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Immunological Detection of *N*-formylkynurenine in Oxidized Proteins

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Abstract

Reactions of tryptophan residues in proteins with radical and other oxidative species frequently lead to cleavage of the indole ring, modifying tryptophan residues into *N*-formylkynurenine (NFK) and kynurenine. Tryptophan modification has been detected in physiologically important proteins and has been associated with a number of human disease conditions. Modified residues have been identified through various combinations of proteomic analyses, tryptic digestion, HPLC and mass spectrometry. Here we present a novel, immunological approach using polyclonal antiserum for detection of NFK. The specificity of our antiserum is confirmed using photooxidation and radical-mediated oxidation of proteins with and without tryptophan residues. The sensitivity of our antiserum is validated through detection of NFK in photooxidized myoglobin (two tryptophan residues) and in carbonate-radical oxidized human SOD1, which contains a single tryptophan residue. Analysis of photooxidized milk also shows our antiserum can detect NFK residues in a mixture of proteins. Results from mass spectrometric analysis of photooxidized myoglobin samples corroborate the immunological data, detecting an increase in NFK content as the extent of photooxidation increases.

Keywords

N-formylkynurenine; Oxidized proteins; Tryptophan; Immunological detection; Antibodies; Superoxide dismutase; Myoglobin; Mass spectrometry

Introduction

Various endogenous and exogenous factors contribute to oxidation of biological macromolecules, with potentially profound effects on the health and longevity of the cells and organisms in which they occur. DNA, lipids, and proteins contain vulnerable sites where

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oxidative and/or radical species can cause structural modification. Proteins are susceptible to both fragmentation of the polypeptide backbone and aggregation by cross-linking between amino acid residues [1-3]. Oxidants and radicals also react with amino acid residues, altering their molecular structure. The sulfur containing residues and the aromatic residues histidine, phenylalanine, tyrosine and tryptophan are the most susceptible to oxidation, and studies have described the interactions they undergo and the resulting molecular consequences [1-3].

N-formylkynurenine (NFK) and kynurenine are formed from the oxidation of tryptophan and tryptophan residues through a number of reactions (Fig.1). ROS interact with tryptophan yielding radicals and other intermediates which rearrange into NFK and kynurenine. Tryptophan radical reacts with superoxide or molecular oxygen to form tryptophan hydroperoxide that ultimately rearranges into NFK and kynurenine. Alternatively, singlet oxygen ($^1\text{O}_2$) or ozone can react directly with tryptophan to ultimately form NFK and kynurenine. Functional effects of tryptophan residue oxidation depend on protein type and residue localization. Ozonation of egg white lysozyme converts two tryptophan residues to NFK, but only one of these residues is required for native protein conformation and enzyme activity, while alteration of the other has no effect [4].

There is growing recognition of the role played by altered and aggregating proteins in neurodegenerative and other human disease [5,6], with tryptophan residue oxidation being correlated with the development of conditions such as neonatal respiratory distress syndrome [7], atherosclerosis [8-10] and amyotrophic lateral sclerosis (ALS) [11-13]. Familial ALS is linked to mutations in the gene for SOD1 leading to the formation of SOD1 protein aggregates [14], and non-familial ALS, which accounts for most occurrences of ALS, is believed to have a similar etiology [15]. In vitro experiments have shown that hSOD1 radical-mediated peroxidase activity can engender protein multimers and that the sole tryptophan residue is the site of modification and is essential for aggregation [11-13].

Proteomic studies have also uncovered tryptophan oxidation products in proteins involved in redox metabolism in normal human heart and plant mitochondria [16,17] and in eye lens crystalline proteins from aged and cataract lenses [18,19]. A recent report describes tryptophan oxidation playing a role in endogenous regulation of protein function [20]. The MopE protein of *Methylococcus capsulatus* binds copper only if a crucial tryptophan residue is oxidized to kynurenine.

Current technology for identification of oxidized tryptophan residues includes electron spin resonance (ESR) and ESR spin trapping for real-time detection of tryptophan radical [21,22] and chromatographic and mass spectrometric techniques to identify the stable end-products. Fluorescence spectroscopy can also be used because tryptophan, NFK and kynurenine each have distinctive spectral characteristics. Fluorescence spectra, however, are often complicated by the influence of neighboring amino acid residues and protein structure and can be relatively non-specific and insensitive due to high fluorescence background. A simpler, more sensitive immunological approach for identification of proteins with oxidized tryptophans for use with complex biological systems would be desirable.

