and then treated with 10 mM DNPH in 2 N HCl at 37°C for 1 h. The resulting hydrazones were precipitated with TCA, washed with ethanol/ethyl acetate, then with acetone, then dissolved in 8 M urea and emulsified with the same volume of Freund's complete or incomplete adjuvant. The emulsion containing 1 mg of the derivatized BSA was injected subcutaneously into Japan White rabbits every 2 weeks. Antibody activity was monitored by specific reaction with oxidized and DNPH-treated BSA using oxidized but DNPH-untreated BSA as a negative control. After 3 injections, the antiserum was obtained. The antibodies in 5 ml of the antiserum were partially purified by ammonium sulfate precipitation at 33% saturation followed by affinity chromatography on a column containing 2 ml of DNPH-coupled Sepharose 4B, and the bound antibodies were eluted with 0.1 M glycine-HCl buffer (pH 2.5) containing 0.1% (v/v) Tween 20. The eluate was neutralized, supplemented with 0.05% NaN_3, and stored at +4°C.

Immunoblot on SDS-PAGE—Proteins precipitated with TCA were suspended and incubated in a solution containing 10 mM DNPH and 2 N HCl for 1 h at 15°C. The resulting protein hydrazones were pelleted in a centrifuge at 11,000×*g* for 5 min. The pellets were washed twice with 1 ml of ethanol-ethyl acetate (1 : 1) and then once with acetone. The final precipitates (1 mg protein) were dissolved in 1 ml of Laemmli's buffer (*23*) containing 8 M urea and 5% 2-mercaptoethanol using a sonicator Silentsonic (Sharp, Tokyo) for 10 min. Duplicate SDS-PAGE of the derivatized proteins was carried out in 12% polyacrylamide gels containing 0.1% SDS, first at 90 V and then at 250 V for about 3 h. One gel was stained with Coomassie Brilliant Blue R-250 (CBB) and the other was processed for immunoblot as follows. The proteins were transferred to PVDF membranes or, in some experiments, to nitrocellulose membranes. The membranes were soaked in PBS containing 3% skim milk, 0.05% Tween 20, and 0.05% sodium azide, then treated with anti-DNPH antibodies in the same buffer. After washing with the buffer without the antibodies, the membranes were treated with ^{125}I-Protein A (0.02 μCi/ml) in the same buffer for 2 h at room temperature, washed with PBS(−) containing 0.05% Tween 20 and exposed to an Imaging Plate (Fuji Film, Tokyo) overnight. Radioactive signals were visualized in a BAS 2000 Bioimaging Analyzer (Fuji Film). Molecular weight markers used were BSA (M_r 66,300), hen egg albumin (M_r 42,700), rabbit glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), bovine carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), soybean trypsin inhibitor (M_r 20,100), and bovine lactoalbumin (M_r 14,200). Some of these proteins were carbonylated when they were purchased and therefore also served as molecular weight markers in immunoblot.

Immunoblot on 2-D PAGE—This was performed essentially as described with some modifications (*24*). Protein samples were treated with DNPH in the same way as for SDS-PAGE described above, and the final precipitates were dissolved in 8.5 M urea solution containing 2% (w/v) NP40, 5% (v/v) 2-mercaptoethanol, and 2% (v/v) carrier Ampholine-Pharmalite mixture (8 : 1 : 1 mixture of Ampholine pH 3-10.5, Ampholine pH 4-6, and Pharmalite pH 8-10.5). Thirty microliters of sample solution was applied on 4.5% (w/v) polyacrylamide gel (120×2 mm) containing 8.5 M urea, 20% (w/v) sucrose, 2% (w/v) NP40, and 2% (w/v) carrier Ampholine-Pharmalite mixture. To protect proteins against possible damage due to the extreme pH of the electrode solution, a solution containing 5 M urea, 2% NP40, 2% Ampholine, and 0.8% (w/v) glutamate was overlaid on the samples. The anode and cathode solutions were 0.02 M phosphoric acid and 1 M NaOH, respectively. The electrophoresis was conducted for 3 h at 150 V, then for 20 h at 300 V and finally for 1.5 h at 450 V. After electrophoresis in the first dimension, the gels were soaked in Laemmli's buffer containing 5% (v/v) 2-mercaptoethanol for 30 min, then subjected to SDS-PAGE for immunoblot as described above.

Sodium Borohydride Reduction—Proteins precipitated with 10% (w/v) TCA were suspended and incubated in 86 mM Tris-HCl buffer (pH 8.5) containing 40 mM sodium borohydride and 0.86 mM EDTA at 37°C for 1 h essentially as described (*14*). The reaction was terminated with 10% (w/v) TCA, and the precipitates were subjected to duplicate SDS-PAGE for immunoblot and CBB staining.

