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Original Research Communication

Development of a Sensitive Assay to Detect Reversibly Oxidized Protein Cysteine Sulfhydryl Groups

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ABSTRACT

Protein sulfhydryl groups can undergo reversible oxidation reactions in response to reactive oxygen and reactive nitrogen species. Sensitive detection of sulfhydryl group oxidation in specific proteins is required to further our understanding of protein redox changes in biological systems. In general, to detect reversible oxidation reactions the oxidized sulfur atom is reduced to a sulfhydryl group followed by a reaction with a quantifiable agent. Our aim was to develop a sensitive method to detect reversibly oxidized protein sulfhydryl groups in a Western blot format. Conjugation of methoxypolyethylene glycol-maleimide (MAL-PEG) to protein sulfhydryl groups was optimized. Once MAL-PEG forms a covalent bond with the protein, the MAL-PEG–protein conjugate can be detected as a band shift by western analysis. The efficiency of MAL-PEG conjugation to protein was determined with creatine kinase. MAL-PEG conjugated to ~100% of the available sulfhydryl groups on creatine kinase within 30 min. Band shift detection sensitivity was measured using the redox-regulated protein p53. MAL-PEG conjugation coupled to western analysis detected a minimum of 0.23 pmol of oxidized p53. The MAL-PEG conjugation method described in this communication can be used to assess the reversible sulfhydryl oxidation status of proteins for which antibodies suitable for western analysis are available. Antioxid. Redox Signal. 3, 1105–1118.

INTRODUCTION

It has been nearly fifty years since Harman first suggested that endogenous oxygen radicals generated in cells could result in cumulative damage (8). Subsequently, it was determined that a number of small molecules that centered on oxygen and nitrogen, known collectively as reactive oxygen species and reactive nitrogen species, could damage biological tissues (26). Although a substantial amount of evidence indicates that this is true, we now know that these small molecules can also mediate biological responses through oxidation of appropriate protein targets (3, 26).

One amino acid residue susceptible to oxidation is cysteine. Recent evidence has indicated that epidermal growth factor (EGF)-mediated and some nitric oxide-mediated signaling pathways depend on protein sulfhydryl group oxidation reactions to effect biological responses (15, 16, 19, 20). Oxidation of the sulfhydryl group of protein cysteine residues can be either irreversible or reversible. Irreversible oxidation reactions result in sulfinic acid or sulfonic acid. To date, enzymes that reduce such oxidized sulfur atoms within cysteine residues have not been isolated. Reversible cysteine sulfhydryl oxidation reactions include *S*-nitrosylation, *S*-glutathiolation, *S*-

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cysteylation, sulfenic acid formation, and protein cystine residue disulfide bond formation (25). A number of enzymes that can reduce these oxidized sulfhydryl groups to the thiol have been characterized (19). Efforts to understand the emerging field of reversible protein oxidation have been hampered by the fact that current detection methods are often too insensitive to permit direct measurement of protein oxidation reactions in cells and tissues. Recently, we introduced a sensitive method for measuring reversible sulfhydryl group oxidation reactions in specific proteins (30). A major question raised from our previous work was whether this new method was efficient and sensitive.

Methods designed to assess reversible oxidation reactions at protein sulfhydryl groups typically require that other nonoxidized sulfhydryl groups on the protein first be capped with a reagent such as maleimide. After capping, oxidized sulfhydryl groups are reduced with an agent such as dithiothreitol (DTT). Reduced sulfhydryl groups are then reacted with a compound that can be quantified. Quantification commonly relies on spectroscopic and fluoroscopic techniques that have detection limits within the 10–300 pmol range (27), necessitating purification of a significant amount of protein for analysis. In theory, sensitivity of thiol detection can be increased significantly if it can be coupled to western analysis (12, 30, 31), a technique that does not require purification of the protein prior to analysis. In the coupled assay introduced here, the free sulfhydryl group is covalently linked to a maleimide moiety that is part of a large-molecular-weight molecule. The compound increases the molecular mass of the protein upon adduct formation. The large protein–conjugate complex is then detected as a band shift by western analysis. However, it is not clear if such large maleimide compounds can efficiently react with protein sulfhydryl groups.

To address this critical issue, a maleimide moiety linked to polyethylene glycol (MAL-PEG) was conjugated to creatine kinase (CK), a protein with a single thiol group exposed on the surface of the protein (18). MAL-PEG has a molecular mass of 5,000 Da, and thus adduct formation with a target polypeptide of average molecular mass should significantly retard its mobility on standard protein gels. We compared the number of CK thiol groups that conjugated to MAL-PEG to the total number of CK thiol groups that were available for conjugation, as determined by isoelectric focusing (IEF) analysis. We found that MAL-PEG was able to conjugate to 100% of the available thiol groups on CK. Western analysis of reversibly oxidized p53 protein indicated that the MAL-PEG method allows direct detection of as little as 0.23 pmol of oxidized protein. This constitutes a 20–50-fold increase in sensitivity compared with other direct detection techniques.

EXPERIMENTAL PROCEDURES

Reagents

Rabbit muscle creatine phosphokinase (CK) was purchased from CalBiochem–Novabiochem (San Diego, CA, U.S.A.) or Sigma–Aldrich (St. Louis, MO, U.S.A.). CK monomer concentration was determined using a calculated extinction coefficient of 40,152 $M^{-1}cm^{-1}$ at 280 nm. Methoxypolyethylene glycol-maleimide, MW 5,000 (MAL-PEG), and methoxypolyethylene glycol-maleimide, MW 2,000 (MAL-PEG-2000), were purchased from Shearwater Polymers, Inc. (Huntsville, AL, U.S.A.). 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB), guanidine hydrochloride (Gu-HCl), *N*-ethylmaleimide (NEM), 2-hydroxyethyldisulfide (HED), β-glycerophosphate, horse cytochrome *c*, iodoacetamide (IAM), DTT, and Coomassie Brilliant Blue were purchased from Sigma–Aldrich. All other common reagents were purchased from Fisher Scientific (Tustin, CA, U.S.A.). Dialyses were performed in Slide-A-Lyzer Mini Dialysis Units, 10,000 molecular weight cutoff (Pierce, Rockford, IL, U.S.A.).

MAL-PEG conjugation to CK

CK was dissolved to a final concentration of 25 μM in 0.1 *M* sodium phosphate, pH 7.4, 5 m*M* EDTA, 5 m*M* EGTA, 0.1% Nonidet P-40 (SEEN) buffer or 20 m*M* β-glycerophosphate buffer, pH 7.4. To denature CK, Gu-HCl was added to a final concentration of 6 *M* as a solid, which lowered the pH of the solution to 6.2. MAL-PEG was added to an appropriate final

concentration, and the sample was allowed to incubate for 15 min. Five percent of the total volume was quenched by the addition of excess hot sodium dodecyl sulfate (SDS) sample buffer (14), incubated at ~95°C for 1–3 min, and stored at 0–4°C until analysis. For SDS–polyacrylamide gel electrophoresis (PAGE) analysis, the sample was reheated to 95°C and immediately loaded onto a discontinuous denaturing gel. In some cases, one-fourth volume of SEEN was added just prior to gel loading to reduce the viscosity of the solution. Gels were stained with Coomassie Brilliant Blue R and destained according to standard protocols. Stained gels were equilibrated in water, dried in a 5% glycerol/95% water solution, and electronically scanned. Densitometry was performed on the scanned images with UN-SCAN-IT gel software (Version 5.1, Silk Scientific Corp., Orem, UT, U.S.A.) set at the logarithm setting. Data were compiled onto a spreadsheet with Excel software (Microsoft Corp., Redman, WA, U.S.A.), and graphs were created with Microsoft Excel and ProStat software (Poly Software International, Salt Lake City, UT, U.S.A.).