In this paper we describe the development of antiserum to the tryptophan oxidation product NFK using a synthetic hapten 4-(2-formamidobenzoyl)-butyric acid (Fig. 2). We validate its utility in western and ELISA analyses using both single proteins and protein mixtures and by evaluating proteins oxidized via both photosensitization and radical chemistry. We also demonstrate that our anti-NFK antiserum is sensitive enough to detect the oxidation of the single tryptophan residue of hSOD1 and for detecting NFK in a mixture of proteins. Furthermore, mass spectrometric analyses demonstrate that the amounts of NFK detected by our antiserum correlate closely with those detected using LC/MS of photooxidized protein.

Material and Methods

Antibody production

4-(2-formamidobenzoyl)-butyric acid (Fig. 2) (synthesized by Cerilliant Corporation, Round Rock, TX) was conjugated to ovalbumin using carbodiimide chemistry as described [23]. After dialysis, 500 µg of the conjugated protein in Freund's complete adjuvant was used to immunize New Zealand white rabbits (Harlan Bioproducts, Madison WI) and serum was collected by exsanguination at day 21.

Chemicals and materials

N-bromosuccinimide (NBS), rose bengal, riboflavin, human and bovine SOD (from erythrocytes), diethylenetriaminepentaacetic acid (DTPA), kynurenine, and ovalbumin were from Sigma (St. Louis, MO). Reagent grade H₂O₂ was from Fisher Scientific Co. (Fair Lawn, NJ) and the concentration determined at 240 nm ($\epsilon_{240} = 43.6 \text{ M}^{-1}\text{cm}^{-1}$). Horse heart myoglobin was from USB Corp. (Cleveland, OH), alkaline-phosphatase conjugated anti-rabbit IgG was from Pierce (Rockford, IL), anti-horse heart myoglobin antibody was from Bethyl Laboratories (Montgomery, TX) and sodium bicarbonate was from Alfa Aesar (Ward Hill, MA). Non-fat milk (Pet, St. Louis, MO) was purchased from the NIEHS cafeteria. Phosphate buffer was treated with Chelex 100 resin (Bio-Rad, Hercules, CA) to remove contaminating transition metals.

NFK synthesis

NFK was synthesized by modifying the procedure described by Dalglish [24]. Acetic anhydride (1.5 mL) was mixed with formic acid (3 mL) on ice and then allowed to warm to room temperature. This mixture was then added to 2 g of kynurenine in 4.5 mL formic acid. After remaining overnight at room temperature, an aliquot analyzed using mass spectrometry indicated the formylation reaction was complete. A large excess of ether was then added, and the mixture was allowed to stand on ice for 1 h. After carefully decanting the ether, absolute ethanol was added, and the NFK was kept on for ice an additional hour. The NFK was then recovered by removal of the ethanol by pipetting and drying with a gentle stream of argon. The concentration of NFK in solution was measured using its absorption ($\epsilon_{320} = 3,100 \text{ M}^{-1}\text{cm}^{-1}$) [25].

Photooxidation of myoglobin

Oxidation of myoglobin by ¹O₂ was performed similarly to the ¹O₂-induced oxidation of the B6 vitamers, as reported [26]. Twenty µM aliquots of horse heart myoglobin with 10 µM rose bengal were placed in a 1 cm path length cuvette and were stirred continuously during irradiation. Irradiations were carried out at room temperature using a 450 Watt xenon-mercury lamp with a heat absorbing filter and a cutoff filter combination of 389 and 450 nm. Fluorescence spectra were measured using a FluoroLog 3 spectrofluorometer (Horiba Jobin Yvon, Edison, NY).

NBS modification of myoglobin and hSOD1

Solutions of horse heart myoglobin (100 µM) were incubated in the dark with either no *N*-bromosuccinimide (NBS) or with a two-, five- or tenfold molar excess of NBS for 10 minutes in a 50 mM sodium acetate buffer, pH 4.5. The reactions were stopped by addition of 10 mM free tryptophan, and the treated proteins were dialyzed 4 times against PBS, pH 7.4. These myoglobin samples were then oxidized for 30 min in the presence of rose bengal as described above. Human SOD1 modified with a twofold excess of NBS was prepared as described above for myoglobin.