Limited Protease Digestion of DNPH-Treated Proteins—DNPH-derivatized ovalbumin precipitates (0.25 mg) were dissolved in 0.25 ml of 125 mM Tris-HCl buffer (pH 6.8) containing 0.5% (w/v) SDS and 10% (v/v) glycerol in a boiling bath for 2 min. Staphylococcal protease V8 (15 or 25 μg) dissolved in 25 μl of the buffer was added to the solution, and the mixtures were incubated at 37°C for 1 h. The reaction was terminated by the addition of SDS and 2-mercaptoethanol to final concentrations of 2% and 5%, respectively. The resulting digests were examined by SDS-PAGE followed by CBB staining and immunoblot.

Oxidative Modification of Lysozyme—Chicken lysozyme (1 mg/ml) dissolved in 50 mM phosphate buffer (pH 7.5) containing 25 mM ascorbate, 0.1 mM $FeCl_3$, 1 mM EDTA, and 90 mM KCl was incubated at 37°C for 1 to 6 h. The oxidized lysozymes were precipitated with 10% (w/v) TCA, treated with 10 mM DNPH and analyzed by immunoblot. To monitor the extent of oxidation, the carbonyl content of each sample was determined as described above.

Treatment of Catalase with Glucose—Catalase was dissolved in 1 ml of PBS (1 mg/ml, pH 7.4) containing 0, 0.8, 1.6, or 160 mg per ml of glucose and 0.05% (w/v) NaN_3, and the mixture was incubated either in the presence or absence of 5 mM EDTA at 37°C for 9 days. The catalase thus treated was precipitated and washed with 10% (w/v) TCA twice and processed for immunoblot after derivatization with DNPH.

Modification of Lysozyme with Acetaldehyde—Five milligrams of chicken lysozyme was dissolved in 10 ml of PBS (pH 7.4) containing 240 mM acetaldehyde as described (*25*), and the solution was incubated at 20°C for 5 s, 20, 60, or 120 min. The resulting modified lysozymes were precipitated with TCA, treated with DNPH, then subjected to immunoblot.

RESULTS

Specificity of the Antibodies—The reaction of the purified antibodies with rat brain soluble proteins treated with DNPH yielded multiple positive signals in immunoblot but none at all in proteins untreated with DNPH (Fig. 1). The signals were completely abolished when the immune reaction was performed in the presence of an excess amount of free DNPH. These results demonstrate the specificity of the antibodies for 2,4-dinitrophenyl hydrazones of proteins. It was noted that although some free DNPH was strongly absorbed by the nitrocellulose membranes in the competition experiment, no background signals were detected, while significant signals were observed when PVDF membranes were used.

The above results, however, do not prove that DNPH reacted specifically with carbonyls in proteins under our experimental conditions. Because of the high sensitivity of the immunological detection used here it was conceivable that the reagent reacted, albeit weakly, with other moieties in proteins, and hence the method might not have detected carbonyls exclusively. We therefore examined the effect of prior treatment of proteins with reducing reagent on the signal intensity. After sodium borohydride treatment, the intensity was reduced to less than 30% of the untreated preparation without any detectable changes in the staining pattern (Fig. 2, A and B). Incubation at higher concentrations of sodium borohydride completely abolished the signals with apparent fragmentation of proteins (data not shown). Thus, it is unlikely that DNPH reacted with moieties other than carbonyls in tissue proteins under our experimental conditions.

Detection of Carbonyls in Fragments of Proteins—To further verify the specificity of the reaction of DNPH with proteins, we attempted to see if specific proteolytic fragments of a carbonylated protein react more strongly than others. Commercially available ovalbumin which apparent-

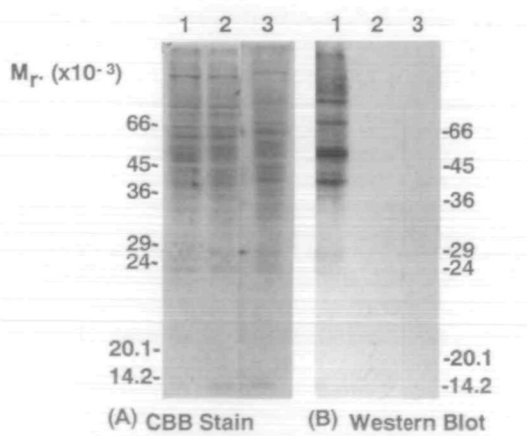

Fig. 1. **Specificity of anti-DNPH monospecific antibodies.** Panels A and B represent CBB staining of SDS-PAGE and its immunoblot, respectively. Proteins with (lane 1) and without (lane 2) prior treatment with DNPH were applied. The sample for lane 3 was incubated with the antibodies in the presence of large excess of free DNPH.