Isolation of the reduced form of CK

CK was dissolved to 8 mg/ml in 20 m*M* Tris, 10 m*M* DTT, pH 7.6, and the solution was incubated at 37°C for 30 min to remove possible DTT-reversible modifications. DTT was removed by dialysis against 20 m*M* Tris, pH 7.6, and the extent of DTT removal was assessed by testing the dialysate with DTNB (6). CK was treated with 20 m*M* HED at 37°C for 30 min to block reactive cysteine residues with the uncharged adduct-mercaptoethanol group (MEOH). Any cysteine residues irreversibly oxidized to sulfinic or sulfonic acid are negatively charged and do not react with HED (data not shown). HED-treated CK was centrifuged at 15,000 *g* for 5 min and filtered by centrifugation through a 0.22-μm cellulose acetate filter (Spin-X column, Costar, Acton, MA, U.S.A.). CK-MEOH was separated from irreversibly oxidized CK on a 10 × 64 mm Bioscale DEAE-5 anion–exchange column (Bio-Rad, Hercules, CA, U.S.A.). Proteins were eluted with an isocratic gradient of 20 m*M* Tris, pH 7.6, at a flow rate of 1.5 ml/min. The column was washed for 5 min with 20 m*M* Tris, pH 7.6, 100 m*M* NaCl and reequilibrated with 20 m*M* Tris, pH 7.6, for 10 min between each injection. Collected fractions containing CK-MEOH were pooled, concentrated to 2–3 mg/ml, and stored at −80°C. Reduced CK was prepared by incubation in 10 m*M* DTT, 20 m*M* β-glycerophosphate, pH 7.4, for 30 min at 37°C followed by overnight dialysis against 20 m*M* β-glycerophosphate.

IEF was also used to separate oxidized CK from CK alkylated with either IAM or NEM (17). The pI of each IEF-separated CK protein was determined by excising protein-containing gel slices from the gel. The gel slices were incubated in water to allow the ampholytes to diffuse out of the gel, and the pH of the water/ampholyte mixture was measured.

Calculation of the average number of MAL-PEG adducts per polypeptide

The following calculation was used to determine the average number of MAL-PEG molecules covalently bound to each polypeptide from gel densitometry measurements:

$$\frac{n(D_n) + (n-1)(D_{n-1}) + (\mathrm{n}-2)(D_{n-2}) + \ldots + (0)(D_0)}{100}$$

where n is the total number of thiol groups available for adduct formation on the CK polypeptide and D_n is the percentage of protein band density within a single gel lane bound to n MAL-PEG molecules.

Spectrophotometric analysis of MAL-PEG breakdown

MAL-PEG was dissolved to 2 m*M* in ice-cold SEEN buffer without Nonidet P-40. The solution was placed at the appropriate temperature using a water bath, and its 400 nm–200 nm absorbance spectrum was measured on a UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan). The absorbance at 305 nm was read from each graph.

Purification of recombinant human p53

A slight modification of the recombinant p53 purification protocol of Delphin *et al.* was used

(2). In brief, at 72 h after infection with recombinant human p53-expressing baculovirus (received as a gift from G. Zambetti, St. Jude's Hospital, Memphis, TN, U.S.A.), 20 75-mm^2 flasks with confluent Sf9 cells were harvested. Cells were washed twice with ice-cold 10 m*M* Na_2HPO_4, pH 7.2, 130 m*M* NaCl buffer and resuspended in 100 ml of 10 m*M* Tris-Cl, pH 7.6, 12% sucrose, 2 m*M* EGTA, 2 m*M* phenylmethylsulfonyl fluoride, 5 m*M* DTT (buffer A). Triton X-100 was added to a final concentration of 0.2% (vol/vol), and cells were centrifuged at 1,000 *g* for 3 min. The pellet was resuspended in 100 ml of buffer A supplemented with 0.1% Triton X-100. The resuspended nuclei were centrifuged again at 1,000 *g* for 3 min. Nuclei were lysed in 30 ml of 20 m*M* Tris-Cl, pH 7.6, 2 m*M* EGTA, 10 m*M* DTT, 2 m*M* phenylmethylsulfonyl fluoride (buffer B) supplemented with 0.5 *M* NaCl on ice for 15 min. Nuclear extracts were centrifuged at 100,000 *g* for 60 min. The supernatant was diluted fivefold with buffer B to a final concentration of 0.1 *M* NaCl. After centrifugation at 20,000 *g* for 30 min, the supernatant was applied to a 3-ml Q-Sepharose column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was washed with 40 m*M* Tris-HCl, pH 7.6 (buffer C) supplemented with 0.2 *M* NaCl. The p53 protein was eluted from the column with buffer C supplemented with 0.3 *M* NaCl and was >90% pure as judged by SDS-PAGE analysis and Coomassie Blue staining. The data indicated that the p53 obtained from this procedure was mildly oxidized due to the lack of reducing agent in the elution buffer. Protein was quantified by comparison with known amounts of bovine serum albumin protein on Coomassie-stained SDS-PAGE gels.

MAL-PEG conjugation method to detect oxidized p53

The purified p53 protein, at 0.2 mg/ml, was incubated with 10–500 μM hydrogen peroxide (H_2O_2) in SEEN buffer for 1 h at 0°C. The reaction was quenched with 10 m*M* NEM to cap remaining free thiol groups on p53. After 2 h, the mixture was dialyzed against SEEN. DTT was then added to each sample to a final concentration of 20 m*M* to create free thiol groups on p53. After exhaustive dialysis, 300 μM MAL-PEG-2000 was added to p53, and the mixture was allowed to incubate for 1 h at 0°C. The reaction was quenched by the addition of SDS sample buffer, and p53-MAL-PEG-2000 protein was separated by SDS-PAGE and detected by Coomassie staining.

For western analysis, a solution containing 0.8 μg/ml oxidized p53, 1.0 mg/ml cytochrome *c* (as carrier), 1 m*M* NEM, and 6 *M* Gu-HCl in SEEN, pH 7.4, was incubated for 15 min at 37°C to cap all reactive sulfhydryl groups. The solution was dialyzed against a 400-fold excess of 6 *M* Gu-HCl in SEEN, pH 7.4, for a minimum of 4 h at room temperature. DTT was added to a final concentration of 1 m*M* and incubated for 15 min at 37°C. The sample was dialyzed against 6 *M* Gu-HCl in SEEN, pH 7.4, overnight at 4°C and then incubated with 2 m*M* MAL-PEG for 15 min at 37°C. The MAL-PEG tagging reaction was quenched by the addition of an equal volume of hot SDS sample buffer. The sample containing p53 was boiled for 3 min, diluted into sample buffer to achieve the indicated amount of protein, and separated by SDS-PAGE. Western analysis using DO-1 antibody (CalBiochem–Novabiochem) was performed as previously described (30).

RESULTS

Analysis of CK-MAL-PEG reaction kinetics

CK is a homodimer in which each polypeptide subunit has one cysteine residue at its active site on the surface of the protein (18). Three other cysteine sulfhydryl groups within the subunit are solvent-inaccessible. To assess the ability of MAL-PEG to form a conjugate with the single exposed cysteine residue (Cys^{283}), CK was incubated at 0°C with a 10-fold molar excess of MAL-PEG, relative to the four CK thiol groups. Within 60 min, the majority of CK formed a single adduct with MAL-PEG (Fig. 1A, lanes 7 and 8). Densitometry measurements of the protein bands indicated that 70% of CK was bound to at least one MAL-PEG adduct and that the average number of MAL-PEG adducts per CK monomer was 0.90 after 24 h (Fig. 1B). This average was calculated based on