Reactions of SOD with H₂O₂ and bicarbonate

Human, NBS-modified human and bovine Cu, Zn SOD-1 (1 µg/µl) were incubated for 1 hour at 37° C in a (chelexed) 100 mM sodium phosphate buffer, pH 7.4, in the presence 25 mM NaHCO₃ and increasing concentrations of H₂O₂ (0-10 mM). Control aliquots were incubated without either NaHCO₃ or H₂O₂, and a second control contained 10 mM H₂O₂, but no bicarbonate. All reaction mixtures contained 100 µM DTPA.

Photooxidation of milk

Aliquots of non-fat milk were either supplemented with 20 µg/ml riboflavin or were used as is. Five mL samples were illuminated using a table lamp (light intensity approximately 35 µEinsteins) for 1.5, 4 or 6 h at room temp or were stored in the dark for 6 h at room temp. Milk maintained at 4° C was used as an additional control. The acid-soluble proteins were recovered by decreasing the sample pH to 4.6, followed by centrifugation in a microfuge for 10 min at 4° C and recovery of the supernatant.

ELISA and western analyses

ELISA and western analyses were performed essentially as previously described [27], except that for the myoglobin ELISA, 2.5 µg of protein were adsorbed to each well in 0.1 M NaHCO₃, pH 9.6. ELISAs and westerns were done using a 1:1000 dilution of anti-NFK antiserum and a 1:5000 dilution of anti-rabbit IgG-alkaline phosphatase (Pierce), and the western blots were incubated for 2 hours at room temperature with anti-NFK antiserum. Western analysis of myoglobin samples contained 2.5 µg protein per lane while western analysis of all SOD and milk samples contained 10 µg per lane. Competition ELISA was performed using myoglobin samples (2.0 µg/well) irradiated for 30 min with rose bengal as described above. The anti-NFK serum (1:1000) was mixed with appropriate dilutions of the test compounds and then used in a standard ELISA assay.

Mass Spectrometry

Twenty µL aliquots of the myoglobin photoreaction mixtures (above) were desalted using C4-Zip Tips (Millipore Corp, Billerica, MA), lyophilized and resuspended in 75 µL of 25 mM ammonium bicarbonate (pH 7.4). Trypsin was added in an enzyme/substrate ratio of 1:30 and the digestion performed overnight at 37° C. Prior to LC-MS analyses, 20 µL aliquots of each digestion were diluted with an equal volume of 0.1% formic acid. LC/MS analyses were performed using a Waters Q-TOF Premier mass spectrometer equipped with a nanoAcquity UPLC system (Waters, Milford, MA). Separations were performed on a 100 µm × 100 mm, Atlantis 3 µm dC18 column (Waters, nanoAcquity), using a flow rate of 300 nL/min. A C18 trapping column (180 µm × 20 mm) with a 5 µm particle size (Waters, nanoAcquity) was positioned in-line of the analytical column. A 2 µL aliquot of the digest sample was injected, trapped onto the column, and peptides were eluted using a linear gradient of 98% solvent A (0.1% formic acid in water (v/v)) and 2% solvent B (0.1% formic acid in acetonitrile (v/v)) to 40% solvent B over 90 minutes. Instrument settings for the MS analyses were: capillary voltage, 3.5 kV; cone voltage, 30 V; collision energy, 8.0 V; and source temperature, 80° C. The mass spectra were acquired over the mass range 200 – 2000 Da. For determination of the relative abundance of the oxidized peptides, the mass spectrometer was operated in the MS-only mode. MS/MS data were acquired separately for each target ion. A collision energy ramp from 30 V to 40 V was employed to obtain fragmentation of the precursor ions. An external lock mass using a separate reference sprayer (LockSpray) and a solution of Glu-Fibrinogen peptide (300 fmol/µL) in water/acetonitrile 80:20 (v/v) and 0.1% formic acid, with a mass of 785.8496 (2+), was used for calibration. Data analyses were performed using MassLynx 4.0 software (Waters, Milford, MA).