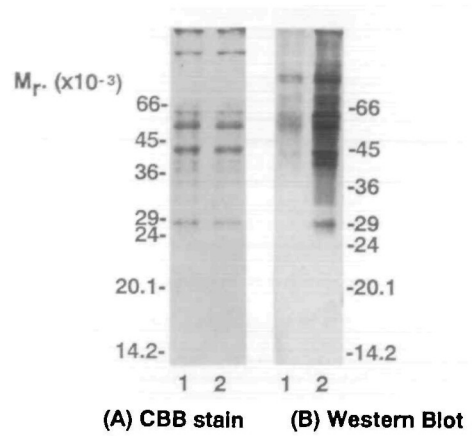

Fig. 2 **Effect of treatment with sodium borohydride on immunoblot of rat kidney soluble proteins.** The proteins were treated with sodium borohydride before the reaction with DNPH as described in "MATERIALS AND METHODS" and analyzed by immunoblot Proteins treated with sodium borohydride and untreated control proteins were run on lanes 1 and 2, respectively. Lane 3 is for molecular weight markers, some of which are obviously carbonylated and served as markers for both staining and immunoblot (A) CBB staining and (B) immunoblot.

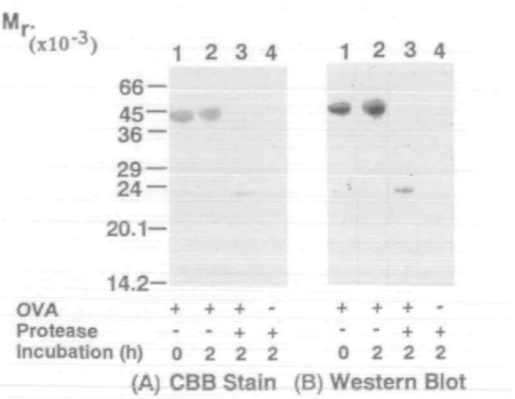

Fig 3. **Distribution of carbonyls in proteolytic fragments of DNPH-ovalbumin.** Ovalbumin (0.25 mg) was derivatized with DNPH and subjected to limited digestion with (lane 3) or without (lane 2) 25 μg of V8 protease for 2 h in 125 mM Tris-HCl buffer containing 0.5% SDS and 10% glycerol After inactivation of the enzyme by boiling the reaction mixture in 2% SDS and 5% 2-mercaptoethanol, the protein fragments were subjected to SDS-PAGE followed by CBB staining and immunoblot The V8 protease only was similarly processed and run in lane 4 Ovalbumin not incubated with the protease was run in lane 1.

ly had undergone some oxidative modifications was treated with DNPH and then digested with V8 protease. The resulting fragments were analyzed by SDS-PAGE followed by CBB staining and immunoblot (Fig. 3). The derivatized ovalbumin was cleaved to generate two major fragments with molecular masses of 40 and 23 kDa, the signal intensities of which were significantly different from each other, that of the lower molecular weight component being stronger (Fig. 3). Thus, moieties reactive with DNPH do not appear to be distributed evenly but localized in a protein. Similar observations were made with bovine catalase (data not shown). This finding provides further support for the interpretation that DNPH reacts with specific sequences of a polypeptide that contain carbonyls.

Detection of Carbonyls on 2-D PAGE—For two-dimensional PAGE, the derivatized proteins had to be solubilized with nonionic solvent because proteins treated with DNPH were precipitated with acid to remove the free reagent. The acid precipitates were washed with acetone, then dissolved in the sample solution for isoelectric focusing containing 8.5 M urea, usually with no sedimentable remnants appearing after centrifugation at 8,000×*g* for 5 min. More than 25 signals were detected on 2-D PAGE followed by immunoblot of soluble proteins from rat kidney and liver (Fig. 4). It was noted that much fewer signals were found in the brain. Interestingly, apart from a protein of M_r 67,000 in the kidney, which is probably serum albumin, the proteins showing prominent signals were minor components in the staining. It should also be pointed out that while spots on CBB staining tended to locate in more alkaline regions, the majority of immunoblot signals are found in more acidic regions.