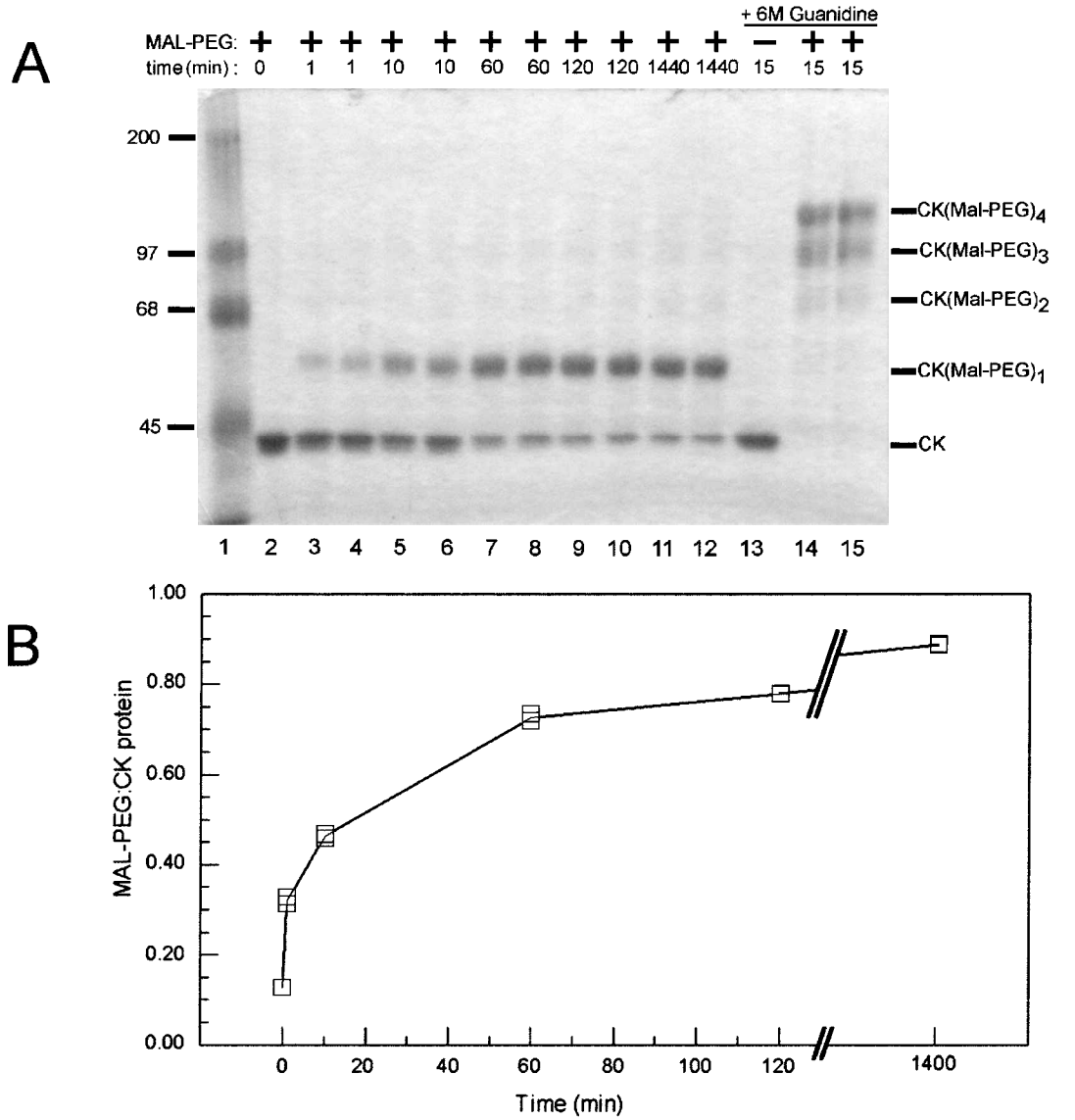

FIG. 1. Time-course study of CK reaction with MAL-PEG. CK was incubated with a 10:1 ratio of MAL-PEG to protein sulfhydryl groups for the indicated time periods. The reaction, carried out in SEEN buffer, was quenched by the addition of SDS-PAGE sample buffer, and the CK-MAL-PEG protein adducts were separated by SDS-PAGE on an 8% gel. Proteins were visualized by staining with Coomassie Blue. (**A**) Lane 1, protein molecular mass standards (in kDa); lanes 2–12, time-course reaction incubated at 0°C; lane 13, CK incubated in the absence of MAL-PEG and in the presence of 6 *M* Gu-HCl for 15 min; lanes 14 and 15, CK incubated in the presence of MAL-PEG and 6 *M* Gu-HCl for 15 min. For lanes 13–15, the reaction was carried out at 37°C. (**B**) Quantification of time-course reaction by densitometry. *y*-axis, calculated number of MAL-PEG adducts bound per CK polypeptide; *x*-axis, time of reaction (in min).

the amount of each CK-MAL-PEG conjugate and unconjugated CK (see Experimental Procedures). The majority of CK (56%) was bound to one MAL-PEG adduct. Thirty percent of the CK did not react with MAL-PEG, and 14% of the CK formed covalent linkages with two or more MAL-PEG molecules. Presumably, one of these latter linkages occurred at Cys^{283}, suggesting that the efficiency of conjugation at that site was 70%.

To assess the ability of MAL-PEG to react with the three other cysteine sulfhydryl groups in the CK monomer, the reaction mixture was incubated in the presence of the denaturant, Gu-HCl, at 37°C (Fig. 1A, lanes 14 and 15). In the presence of Gu-HCl, 42% of CK formed a

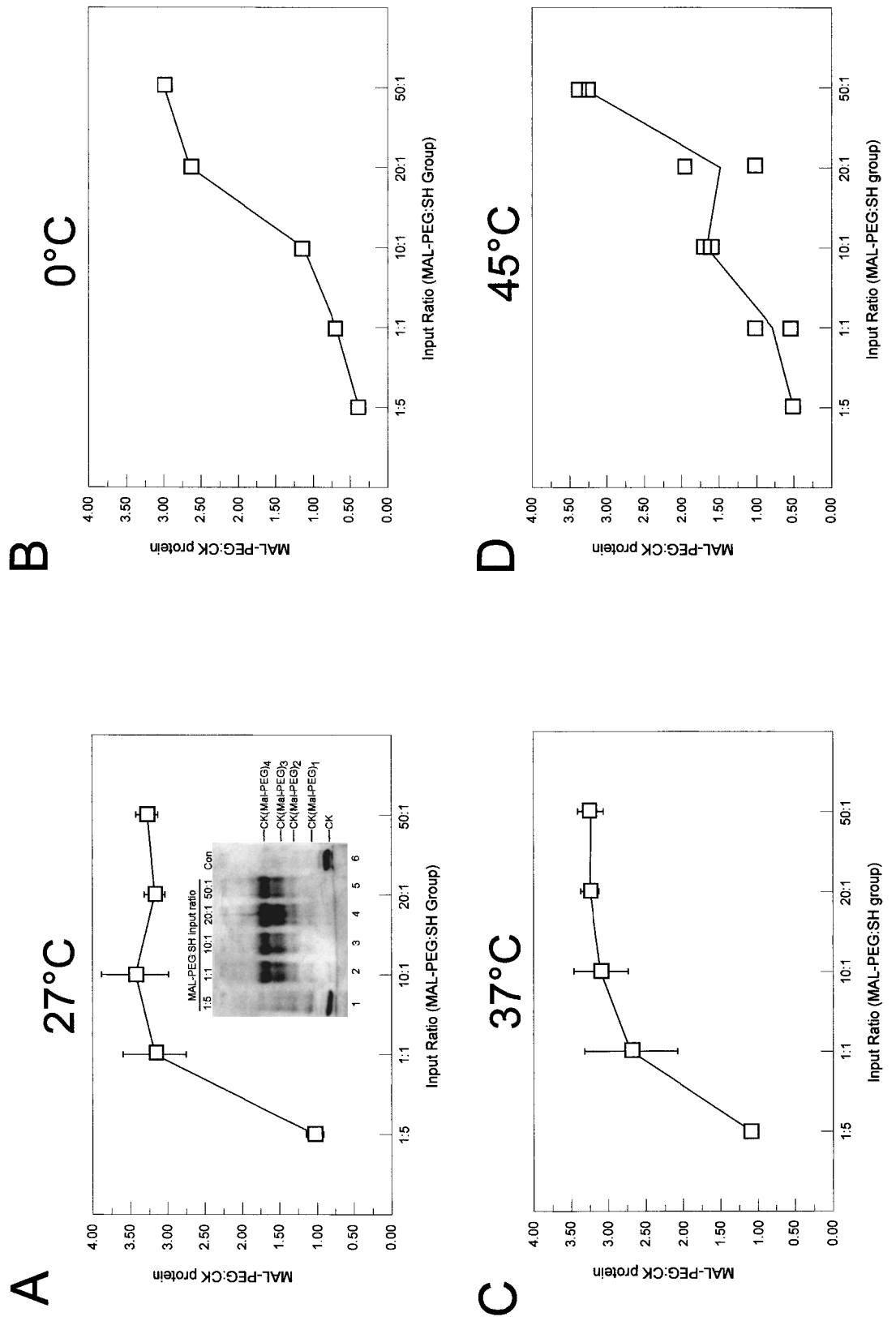

FIG. 2. Temperature dependence of MAL-PEG conjugation to CK. CK was incubated with the input ratio of MAL-PEG to protein sulfhydryl group, as indicated on the x-axis. In each case, the reaction was conducted for 15 min in the presence of Gu-HCl. The amount of MAL-PEG conjugation calculated from densitometry is represented on the *y*-axis. (**A**) 27°C, $n = 3$. The **inset** is an image of a stained gel of one representative experiment. (**B**) 0°C, $n = 1$. (**C**) 37°C, $n = 3$. (**D**) 45°C, $n = 2$. Bars indicate standard deviation.