Results

Titering of polyclonal antiserum

To test antiserum titer we used both ELISA and western analyses of horse heart myoglobin irradiated in the presence of the $^1\text{O}_2$ generating dye rose bengal. We used two negative controls for these experiments: 1) Unirradiated myoglobin lacking rose bengal (C) and 2) unirradiated myoglobin containing rose bengal ("0") which was exposed to ambient light only. Fluorescence spectra indicated that decreased tryptophan fluorescence and parallel increased NFK and kynurenine fluorescence correlated with irradiation time (data not shown). ELISA analysis (Fig. 3, bottom panel) showed the anti-NFK polyclonal serum had little cross-reactivity to unirradiated myoglobin but that increasing $^1\text{O}_2$ exposure increased cross-reactivity. Two minutes of $^1\text{O}_2$ exposure produced ELISA results statistically different from the no-rose-bengal control (C) at the 1% level of confidence. Western analysis (middle panel) corroborated the ELISA data. The control sample (C) shows little, if any, cross-reactivity, while NFK is detected in the 2-minute sample, and there is clearly increasing immunoreactivity with increasing $^1\text{O}_2$ exposure. The western blot also shows that protein aggregation increases with increased $^1\text{O}_2$. Coomassie blue staining (Fig. 3, top panel) confirms comparable loading in all lanes and also demonstrates the structural modification generated by $^1\text{O}_2$ oxidation.

Mass spectrometric analysis of photooxidized myoglobin

Mass spectrometry was used to independently assess the accumulation of tryptophan oxidation products in some of the photooxidized myoglobin samples shown in Fig. 3. Horse heart myoglobin contains two tryptophan residues, Trp 7 and Trp 14, both of which are contained in the N-terminal tryptic peptide. Oxidative modification of the two tryptophan residues in this tryptic peptide (1-16) results in the accumulation of five distinct products, containing either no modified residues or with tryptophan being oxidized to either one or two NFKs or kynurenines, or a mixture of NFK and kynurenine. The identity of each of these products was confirmed by tandem MS, and the abundance of the oxidized species was determined from LC-MS analyses (Fig. 4). These analyses indicated that the abundance of NFK-containing peptides was proportional to $^1\text{O}_2$ exposure, corroborating both the ELISA and western analyses of the same samples (Fig. 3).

NBS modification of myoglobin tryptophan residues

It is clear from Fig. 3 that $^1\text{O}_2$ exposure results in extensive structural rearrangements of the native myoglobin monomer. Amino acid residues other than tryptophan interact with $^1\text{O}_2$ and, to determine if our antiserum provides a specific tool for recognition of oxidized tryptophan rather than a general means of identifying of $^1\text{O}_2$ -exposed proteins, we treated myoglobin with NBS to chemically modify the tryptophan residues to oxindoles. Myoglobin reacted with increasing molar concentrations of NBS, dialyzed, and irradiated in the presence of rose bengal was subjected to western analysis using anti-horse heart myoglobin (top panel) and anti-NFK antisera (bottom panel) (Fig. 5). The anti-NFK western clearly shows that alteration of myoglobin tryptophan residues into a form resistant to indole ring modification to NFK results in decreased cross-reactivity. Myoglobin reacted with 2-fold molar NBS shows reduced cross-reactivity while the sample exposed to 5-fold excess shows only background signal. A parallel western using anti-myoglobin IgG confirms $^1\text{O}_2$ exposure of all samples and that the loss of anti-NFK immunoreactivity in the NBS-treated samples is not due to a deficiency of myoglobin in those samples.

Bicarbonate and hydrogen peroxide-mediated oxidation of the tryptophan residue of human SOD1

Zhang et al. showed that hSOD1 aggregation in the presence of bicarbonate and H₂O₂ requires tryptophan oxidation [12,13]. Parallel experiments with recombinant human SOD1 containing phenylalanine substituted for the sole tryptophan residue, with highly homologous but tryptophan free bovine SOD1, and the wild type enzyme showed dimerization occurred only in tryptophan-containing hSOD1. Spin-trapping analysis identified a radical trapped by the tryptophan residue of hSOD1. Mass spectrometry [13] identified NFK, kynurenine and kynurenine-type products in hSOD1 following bicarbonate/H₂O₂ treatment.

We therefore chose hSOD1 to further test the sensitivity and specificity of our antiserum, performing reactions comparable to those described in Zhang et al. [12]. The Coomassie stained gel (Fig. 6A) parallels the data published by Zhang et al [12], with dimerization of hSOD1 visible at 0.1 mM H₂O₂ and fragmentation occurring at higher H₂O₂ concentrations. Western analysis of these reactions detected NFK only in the samples in which peroxidase activity would be expected, i.e., those containing both bicarbonate and H₂O₂. NFK was detected in hSOD1 monomers reacted with bicarbonate and 0.5-10 mM H₂O₂ and in hSOD1 dimers resulting from incubation with bicarbonate plus 0.1 to 10 mM H₂O₂. In comparable reactions with bSOD1 and hSOD1 treated with NBS to modify the tryptophan residue, no dimers were detected in the Coomassie stained gels (Fig. 6B), confirming the role of tryptophan-32 in hSOD1 dimerization. Parallel western analyses of these samples also failed to detect NFK (data not shown).