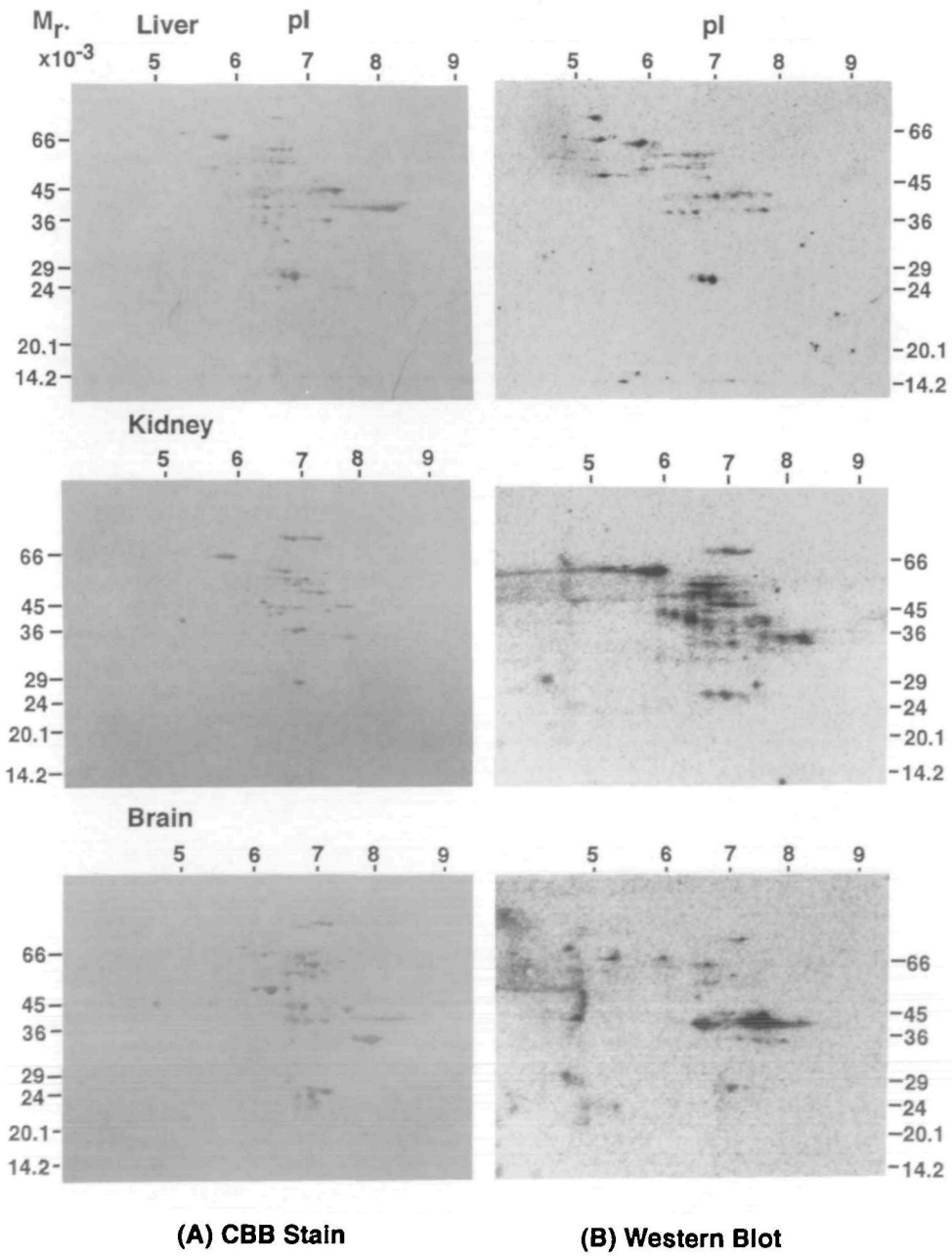

Fig 4. **2-D PAGE followed by immunoblot of rat tissue soluble proteins.** Soluble proteins of rat liver, kidney, and brain were derivatized with DNPH as described in "MATERIALS AND METHODS." The final precipitates were washed with cold acetone and dissolved in the sample buffer for isoelectric focusing supplemented with 2% NP40, 5% 2-mercaptoethanol, and 8 5 M urea. Fifty micrograms of protein was run on the first dimensional disc gel electrophoresis in the range of pH 3.5 and 10.5. The second dimensional gel electrophoresis was performed in 12% SDS-PAGE. (A) CBB staining and (B) immunoblot. Upper panel, liver; middle panel, kidney, lower panel, brain.

Carbonyls in Oxidatively Modified Chicken Lysozymes—While negligible signals were detected in immunoblot of the commercial lysozyme, the signal intensity increased linearly with time in parallel with the spectrophotometrically determined carbonyl content upon incubation with $FeCl_3$ and ascorbate (Fig. 5). In immunoblot the lowest limit of detection was at least 1 pmol.

DNPH-Reactivity of Catalase Treated with Glucose—The catalase incubated with 0.8 mg/ml (physiological concentration) or 1.6 mg/ml (a mild diabetic concentration) of glucose showed no detectable increase of immunoreactivity to the antibodies. At glucose concentration of 160 mg/ml, however, signal intensity for the immunoreactivity increased 2.5- and 1.5-fold of the control (without glucose) in the presence and absence of EDTA, respectively (Fig. 6B). These results suggest that half of the carbonylation of catalase in the presence of glucose may involve metal-catalyzed oxidation of the protein (see "DISCUSSION").