conjugate with four MAL-PEG molecules, 35% formed a conjugate with three MAL-PEG molecules, and the remaining 22% of CK formed conjugates with two MAL-PEG molecules or fewer. The average number of MAL-PEG adducts conjugated to denatured CK was 3.2. Thus, MAL-PEG is capable of forming a covalent linkage with all four cysteine thiol groups in denatured CK, albeit at <100% efficiency.

Effect of MAL-PEG:sulfhydryl group ratio on MAL-PEG conjugation to CK

To determine if the efficiency of MAL-PEG conjugation to sulfhydryl groups could be improved, CK was incubated with increasing levels of MAL-PEG in the presence of Gu-HCl. At 27°C, when the input ratio of MAL-PEG to CK sulfhydryl groups was 1:5, the average number of MAL-PEG molecules covalently bound to each CK subunit was ~1.0 (Fig. 2A). The maximum number of MAL-PEG molecules that can theoretically bind to CK at the 1:5 input ratio is 0.80 because MAL-PEG is limiting. This indicates that MAL-PEG binding to sulfhydryl groups is extremely efficient at 27°C and is stable to postreaction procedures. Furthermore, the high efficiency demonstrates that the amino group of the guanidine in the buffer does not compete, to a significant extent, with protein thiol groups for the maleimide moiety of MAL-PEG. When the input ratio of MAL-PEG to CK sulfhydryl groups was increased to 1:1, the average number of MAL-PEG molecules bound to CK increased to an average of 3.2. When the input ratio of MAL-PEG to CK sulfhydryl groups was increased to 50:1, there was no further increase in the number of MAL-PEG molecules bound to CK. The relative percentages of one-, two-, three-, or four-adducted protein also did not change significantly. The overall efficiency of MAL-PEG conjugation was 80% in the presence of the denaturant. The data suggest that all of the MAL-PEG is capable of reacting with CK, but that a subpopulation of CK cannot react completely with MAL-PEG.

Effect of temperature and pH on MAL-PEG conjugation to CK

To test further if the efficiency of MAL-PEG conjugation to CK could be improved, the reaction was performed for 15 min at 0°C, 37°C, and 45°C (Fig. 2B–D). At 0°C, the reaction was inefficient except at the highest concentration of MAL-PEG. When the ratio of MAL-PEG to sulfhydryl groups was 1:1, the reaction at 27°C was slightly more efficient than at 37°C. However, when MAL-PEG was at 10-fold molar excess or more, the average number of MAL-PEG molecules bound to CK was nearly identical at these two temperatures. Interestingly, at 45°C,

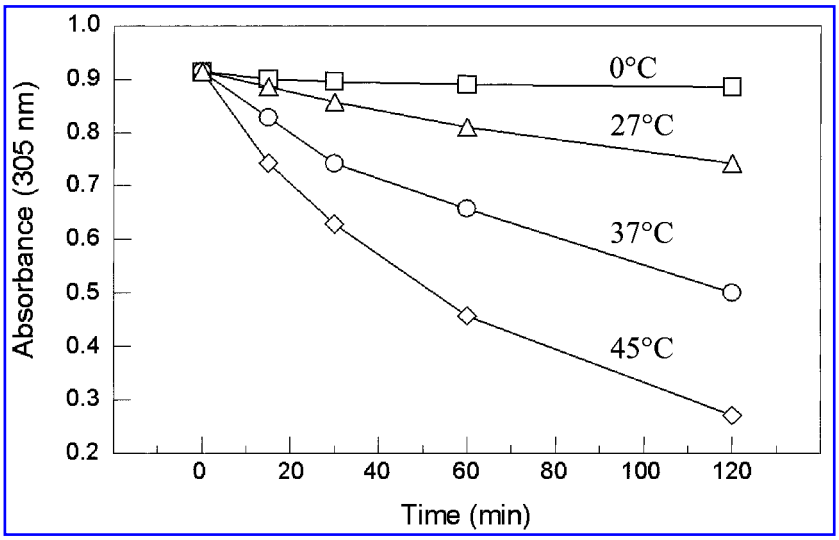

FIG. 3. Temperature dependence of conversion of MAL-PEG to maleamic acid. MAL-PEG was incubated at pH 7.4 for 0, 15, 30, 60, and 120 min. At the indicated time and temperature, absorbance at 305 nm was obtained. Absorbance at 305 nm is a measure of the integrity of the carbon–carbon double bond in MAL-PEG.

MAL-PEG conjugation to CK was significantly inhibited except at the highest concentration of MAL-PEG. It is possible that the reaction becomes less efficient at 45°C because the carbon–carbon double bond in MAL-PEG hydrolyzes prior to nucleophilic attack by the thiol group.

Water can compete with thiolate ions to add to the carbon–carbon double bond in maleimides, resulting in conversion of maleimide to maleamic acid (9). This reaction can be monitored spectrophotometrically by measuring the absorbance of the double bond at 305 nm (21). To determine if MAL-PEG is susceptible to water addition at the reaction temperatures used in this study, MAL-PEG conversion to maleamic acid was monitored spectrophotometrically in aqueous buffer at 0°C, 27°C, 37°C, and 45°C. The data show that an increase in temperature significantly increased the rate of maleamic acid production (Fig. 3). This result partially explains why, at 45°C, MAL-PEG failed to conjugate CK efficiently (see Fig. 2D). However, it is likely that there are other reasons for the lack of reactivity as well. In summary, MAL-PEG conjugated CK at 27°C and 37°C at 80% efficiency, but not at 45°C. At 0°C, the MAL-PEG did not form significant amounts of maleamic acid. However, the decreased reaction kinetics of MAL-PEG at 0°C (Fig. 2B) indicates that a reaction time of 60 min is required for high conjugation efficiency (see Fig. 1).