Other antisera

We also tested two commercially available anti-kynurenine polyclonal antisera described as being specific for conjugated kynurenine. Neither was able to distinguish irradiated from unirradiated myoglobin in western blots using 1:1000 or 1:500 dilutions (data not shown). These experiments corroborated our own unpublished work in which we attempted to develop anti-NFK/anti-kynurenine antiserum by creating immunogens with kynurenine and NFK. None of the antisera developed using these haptens could recognize proteins containing oxidized tryptophan residues (data not shown).

Competition ELISA

To determine the relative affinity of our anti-NFK antiserum to the hapten to which it was raised (Fig. 2), to NFK, and to related chemicals, we performed competition ELISA using irradiated myoglobin adsorbed to the ELISA plate and tenfold dilution series of the compounds of interest. Three hundred μ M NFK inhibited antiserum binding by 50% while the hapten used to create the immunogen inhibited binding by 50% at a concentration of 3 μ M. Kynurenine affected binding to a small degree, inhibiting antiserum binding by 40% at 10 mM with concentrations lower than 1 mM having no effect. Ten mM tryptophan had no effect on anti-NFK binding.

Detection of NFK in photooxidized milk

To test the efficacy of if our anti-NFK antiserum in a mixture of proteins, we examined photooxidation of milk. Milk contains dozens of proteins with relatively high percentages of tryptophan residues, but approximately eighty percent of these are the acid-insoluble caseins (mw. ca. 25-35 kDa.) Milk also contains an endogenous photosensitizer, riboflavin (1.7 μ g/ml). Non-fat milk used as is or supplemented with additional 20 μ g/ml riboflavin was exposed to lamp light at room temperature for 1.5, 4 and 6 hours. Controls were milk from the original container maintained at 4° C and aliquots kept in the dark at room temperature for 6 hours.

Samples were also subjected to acid precipitation to separate the acid soluble fraction from the (acid-insoluble) caseins.

In the middle of the anti-NFK western (from ca 36-50 kDa) of total milk protein (Fig. 7, top panel) there are bands corresponding to the higher molecular weight caseins visible in all samples. Increasing photooxidation via either increased light exposure or increased riboflavin yielded an increase in NFK-containing protein aggregates between 64 and 148 kDa. The western blot of acid-soluble milk proteins (Fig. 7, bottom panel) lacked the 64 to 148 kDa NFK-containing proteins, suggesting they are aggregated caseins. The analysis of acid-soluble milk proteins also indicated that removal of caseins allows the detection of NFK in this group of milk proteins comprising only about 20% of the total.

Discussion

In this work we have developed and validated an immunological method for detection of proteins containing tryptophan residues oxidized to NFK. The hapten used to produce the antiserum retains the distinctive cleaved indole ring of NFK, but lacks the α -amine (Fig. 2), allowing us to use carbodiimide chemistry for ovalbumin conjugation without formation of hapten oligomers that can impede conjugation of hapten to carrier. The extra methylene group was added to facilitate the exposure of the characteristic oxidized, cleaved indole ring for production of NFK recognizing antibodies. Our previous work (unpublished) made us aware that production of antiserum to NFK and kynurenine was not achievable by the simple expedient of conjugating either NFK or kynurenine to a carrier protein.

Western blots with two commercially available antisera confirmed these observations. Although neither was promoted as a detection agent for NFK or kynurenine, it seemed prudent to test them. Not surprisingly, neither could detect NFK in photooxidized myoglobin (data not shown). A recent paper [28] describes an antibody produced by blocking the α -amine group of tryptophan, oxidizing it with $^1\text{O}_2$ and then conjugating the resulting product to a carrier protein. This antiserum recognized photooxidized protein, but was used at a dilution of 1:50, a concentration likely to prohibit its wide-spread application due to non-specific binding in complex protein mixtures. Also, no experiments were presented to discriminate between recognition of oxidized tryptophan and general recognition of photooxidized protein.