DNPH-Reactivity of Acetaldehyde-Modified Lysozymes—It is reported that not only bifunctional but also monofunctional aldehydes can generate carbonyl groups by reaction with proteins (*26*). We therefore examined whether chicken lysozyme treated with acetaldehyde is modified to give rise to carbonyls reactive to DNPH. Upon reaction with acetaldehyde, the dimer and trimer of the protein were clearly visible within half a minute but their amounts did not increase significantly on further incubation up to 2 h as shown in Fig. 7A. The results of immunoblot demonstrate the appearance of remarkable DNPH-reactivity in high molecular weight aggregates with time when the incubation was carried out with the aldehyde (Fig. 7B). The signals in monomer and dimer/oligomers were lower but exhibited significant increase with time. It is interesting to note that the signal intensity did not appear to increase in parallel with the amount of each form of the protein as seen from the comparison between CBB staining and the signals, suggesting that lysozyme-aldehyde adducts undergo transformation of structures with time and giving rise to carbonyls.

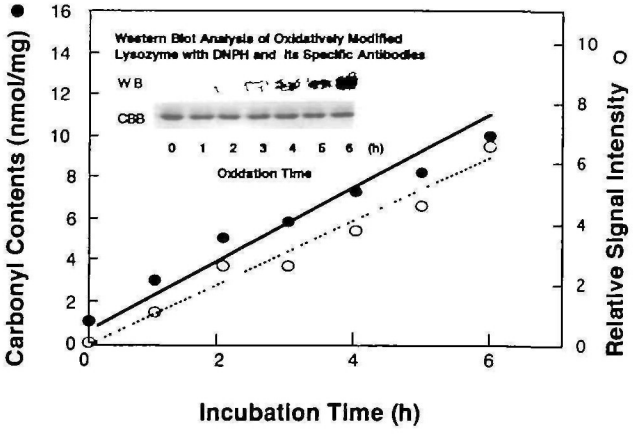

Fig. 5. **Immunoblot of lysozyme oxidatively modified with Fe^{3+}/ascorbate for the indicated times and derivatized with DNPH.** Three micrograms of the protein was loaded on SDS-PAGE and blotted to a PVDF membrane for the immune reaction. Signals intensified in an imaging plate were determined with a Bas 2000 analyzer (Fuji Film). The protein carbonyls were determined as described in "MATERIALS AND METHODS."

DISCUSSION

Carbonyl moieties in tissue proteins are believed to be formed primarily by oxidative modifications of amino acid side chains (*2-4, 10, 12*). In most of these studies the carbonyls were measured spectrophotometrically using DNPH, or in fewer cases by incorporation of tritium into proteins upon reaction with [^{3}H]sodium borohydride (*1, 15*). In these studies, however, no attempt was made to identify individual proteins with carbonyls or the chemical structures of the carbonyl moieties.

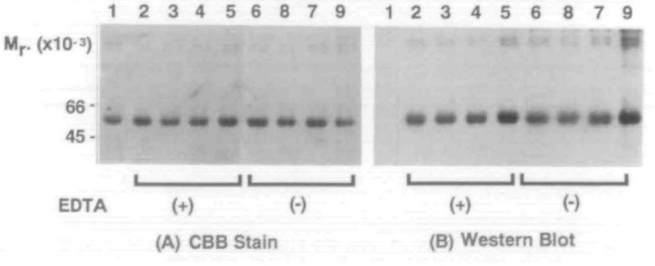

Fig. 6. **Immunoblot analysis of glycated catalase.** Bovine catalase was incubated in 0 (lanes 2, 6), 0.8 (lanes 3, 7), 1.6 (lanes 4, 8), or 160 mg/ml (lanes 5, 9) glucose in the presence or absence of EDTA at pH 7.4, 37°C for 9 days. 0.5 μg of each DNPH-derivatized protein was subjected to SDS-PAGE for CBB staining (A) and immunoblot (B). Lane 1, catalase without DNPH treatment.