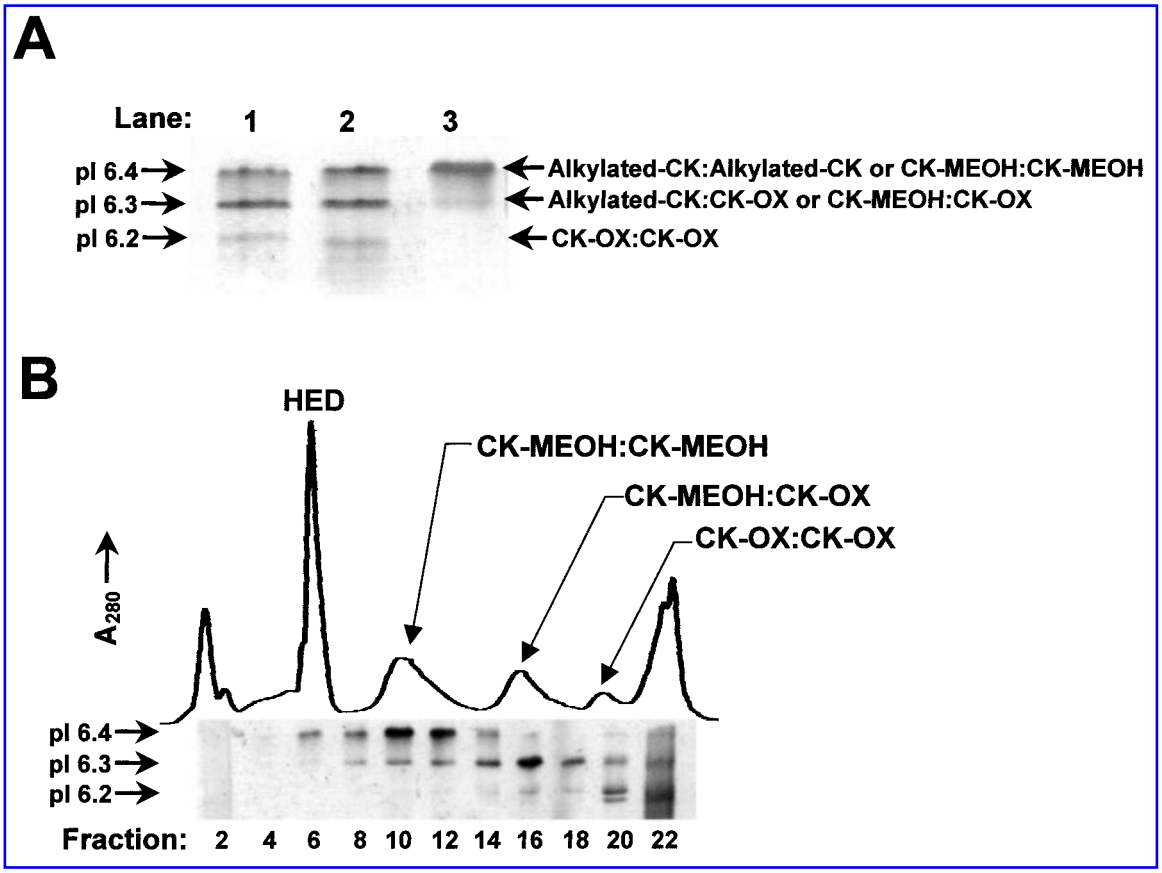

FIG. 4. IEF analysis of commercial and purified CK. (**A**) Two micrograms of CK was applied to IEF gels and analyzed for the presence of irreversibly oxidized CK. Samples were treated with 10 m*M* DTT for 30 min at room temperature in 20 m*M* β-glycerophosphate, pH 7.4, to remove potential trace reversible oxidation reactions. Samples were then alkylated with 40 m*M* IAM for 20 min at room temperature shielded from light. Lane 1, CK received from CalBiochem–Novabiochem; lane 2, CK received from Sigma–Aldrich; lane 3, CK-MEOH isolated by anion–exchange chromatography. (**B**) Purification of CK-MEOH from irreversibly oxidized forms of CK. Approximately 2–3 mg of HED-treated CK was separated by anion–exchange chromatography. Elution positions of unreacted HED, CK-MEOH, and irreversibly oxidized CK (CK-OX) are shown. Indicated column fractions were analyzed by IEF immediately after chromatographic separation. The IEF gels were stained with Coomassie Blue. Note that CK-MEOH and CK conjugated to IAM both have a pI of 6.4.

The ability of the thiol group to attack the maleimide moiety of MAL-PEG depends on its ability to form a thiolate anion. Amino acid residues in close proximity to the thiol groups within CK can influence the pK_a of the sulfhydryl group (25). Thus, the pH of the reaction solution can influence the efficiency of MAL-PEG conjugation. In general, optimal reaction conditions for maleimides range from pH 6.5 to pH 7.5 (9). At pH > 8.0, some maleimides can react with primary amines. Another consideration is the conversion of maleimide to maleamic acid via water attack, which can occur at appreciable rates in aqueous solution at higher pH (9). We tested whether CK reactivity toward MAL-PEG was influenced by pH over a range of 6.2–8.0 in the presence of Gu-HCl, but observed no significant difference in the level of conjugation of MAL-PEG conjugation to CK in this pH range (data not shown). Thus, MAL-PEG coupling to CK was not altered within the pH range of 6.2–8.0.

Analysis of CK oxidation status

We next attempted to explore the possible reasons why 100% of the CK did not react with MAL-PEG. It is possible that some of the CK is oxidized to sulfenic acid or disulfide and therefore incapable of reacting with MAL-PEG. To determine if this was the case, CK was incubated with an equimolar ratio of DTT to protein sulfhydryl groups in the presence of excess MAL-PEG. This failed to increase the MAL-PEG conjugation efficiency (data not shown). However, this did not rule out the possibility that a subpopulation of CK is irreversibly oxidized to sulfinic or sulfonic acid.

To test if a subpopulation of CK is irreversibly oxidized, we treated the CK with an alkylating agent. The alkylating agent covalently modifies the reduced form of CK, but not the irreversibly oxidized form of CK. The alkylated CK can be separated from irreversibly oxidized CK by nondenaturing IEF (17). The alkylated CK homodimer has a pI of 6.4. When one subunit within the dimer contains an irreversibly oxidized cysteine residue and the other subunit is alkylated, the pI is lowered to 6.3. When both subunits are irreversibly oxidized, the pI is lowered further to 6.2. IEF analysis indicated that ~30% of the CK subunits from two commercial sources are irreversibly oxidized (Fig. 4A). This irreversibly oxidized CK likely accounts for the 70% efficiency of MAL-PEG tagging detected previously (see Fig. 1).

We proceeded to isolate the reduced form of CK to test for MAL-PEG conjugation efficiency. CK was treated with the sulfhydryl exchange reagent HED to modify the thiol group on

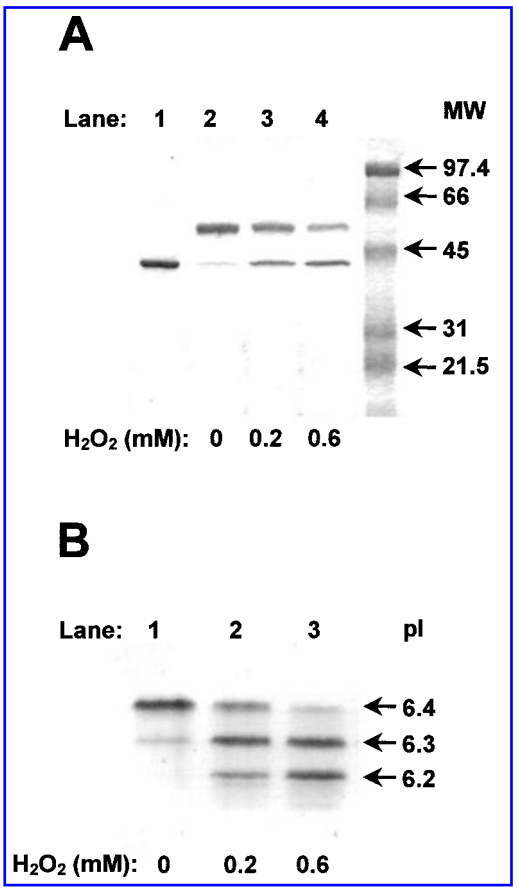

FIG. 5. Comparison of products of H_2O_2 treatment of CK by IEF and MAL-PEG conjugation. Purified reduced CK at 25 μM was treated with 0, 0.2, or 0.6 mM H_2O_2 in 20 mM β-glycerophosphate buffer, pH 7.4, for 10 min at 37°C. The reactions were quenched with 2 mM DTT and incubated for an additional 15 min to remove any reversible modifications. The reactions were treated with 4 mM MAL-PEG and analyzed by SDS-PAGE on a 10% gel (**A**) or 4 mM NEM and analyzed by IEF (**B**). Samples were diluted fourfold with buffer prior to alkylation reactions in order to decrease the DTT concentration. Reactions with MAL-PEG and NEM were carried out at room temperature for 30 min. Lane 1 of A represents purified reduced CK that was not modified with MAL-PEG. Gels were stained with Coomassie Blue.

CK. HED-modified CK (CK-MEOH) was separated from irreversibly oxidized CK by anion-exchange chromatography (Fig. 4B). This isolation procedure resulted in the purification of CK that was 95% CK-MEOH and 5% irreversibly oxidized CK (Fig. 4A, lane 3). CK-MEOH was treated with DTT and alkylated with MAL-PEG. Approximately 94% (±1) of the purified CK reacted with MAL-PEG (Fig. 5A, lane 2; Table 1). NEM treatment of purified CK followed by IEF showed that 94% (±1) of the CK was in the reduced state and therefore available for conjugation (Fig. 5B, lane 1; Table 1). This indicates that MAL-PEG forms a covalent linkage with 100% of the available thiol groups in CK.