In contrast, we have shown that our anti-NFK antiserum is both highly specific to tryptophan residues with oxidized, cleaved indole rings, and highly sensitive. Myoglobin and hSOD1 samples modified so the tryptophan residues can no longer form NFK concomitantly lose cross-reactivity (Figs. 5 and 6). Control samples of myoglobin, hSOD1 and tryptophan free bSOD1 all exhibit little or no anti-NFK immunoreactivity (Figs. 3 and 6). The competition ELISA determined that our antiserum does not detect tryptophan residues with intact indole rings. We also saw very little competition from kynurenine, suggesting that our antiserum specifically recognizes NFK and NFK-like antigens.

Not surprisingly, competition ELISA also shows that recognition of the actual hapten occurs at concentrations 100-fold lower than that of NFK itself. These data, however, do not adequately reflect the actual affinity of our antiserum to proteins containing NFK. The myoglobin used in our experiments contains two tryptophan residues out of a total of 154 amino acid residues, representing just 1.3% of the total protein. hSOD1, a protein of comparable mw to myoglobin, contains but a single tryptophan, yet NFK detection in both proteins occurs at levels unquestionably above background cross-reactivity.

If the two tryptophan residues of the myoglobin were completely oxidized to NFK, the 2.5 μg protein sample used in both the western blot and ELISA of Fig. 3 would result in the production of a total of 285 pmoles of NFK. The samples oxidized for only 2 minutes contain

only a fraction of the NFK in the 60 min sample (ca. 20% by ELISA analysis), yet our antiserum definitively detected NFK above background levels. This suggests that we can detect ≤ 57 pmoles of NFK within 2.5 μg of total protein.

Because mass spectrometry is a widely accepted technique for NFK identification and quantification, we also used it as an independent measure of NFK content in photooxidized myoglobin (Fig. 4). A subset of the myoglobin samples used for the ELISA and western analyses in Fig.3 were desalted, lyophilized, resuspended, digested with trypsin, and then subjected to LC/MS. The data resulting from this analysis closely corroborate our ELISA and western data, showing increased accumulation of NFK with increased photooxidation. Our immunological assay for NFK, however, is not only as sensitive as mass spectrometry, but is more amenable to analysis of a greater number of samples in a shorter time with fewer steps.

Zhang et al. [13] used mass spectrometry to detect the conversion of the single tryptophan residue of hSOD1 to NFK and kynurenine. Our work confirms that of Zhang et al. [12,13], but was achieved with the much simpler techniques of immunochemistry. The higher throughput technology of immunochemistry should allow a more detailed dissection of the conditions that produce oxidation of tryptophan to NFK in a much shorter time. It is also feasible that the anti-NFK antiserum can be used for immunoprecipitation of proteins containing NFK residues, facilitating mass spectrometric and other analyses.

Additionally, immunological detection of NFK uses a relatively low level of processing and technology, which in turn reduces the possibilities for producing artifacts. The validity and utility of a number of oxidative stress assays have come into question due to the potential for creating artifacts during sample processing and analyses [29-31]. Proteins can be recovered from cells and organisms with a minimum of manipulation, and the reducing conditions employed for SDS-PAGE do not facilitate tryptophan oxidation or alteration of the end product. This is clear from the myoglobin photooxidation experiment (Fig. 3) in which the ELISA and western results are unambiguously equivalent.

The milk oxidation experiments (Fig.7) validate the use of this antiserum for detection of tryptophan oxidation products in protein mixtures. In conjunction with the simple purification step of acid precipitation, we derived a tentative identification for the oxidized protein(s). These westerns also illustrate a caveat important in all immunochemistry. The milk protein westerns cannot differentiate between endogenous pre-existing tryptophan oxidation in the samples and non-specific background. ELISA analysis of these samples without western data would likely lead to the conclusion that the background cross-reactivity in milk is too high to allow detection of specific protein bands containing NFK. In cases where the background cross-reactivity is low enough, however, ELISA allows quantitative corroboration of western data.

The experiments presented here provide compelling evidence that we have developed a practical and efficient approach for detection of proteins with tryptophan residues oxidized to NFK which can be used with both single proteins as well as protein mixtures. Use of this antiserum could prove to be a valuable and versatile tool applicable not only to *in vitro* analysis of oxidized proteins but potentially to analysis of cells, organs and organisms and should provide a straightforward, facile and expedient method for initial assays of oxidative protein modifications and, possibly, organismal oxidative stress. Western analysis could be used to identify single protein bands exhibiting cross-reactivity, which would greatly facilitate any further analysis using MS techniques, in some cases circumventing the need for large-scale proteomic mass spectrometric experiments using methodology not readily accessible to all. The low amount of background found in these experiments also suggests that our anti-NFK antiserum may be very useful in immunohistological study of cells undergoing oxidative and/or radical stress.