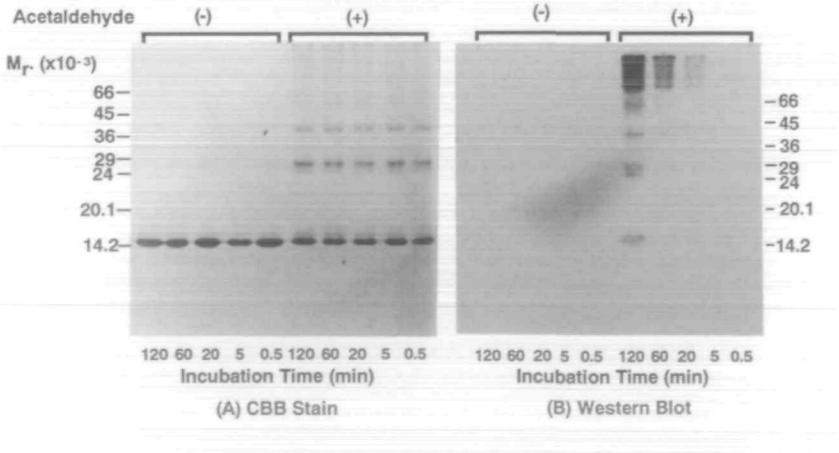

Fig. 7. **Immunoblot analysis of lysozyme modified with acetaldehyde.** Chicken lysozyme was incubated with or without 240 mM of acetaldehyde at 20°C for 5 s, 20, 60, and 120 min. The modified and control lysozymes were derivatized with DNPH and 2 μg of each protein was subjected to SDS-PAGE for CBB staining (A) and immunoblot analysis (B).

In the present work we developed and characterized an indirect immunological method to investigate protein carbonyls by immunoblot on either one- or two-dimensional polyacrylamide gel electrophoresis. Since DNPH is a potent immunogen, monospecific antibodies against the reagent bound to BSA could readily be prepared. The specificity of affinity-purified antibodies for carbonyls was demonstrated by the experiment in which positive signals on immunoblot were totally abolished by an excess of free DNPH (Fig. 1). The specificity of the method was further verified by the findings that reduction of carbonyls with sodium borohydride greatly diminished the intensity of the signals (Fig. 2). Since the immunological detection is highly sensitive, we explored whether even a minor reaction with DNPH of groups such as one in a peptide bond, if it occurs, could give positive signals. This possibility was excluded, however, because the reactive groups were found to be localized unevenly in different peptide fragments generated by protease digestion of a protein containing carbonyl groups (Fig. 3). This result would not be expected if DNPH had reacted with chemical structures commonly found in protein sequences in general. Other possible sites of reaction of DNPH with proteins are polysaccharide moieties in glycoproteins. This possibility was examined using ovalbumin, a glycoprotein with a single polysaccharide chain, which contained some carbonyls when purchased (see Fig. 3). No significant change in the signal intensity of immunoblot was observed after endoglycosidase F digestion, which resulted in apparent reduction of molecular mass on SDS-PAGE, proving that the carbohydrate chain had been removed (data not shown). Thus, carbohydrate moieties do not appear to contribute to the hydrazone formation upon reaction with DNPH. This is consistent with a recent report that fibrinogen subunits containing no glycosylated residues were more associated with carbonyl groups than glycosylated subunits (*27*). Recently, Levine and his collaborators reported that carbonyl groups in human plasma proteins oxidized *in vitro* and rat liver proteins can be examined by SDS-PAGE followed by immunoblot using an approach similar to that described here (*18, 19*). They found that plasma fibrinogen is more susceptible to oxidative modifications in iron/ascorbate system than other plasma proteins (*18*) and carbonic anhydrase III is one of the most highly oxidized hepatic proteins (*19*). To better characterize or identify individual carbonylated proteins it is desirable to separate derivatized proteins on 2-D PAGE. Since they derivatized the proteins with DNPH in the presence of SDS and trifluoroacetic acid followed by neutralization with Tris, it would not be possible to study those proteins in 2-D PAGE since that involves isoelectric focusing. In the present work, we developed a method for 2-D PAGE followed by immunoblot of carbonylated proteins derivatized with DNPH. Since we failed in our attempt at post-blotting derivatization of separated proteins with DNPH for immune staining, proteins were treated with the reagent in 2 N HCl prior to the electrophoresis. To asure good separation of proteins, precipitates formed after DNPH treatment in the acid were dissolved in the sample solution for isoelectric focusing containing 8.5 M urea. More than 70 separate spots were detected in CBB staining without significant difference of the patterns between samples treated and untreated with DNPH (data not shown). As described in the "RESULTS," while a few immunoblot signals coincided with stained spots, some signals did not appear to correspond to major visible spots in CBB staining and were found to be localized in more acidic regions than the stained spots. These results suggest that the proteins with positive signals are skewed from the positions of noncarbonylated original forms, which are probably more positively charged, and hence tend to be localized in more alkaline regions, due to the presence of unmodified amino groups. Without specific probes such as antibodies for a protein in question, however, it would not be possible to prove this. It should be mentioned here that the pI of a carbonylated protein should not change after the reaction with DNPH since neither carbonyls nor dinitrophenyl hydrazones carry charges, although carbonylation itself could change the charge of a protein if it is generated at the expense of an amino group.