If irreversible oxidation prevents MAL-PEG reactivity to thiol groups, then treatment of CK with H_2O_2 should decrease the efficiency of MAL-PEG reactivity. H_2O_2 oxidizes the thiol groups to sulfenic acid, sulfinic acid, and sulfonic acid. The latter two acids are irreversibly oxidized forms and, therefore, after DTT treatment CK should be recalcitrant to MAL-PEG conjugation. After treatment with 0.2 m*M* H_2O_2 followed by DTT treatment, only 62% of the CK reacted with MAL-PEG (Fig. 5A, lane 3; Table 1). Treatment with 0.6 m*M* H_2O_2 resulted in only 39% of the CK remaining reactive with MAL-PEG (Fig. 5A, lane 4; Table 1). IEF analysis appeared to detect 10–20% higher levels of oxidized CK than the MAL-PEG conjugation method after H_2O_2 treatment. As CK contains three other thiols, the higher level of oxidized CK detected by IEF analysis may be due to a low level of irreversible oxidation reactions at these sites. The data strongly suggest that the MAL-PEG can be a useful tool to detect reversible oxidation reactions and that MAL-PEG fails to react with irreversibly oxidized thiols.

Determination of the sensitivity of MAL-PEG conjugation method in the detection of oxidized protein sulfhydryl groups

The benefit of using MAL-PEG to tag reversibly oxidized sulfhydryl groups is the increased sensitivity of detection. To determine the limit of sensitivity, we used a protein that is susceptible to oxidation and, in addition, has a commercially available antibody that can be used to perform western analysis. The p53 protein contains 10 cysteine residues, some of which are known to be sensitive to oxidation (7, 28). The p53 protein was treated with increasing concentrations of H_2O_2 to increase the level of sulfenic acid, sulfinic acid, and sulfonic acid. In this experiment, the relative level of sulfenic acid produced on p53 was measured. After H_2O_2 treatment, residual nonoxidized thiol groups on the p53 protein were capped with NEM. The protein was treated with DTT to reduce the reversibly oxidized thiol groups, and then the protein was reacted with MAL-PEG. It is expected that H_2O_2 treatment should increase the number of thiols oxidized to sulfenic acid.

MAL-PEG conjugation revealed a significant

Table 1. Comparison of IEF and MAL-PEG Conjugation Detection Methods of Oxidized CK

Method		*Percentage of CK monomer*		
		*0 m*M H_2O_2	*0.2 m*M H_2O_2	*0.6 m*M H_2O_2
MAL-PEG	Adducted CK	94 ± 1	62 ± 2	40 ± 4
	Unadducted CK	6 ± 1	38 ± 2	60 ± 4
IEF	Alkylated CK	94 ± 1	55 ± 3	27 ± 4
	Oxidized CK	6 ± 1	45 ± 3	73 ± 4

Duplicate samples used in Fig. 5 were separated by SDS-PAGE or IEF and stained with Coomassie. Average band densitometry measurements were used to calculate percentage of adducted and unadducted CK for the MAL-PEG conjugation method. To obtain the percentage of alkylated and oxidized CK from IEF analysis, band densities corresponding to CK dimers focusing at pI 6.3 (one subunit alkylated and one subunit oxidized) were divided by two. To obtain the percentage of alkylated CK, this value was added to the measured band densities focusing at pI 6.4 (both subunits alkylated). The halved pI 6.3 density value was also added to the measured band density focusing at pI 6.2 (both subunits oxidized) to obtain the percentage of oxidized CK.

level of reversibly oxidized cysteine residues in p53. Even in the absence of H_2O_2 treatment, p53 was observed to contain a low level of reversibly oxidized sulfhydryl groups. Delphin *et al.* previously reported that purified recombinant human p53 was readily susceptible to intersubunit disulfide bond formation (2). The percentage of p53 shifted indicated that 33% of the total was reversibly oxidized prior to H_2O_2 addition (Fig. 6A and B). Of the total p53, 24% contained one reversibly oxidized cysteine residue per p53 molecule and 9% contained two reversibly oxidized cysteine residues per p53 molecule. The percentage of reversibly oxidized p53 molecules increased from 33% to 50% upon addition of 500 μM H_2O_2. Importantly, MAL-PEG conjugation to p53 is specific for DTT-reducible sulfhydryl groups because in the absence of DTT, p53 could not be tagged (Fig. 6A, lane 6). Although we have stated that MAL-PEG is likely to conjugate to cysteine residues that have been converted to H_2O_2-induced sulfenic acid, it is also possible that MAL-PEG conjugates to p53 after H_2O_2-