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Abbreviations

NFK	N-formylkynurenine
NBS	N-bromosuccinimide
SOD	superoxide dismutase

Fig. 1. Oxidation of tryptophan by ROS to *N*-formylkynurenine (NFK) and kynurenine
Modified from Gieβauf et al. [9].

Fig. 2. Structure of hapten 4-(2-formamidobenzoyl)-butyric acid

Fig. 3. Photosensitized myoglobin cross-reacts with anti-NFK antiserum

Horse heart myoglobin (20 μ M) without rose bengal (C), or with 10 μ M rose bengal was irradiated for increasing durations as described in material and methods, or was exposed to only ambient light ("0"). Duplicate gels containing 2.5 μ g protein were stained with Coomassie blue to visualize protein loading (**top panel**) or were transferred to nitrocellulose for western analysis using a 1:1000 dilution of anti-NFK antiserum and chemiluminescent detection (**middle panel**). ELISA analysis was also performed using 2.5 μ g protein per well and a 1:1000 dilution of anti-NFK antiserum (**bottom panel**). ELISA data are the means $\pm$ SD from three independent determinations. Values significantly different ($p < 0.05$ and $p < 0.01$) from the samples without rose bengal (C) are indicated by one or two asterisks, respectively.

Fig. 4. LC-ESI-MS analysis of photooxidized myoglobin

The N-terminal tryptic peptide 1-16 containing both myoglobin tryptophan residues (Trp 7 and Trp 14) was obtained from a subset of the samples in Fig. 3 and analyzed for tryptophan residue oxidation. The bar for each peptide was generated by dividing the summed ion abundances of the 2+ and 3+ charged peptide ions by the sum of the ion abundances of all observed oxidized peptides at a specific irradiation time point.

Fig. 5. Modification of tryptophan residues eliminates cross-reactivity with anti-NFK antiserum Horse heart myoglobin, either untreated or incubated with 2, 5, or 10-fold molecular equivalents of *N*-bromosuccinide, was extensively dialyzed as described in the material and methods. 20 μ M aliquots of each treatment were then irradiated for 30 min in the presence of 10 μ M rose bengal. Duplicate gels of irradiated and non-irradiated samples containing 2.0 μ g of protein per lane were then subjected to western analysis using anti-horse heart myoglobin (**top panel**) or a 1:1000 dilution of anti-NFK antiserum (**bottom panel**) and chemiluminescent detection.

A hSOD1 **B Coomassie Stained Gels** **Fig. 6. Bicarbonate-mediated oxidation of the single tryptophan residue in hSOD1**

(A) hSOD1 (1.0 $\mu\text{g}/\mu\text{l}$) was incubated for 1 h at 37 C° in the presence of 100 μM DTPA. Addition of H₂O₂ and/or 25 mM Na bicarbonate was as shown. Duplicate gels containing 10 μg of protein per lane were stained with Coomassie blue to visualize protein loading or were transferred to nitrocellulose for western analysis with a 1:1000 dilution of anti-NFK antiserum and chemiluminescent detection. (B) Native bSOD1 and hSOD1 treated with a twofold excess of *N*-bromosuccinide were incubated for 1 h at 37 C° in the presence of 100 μM DTPA and H₂O₂ and/or 25 mM Na bicarbonate as shown. Duplicate gels containing 10 μg of protein were stained with Coomassie blue to visualize protein loading or were transferred to nitrocellulose for western analysis (data not shown) with anti-NFK antiserum and chemiluminescent detection.

Fig. 7. Detection of NFK in photooxidized milk proteins

Aliquots of non-fat milk without (-) or with (+) additional riboflavin (20 $\mu\text{g}/\text{ml}$) were exposed to lamp light (light intensity approx. 35 $\mu\text{Einsteins}$) for the number of hours indicated. The sample labeled C was kept at 4° C in the original container until the end of the experiment, while all other samples, including the non-irradiated controls, were kept at room temperature. Gels containing 10 μg protein per lane of total milk proteins (top panel) and acid soluble milk proteins (bottom panel) were transferred to nitrocellulose for western analysis with a 1:1000 dilution of anti-NFK antiserum and chemiluminescent detection. Molecular weights are given in kDa on the left of each panel.