Among signals in the immunoblot of liver proteins which coincided to stained spots, two with the molecular mass of 27 kDa and pI 6.8 are probably carbonic anhydrase III, which has been shown to be one of the prominent proteins containing carbonyls (*19*). Another notable spot/signal with molecular mass of about 60 kDa and pI 5.2 may be a monoamine oxidase, whose active site is reported to contain covalently bound carbonyl groups, at least in copper-containing enzymes (*28*). In the latter case the carbonyl is not generated as a result of unfavorable modification, but is rather an essential part of the protein. Thus, the existence of carbonyl moieties is not necessarily an indication of damage to proteins. It was noted that the pattern of protein carbonylation is tissue-specific, *e.g.*, the number of detectable carbonylated proteins in the brain being much fewer and some of the proteins in this tissue being more highly carbonylated than others. Most significantly, a protein with approximate molecular mass of 42 kDa and pI 6.5 and another with the same apparent molecular mass and pI 7.2–8.2 were markedly carbonylated in the brain, although both proteins appear to exist in all three tissues examined in similar amount as judged by the intensity of CBB staining. These proteins are remarkable examples of the specificity of the carbonylation, since it is obvious that proteins of the nearby spots with similar or perhaps the same molecular masses are only faintly carbonylated. Whether these highly or barely carbonylated proteins are identical apart from the modification remains to be examined.

The amount of carbonyls measured by the DNPH method is generally believed to reflect the degree of oxidation of proteins (*3, 4, 12, 19*). In this regard it has been pointed out, but to our knowledge not definitely proven, that carbonyl moieties can be introduced into proteins not only by direct oxidation of amino acid side chains but also by reaction with aldehydes such as 4-hydroxynonenal and malonaldehyde formed from oxidation products of polyunsaturated fatty acids (*1, 2*). We found that treatment of proteins with acetaldehyde, a monofunctional aldehyde, can generate carbonyl moieties detectable by immunoblot (Fig. 7), suggesting that the reaction with aldehydes can, in fact, generate protein carbonyls. Glycation, which has been shown to occur in some proteins in aged tissues, can not only generate carbonyl moieties directly but also give rise to reactive oxygen species *via* formation of a Schiff's base or its Amadori products which may, in turn, generate carbonyls (*16, 29, 30*). In the present experiment, while increase in carbonylation of a protein did occur at a high

concentration of glucose, it was not significant even after 9 days of incubation when the glucose concentration was at a physiological (0.8 mg/ml) or a diabetic (1.6 mg/ml) level. Since an intracellular concentration of glucose is lower than that in the serum and intracellular proteins with half-lives longer than 9 days are rare, reaction products with glucose are not likely to contribute significantly to carbonyls in intracellular proteins. It should, however, be mentioned that the suppressive effect of EDTA on carbonylation suggests that generation of protein carbonyls by metal-catalyzed direct oxidation of amino acid side chains may be promoted by glycation, perhaps *via* generation of active oxygens (*31*).

These results suggest that it is important to identify chemical structures to prove that a carbonyl group reactive to DNPH is indeed generated by direct oxidation of an amino acid residue or is formed as a result of amino acid modification by other mechanisms. The premise that carbonyls found in tissue proteins are the consequence of direct oxidation of amino acid residues should be carefully examined for individual carbonylated proteins.