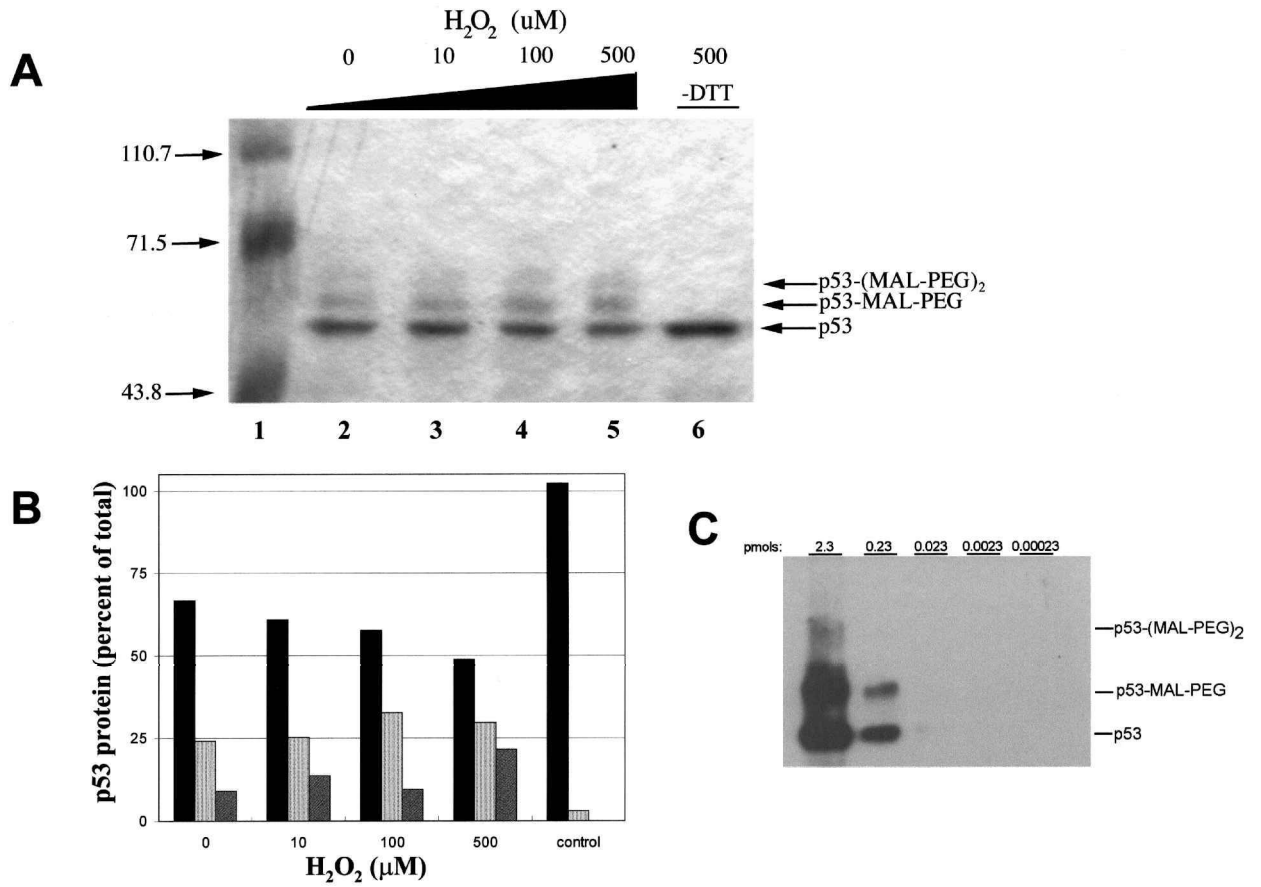

FIG. 6. Analysis of protein products of H_2O_2-treated p53. p53 was incubated with the indicated concentrations of H_2O_2 for 1 h. The reaction was quenched with NEM to cap nonoxidized p53 sulfhydryl groups. The protein was reduced, treated with MAL-PEG-2000, and separated by SDS-PAGE on a 8% gel. (**A**) Lane 1, protein molecular mass markers (measured in kDa); lanes 2–5, p53 treated with 0–500 μM H_2O_2; lane 6, p53 oxidized with 500 μM H_2O_2 and processed for MAL-PEG-2000 tagging in the absence of the DTT reducing step. The gel was stained with Coomassie Blue. (**B**) Band densities from A were quantified, and the total amount of protein in each lane was set to 100%. In each three-column set, the left column represents unconjugated p53, the middle column represents the single-MAL-PEG-2000-conjugated protein, and the right column represents the double-conjugated protein. The percentage of each p53 protein subpopulation was plotted. The apparent percentage of unconjugated p53 protein in the "control" experiment is >100 because the gel background in that particular lane is less dense than the average background density. (**C**) Recombinant human p53 (no H_2O_2 treatment) was capped with NEM, treated with DTT, and then incubated with MAL-PEG for 15 min. The indicated amounts of p53 were loaded onto an 8% gel and separated by SDS-PAGE. Western analysis was performed as described in Experimental Procedures.

induced disulfide bond formation. There are two interesting points to be derived from this experiment. First, p53 appears to contain two cysteine residues per p53 molecule that are capable of undergoing reversible oxidation. Second, our simple conjugation procedure appears capable of tagging the oxidized protein in a fashion that is amenable to western blot analysis. To determine the sensitivity of the oxidation detection procedure, MAL-PEG-conjugated p53 was serially diluted and processed for western analysis (Fig. 6C). The minimum detection limit was ~0.23 pmol of reversibly oxidized p53.

DISCUSSION

Our results indicate that MAL-PEG is an efficient method to assess the reversible oxidation status of cysteine thiol groups in proteins. First, reduced protein sulfhydryl groups are capped with either IAM or NEM. Second, the remaining oxidized sulfhydryl groups are reduced with DTT. Third, MAL-PEG is conjugated to the newly reduced sulfhydryl groups. Fourth, western analysis is used to detect the band shift of low-abundance proteins with an antibody directed to the protein of interest. MAL-PEG was capable of reacting with 100% of the available thiol groups at Cys^{283} of rabbit muscle CK. At 10-fold molar excess, conjugation was complete within 15 min at either 27°C or 37°C. At 0°C, the conjugation reaction requires 60 min to complete. MAL-PEG efficiently conjugated to CK within the pH range of 6.2–8.0. Furthermore, MAL-PEG can be used in the presence of Gu-HCl to conjugate buried sulfhydryl groups.

When used in combination with western analysis, as little as 0.23 pmol of reversibly oxidized p53 was detected. The sensitivity of the detection method will likely vary from protein to protein, depending in large part on the affinity of the antibody for the protein and the efficiency of the enzymatic detection reaction coupled to the secondary antibody. MAL-PEG, however, is incapable of detecting irreversibly oxidized thiol groups and, therefore, other methods must be developed to assess these products. MAL-PEG (with a molecular size of 5,000 Da) is an ideal reagent for conventional SDS-PAGE band shift analysis because, upon adduct formation, it shifts the apparent molecular mass of the polypeptide by 22 (±3) kDa on the gel. The large molecular mass shift is due to the fact that polyethylene glycol is extensively hydrated (5, 13). The size of the shift in our study is consistent with size exclusion chromatography experiments indicating that each MAL-PEG increases the effective molecular mass of the protein by ~25 kDa (5, 13). On the other hand, the smaller MAL-PEG, MAL-PEG-2000, only shifted the apparent molecular mass by ~5 kDa. This small band shift limits the use of MAL-PEG-2000 to the detection of reversible oxidation on proteins that are <60 kDa. A method to use protein band shifts to detect reversible protein oxidation reactions was also developed by Jakob *et al.* (11), who used 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate (AMS) to conjugate to protein thiol groups. AMS has a molecular mass of only 536 Da and, therefore, is likely to be most useful in the analysis of very small molecular mass proteins.

MAL-PEG conjugation has already been shown to be capable of detecting reversible oxidations of endogenous protein in cultured cells (30). MAL-PEG conjugation would be predicted to be useful in mapping sites of protein oxidation in cultured cells. It is now standard practice to map sites of oxidation by substituting amino acids at predicted oxidized cysteine residues and measure the biological effect after treatment with the oxidizing agent. However, this method does not directly measure oxidation. If the redox-sensitive cysteine residue is substituted, then, after treatment with the oxidizing agent, the protein should fail to show a band shift after MAL-PEG treatment.

The fact that CK and p53 are readily susceptible to oxidation in the absence of added oxidizing agent indicates that certain precautions must be taken when assessing the efficiency of oxidation detection methods. Our results indicate that MAL-PEG conjugation is prevented if thiols are irreversibly oxidized. In a previous study, only 70% conjugation efficiency was observed when large polyethylene glycol-maleimides were reacted with IgG cysteine residue sulfhydryl groups (22). This likely in-

dicates that a portion of the sulfhydryl groups on IgG was oxidized prior to conjugation. There are other caveats to consider regarding the use of MAL-PEG conjugation to detect reversibly oxidized proteins: (a) the method does not detect the chemical composition of the oxidizing adduct; (b) care must be taken to choose an antibody/epitope combination that is distal to the MAL-PEG adduct site to maximize the potential for efficient western analysis; (c) Two sulfhydryl groups in close spatial proximity may not both efficiently bind MAL-PEG because a single MAL-PEG adduct may occlude MAL-PEG access to a neighboring thiol.

Regarding this last point, MAL-PEG conjugated all four sulfhydryl groups on CK when the reaction was performed in the presence of denaturant. However, the cysteine residues on CK are spaced far apart with the nearest two cysteine residues 29 residues apart in the linear sequence. Further tests must be performed to determine the minimum spacing of sulfhydryl groups that maintains efficient MAL-PEG conjugation. This method opens the possibility of detecting endogenous reversible oxidation reactions on low-abundance proteins in tissues and biological fluids.

Perspective

Proteins can undergo reversible oxidation reactions at cysteine residues that alter their function. An early example of this scenario was shown to occur on heart muscle creatine kinase where it was demonstrated that the kinase was partially inhibited by diamide-mediated oxidation in cultured bovine and rodent heart cells (1). Recently, it was demonstrated that EGF treatment of cultured fibroblasts leads to reversible oxidation of the active-site cysteine residue in the enzyme phosphatase 1B (15). Oxidation of the phosphatase inhibits its activity, which, in turn, leads to hyperphosphorylation of the EGF receptor, strengthening signaling through this pathway. Oxidation can activate proteins as well. Two stress response proteins in *E. coli*, Hsp33 and OxyR, require oxidation to increase their activity (11, 23, 32). Although only a few examples of reversibly oxidized proteins are discussed here, the list of proteins that become reversibly oxidized at cysteine residues in cells is increasing (29). After oxidative stress, oxidized protein cysteine residues likely return back to the reduced state. Enzymatic reduction is catalyzed by glutaredoxin (10), thioredoxin (24), and redox factor 1 (4). These reducing proteins are predicted to become transiently oxidized as they reduce their substrates. However, the substrate specificity of these proteins is not clear. The method described in this communication should aid in determining the conditions required for specific proteins to undergo transient oxidation in cells and tissues.

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ABBREVIATIONS

AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonate; CK, rabbit skeletal muscle creatine kinase (EC 2.7.3.2); DTNB, 5,5-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EGF, epidermal growth factor; Gu-HCl, guanidine hydrochloride; HED, hydroxyethyldisulfide; H_2O_2, hydrogen peroxide; IAM, iodoacetamide; IEF, isoelectric focusing; MAL-PEG, methoxypolyethylene glycol-maleimide (5,000 Da); MEOH, β-mercaptoethanol; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SEEN, sodium phosphate/EDTA/EGTA/Nonidet P-40 buffer.

REFERENCES

1. Collison MW and Thomas JA. *S*-Thiolation of cytoplasmic cardiac creatine kinase in heart cells treated with diamide. *Biochim Biophys Acta* 928: 121–129, 1987.
2. Delphin C, Cahen P, Lawrence JJ, and Baudier J. Characterization of baculovirus recombinant wild-type p53. Dimerization of p53 is required for high-affinity

DNA binding and cysteine oxidation inhibits p53 DNA binding. *Eur J Biochem* 223: 683–692, 1994.
3. Finkel T and Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247, 2000.
4. Fritz G. Human APE/Ref-1 protein. *Int J Biochem Cell Biol* 32: 925–929, 2000.
5. Goodson RJ and Katre NV. Site-directed pegylation of recombinant interleukin-2 at its glycosylation site. *Biotechnology (NY)* 8: 343–346, 1990.
6. Habeeb AF. Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol* 25: 457–464, 1971.
7. Hainaut P and Milner J. Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer Res* 53: 4469–4473, 1993.
8. Harman D. Aging: a theory based on free radical and radiation chemistry. *J Gerontol* 2: 298–300, 1957.
9. Hermanson GT. *Bioconjugate Techniques*, San Diego: Academic Press, 1996, p. 148.
10. Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxid Redox Signal* 2: 811–820, 2000.
11. Jakob U, Muse W, Eser M, and Bardwell JC. Chaperone activity with a redox switch. *Cell* 96: 341–352, 1999.
12. Kim JR, Yoon HW, Kwon KS, Lee SR, and Rhee SG. Identification of proteins containing cysteine residues that are sensitive to oxidation by hydrogen peroxide at neutral pH. *Anal Biochem* 283: 214–221, 2000.
13. Knauf MJ, Bell DP, Hirtzer P, Luo ZP, Young JD, and Katre NV. Relationship of effective molecular size to systemic clearance in rats of recombinant interleukin-2 chemically modified with water-soluble polymers. *J Biol Chem* 263: 15064–15070, 1988.
14. Laemmli UK. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* 277: 680–685, 1970.
15. Lee SR, Kwon KS, Kim SR, and Rhee SG. Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273: 15366–15372, 1998.
16. Marshall HE, Merchant K, and Stamler JS. Nitrosation and oxidation in the regulation of gene expression. *FASEB J* 14: 1889–1900, 2000.
17. Miller RM, Sies H, Park EM, and Thomas JA. Phosphorylase and creatine kinase modification by thiol-disulfide exchange and by xanthine oxidase-initiated *S*-thiolation. *Arch Biochem Biophys* 276: 355–363, 1990.
18. Rao JK, Bujacz G, and Wlodawer A. Crystal structure of rabbit muscle creatine kinase. *FEBS Lett* 439: 133–137, 1998.
19. Rietsch A and Beckwith J. The genetics of disulfide bond metabolism. *Annu Rev Genet* 32: 163–184, 1998.
20. Sen CK. Redox signaling and the emerging therapeutic potential of thiol antioxidants. *Biochem Pharmacol* 55: 1747–1758, 1998.
21. Shafer DE, Inman JK, and Lees A. Reaction of tris(2-carboxyethyl)phosphine (TCEP) with maleimide and alpha-haloacyl groups: anomalous elution of TCEP by gel filtration. *Anal Biochem* 282: 161–164, 2000.
22. Shahinian S and Silvius JR. A novel strategy affords high-yield coupling of antibody Fab′ fragments to liposomes. *Biochim Biophys Acta* 1239: 157–167, 1995.
23. Storz G, Tartaglia LA, and Ames BN. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* 248: 189–194, 1990.
24. Tanaka T, Nakamura H, Nishiyama A, Hosoi F, Masutani H, Wada H, and Yodoi J. Redox regulation by thioredoxin superfamily: protection against oxidative stress and aging. *Free Radic Res* 33: 851–855, 2001.
25. Thomas JA, Poland B and Honzatko R. Protein sulfhydryls and their role in the antioxidant function of protein *S*-thiolation. *Arch Biochem Biophys* 319: 1–9, 1995.
26. Trush MA and Kensler TW. Role of free radicals in carcinogen activation. In: *Oxidative Stress: Oxidants and Antioxidants*, edited by Sies H. London, U.K.: Academic Press, 1991, pp. 277–318.
27. Wright SK and Viola RE. Evaluation of methods for the quantitation of cysteines in proteins. *Anal Biochem* 265: 8–14, 1998.
28. Wu HH and Momand J. Pyrrolidine dithiocarbamate prevents p53 activation and promotes p53 cysteine residue oxidation. *J Biol Chem* 273: 18898–18905, 1998.
29. Wu HH, Sherman M, Yuan YC, and Momand J. Direct redox modulation of p53 protein: potential sources of redox control and potential outcomes. *Gene Ther Mol Biol* 4: 119–132, 1999.
30. Wu HH, Thomas JA, and Momand J. p53 protein oxidation in cultured cells in response to pyrrolidine dithiocarbamate: a novel method for relating the amount of p53 oxidation *in vivo* to the regulation of p53-responsive genes. *Biochem J* 351: 87–93, 2000.
31. Wu Y, Kwon KS, and Rhee SG. Probing cellular protein targets of H_2O_2 with fluorescein-conjugated iodoacetamide and antibodies to fluorescein. *FEBS Lett* 440: 111–115, 1998.
32. Zheng M, Aslund F, and Storz G. Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279: 1718–1721, 1998.

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2. Sarah L. Cuddihy , Christine C. Winterbourn , Mark B. Hampton . 2011. Assessment of Redox Changes to Hydrogen Peroxide-Sensitive Proteins During EGF Signaling. *Antioxidants & Redox Signaling* **15**:1, 167-174. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links] [Supplemental material]
3. Joseph Shlomai . 2010. Redox Control of Protein–DNA Interactions: From Molecular Mechanisms to Significance in Signal Transduction, Gene Expression, and DNA Replication. *Antioxidants & Redox Signaling* **13**:9, 1429-1476. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
4. Ning Xia, Lin Liu, Xinyao Yi, Jianxiu Wang. 2009. Studies of interaction of tumor suppressor p53 with apo-MT using surface plasmon resonance. *Analytical and Bioanalytical Chemistry* **395**:8, 2569-2575. [CrossRef]
5. Hadas Peled-Zehavi , Shira Avital , Avihai Danon Methods of Redox Signaling by Plant Thioredoxins 251-256. [Abstract] [Summary] [Full Text PDF] [Full Text PDF with Links]
6. Sarah L. Cuddihy, Kristin K. Brown, Susan J. Thomson, Mark B. Hampton. 2008. Induction of apoptosis by phenethyl isothiocyanate in cells overexpressing Bcl-XL. *Cancer Letters* **271**:2, 215-221. [CrossRef]
7. Philip Eaton. 2006. Protein thiol oxidation in health and disease: Techniques for measuring disulfides and related modifications in complex protein mixtures. *Free Radical Biology and Medicine* **40**:11, 1889-1899. [CrossRef]
8. 2003. Trend of Most Cited Papers (2001-2002) in ARS. *Antioxidants & Redox Signaling* **5**:6, 813-815. [Citation] [Full Text PDF] [Full Text PDF with Links]
9. Xiu Zhu Sun , Christopher Vinci , Linna Makmura , Shubo Han , Dung Tran , John Nguyen , Michael Hamann , Sandra Grazziani , Shelether Sheppard , Margarita Gutova , Feimeng Zhou , James Thomas , Jamil Momand . 2003. Formation of Disulfide Bond in p53 Correlates with Inhibition of DNA Binding and Tetramerization. *Antioxidants & Redox Signaling* **5**:5, 655-665. [Abstract] [Full Text PDF] [Full Text PDF with Links]
10. Dipak K. Das Methods in Redox Signaling . [Citation] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]