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REFERENCES

1. Oliver, C.N., Ahn, B., Moerman, E.J., Goldstein, S., and Stadtman, E.R. (1987) Age-related changes in oxidized proteins. *J. Biol. Chem.* **262**, 5488-5491
2. Stadtman, E.R. (1992) Protein oxidation and aging. *Science* **257**, 1220-1224
3. Sohal, R.S., Agarwal, S., Dubey, A., and Orr, W.C. (1993) Protein oxidative damage is associated with life exprectancy of houseflies. *Proc. Natl. Acad. Sci. USA* **90**, 7255-7259
4. Smith, C.D., Carney, J.M., Starke-Reed, P.E., Oliver, C.N., Stadtman, E.R., Floyd, R.A., and Markesbery, W.R. (1991) Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **88**, 10540-10543
5. Ottnad, E., Parthasarathy, S., Sambrano, G., Ramprasad, M., Quehenberger, O., Kondratenko, N., Green, S., and Steinberg, D. (1995) A macrophage receptor for oxidized low density lipoprotein distinct from the receptor for acetyl low density lipoprotein: Partial purification and role in recognition of oxidatively damaged cells. *Proc. Natl. Acad. Sci. USA* **92**, 1391-1395
6. Hodge, J.J., Taylor, A., Shang, F., Huang, L.L., and Mura, C. (1994) Oxidative stress to lens crystallins. *Methods Enzymol.* **233**, 512-522
7. Chapman, M.L., Runbin, B.R., and Gracy, R.W. (1989) Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. *J. Rheumatol.* **16**, 15-18
8. Levine, R.L. (1983) Oxidative modification of glutamine synthetase II. Characterization of the ascorbate system. *J. Biol. Chem.* **258**, 11828-11833
9. Davies, K.J.A., Delsignore, M.E., and Lin, S.W. (1987) Protein damage and degradation by oxygen radicals II. Modification of amino acids. *J. Biol. Chem.* **262**, 9902-9907
10. Amici, A., Levine, R.L., Tsai, L., and Stadtman, E.R. (1989) Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation. *J. Biol. Chem.* **264**, 3341-3346
11. Stadtman, E.R. and Berlett, B.S. (1991) Fenton chemistry. Amino acid oxidation. *J. Biol. Chem.* **266**, 17201-17211
12. Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., and Stadtman, E.R. (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol.* **186**, 464-478
13. Levine, R.L., Williams, J.A., Stadtman, E.R., and Shacter, E. (1994) Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233**, 346-357
14. Lenz, A.-G., Costabel, U., Shaltiel, S., and Levine, R.L. (1989) Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohyride. *Arch. Biochem. Biophys.* **177**, 419-425
15. Winter, M.L. and Liehr, J.G. (1991) Free radical-induced carbonyl content in protein of estrogen-treated hamsters assayed by sodium boro [^{3}H]hydride reduction. *J. Biol. Chem.* **266**, 14446-14450
16. Hayase, F., Nagaraj, R.H., Miyata, S., Njoroge, F., and Monnier, V.M. (1989) Aging of proteins: Immunological detection of a glucose-derived pyrrole formed during Mailard reaction in vivo. *J. Biol. Chem.* **264**, 3758-3764
17. Beppu, M., Murakami, K., and Kikugawa, K. (1987) Detection of oxidized lipid-modified erythrocyte membrane proteins by radiolabeling with tritiated borohydride. *Biochim. Biophys. Acta* **897**, 169-179
18. Shacter, E., Williams, J.A., Lim, M., and Levine, R.L. (1994) Differential susceptibility of plasma proteins to oxidative modification: Examination by Western blot immunoassay. *Free Rad. Biol. Med.* **17**, 429-437
19. Cabiscol, E. and Levine, R.L. (1995) Carbonic anhydrase III. Oxidative modification in vitro and loss of phosphatase activity during aging. *J. Biol. Chem.* **270**, 14742-14747
20. Nakamoto, H., Nakamura, A., Goto, S., Hosokawa, M., Fujisawa, H., and Takeda, T. (1994) Accumulation of oxidatively modified proteins in senescence-accelerated mouse SAMP11 and SAMR1 in *The SAM Model of Senescence* (Takeda, T., ed.) pp. 137-140, Elsevier Science B.V., Amsterdam
21. Nakamura, A., Nakamoto, H., and Goto, S. (1994) Western blot analysis of carabonylated proteins in young and old rat tissues. *Biomed. Gerontol. (Japan)* **18**, 300-301
22. Goto, S., Hasegawa, A., Nakamoto, H., Nakamura, A., Takahashi, R., and Kurochkin, I.V. (1995) Age-associated changes of oxidative modification and turnover of proteins in *Oxidative Stress and Aging* (Cutler, R., Packer, L., Bertram, J., and Mori, A., eds.) pp. 151-158, Birkhauser Verlag
23. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
24. O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *Biol. Chem.* **250**, 4007-4021
25. Israel, Y., Hurwitz, E., Niemela, O., and Arnon, R. (1986) Monoclonal and polyclonal antibodies against acetaldehyde-containing epitopes in acetaldehyde-protein adducts. *Proc. Natl. Acad. Sci. USA* **83**, 7923-7927
26. Kikugawa, K., Takayanagi, K., and Watanabe, S. (1985) Polylysines modified with malonaldehyde, hydroperoxylinoleic acid and monofunctional aldehydes. *Chem. Pharm. Bull.* **33**, 5437-5444
27. Lee, Y.-J. and Shacter, E. (1995) Role of carbohydrates in oxidative modification of fibrinogen and other plasma proteins. *Arch. Biochem. Biophys.* **321**, 175-181
28. Suzuki, H., Ogura, Y., and Yamada, H. (1971) Stoichiometry of the reaction by amine oxidase from *Aspergillus niger*. *J. Biochem.* **69**, 1065-1074
29. Mullarkey, C.J., Edelstein, D., and Brownlee, M. (1990) Free radical generation by early glycation products: A mechanism for accelerated atherogenesis in diabetes. *Biochem. Biophys. Res. Commun.* **173**, 932-939
30. Araki, N., Ueno, N., Chakrabarti, B., Morino, Y., and Horiuchi, S. (1992) Immunochemical evidence for the presence of advanced glycation end products in human lens proteins and its positive correlation with aging. *J. Biol. Chem.* **267**, 10211-10214
31. Kaneto, H., Fujii, J., Suzuki, K., Kasai, H., Kawamori, R., Kamada, T., and Taniguchi, N. (1994) DNA cleavage induced by glycation of Cu,Zn-superoxide dismutase. *Biochem. J.* **304**, 219-225