Tyrosine Nitration Impairs Mammalian Aldolase A Activity*

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Protein tyrosine nitration increases in vivo as a result of oxidative stress and is elevated in numerous inflammatory-associated diseases. Mammalian fructose-1,6-bisphosphate aldolases are tyrosine nitrated in lung epithelial cells and liver, as well as in retina under different inflammatory conditions. Using two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, we now show that aldolase A is nitrated in human skin fibroblasts. To reveal the consequences of tyrosine nitration, we studied the impact of peroxynitrite on the glycolytic functions of aldolase A. A peroxynitrite concentration-dependent decrease in fructose-1,6-bisphosphate cleavage activity was observed with a concomitant increase in nitrotyrosine immunoreactivity. Both $V_{max}$ and the $K_m$ for fructose-1,6-bisphosphate decreased after incubation with peroxynitrite. Aldolase nitrotyrosine immunoreactivity diminished following carboxypeptidase Y digestion, demonstrating that tyrosine residues in the carboxyl-terminal region of aldolase are major targets of nitration. Aldolase A contains a carboxyl-terminal tyrosine residue, Tyro363, that is critical for its catalytic activity. Indeed, tandem mass spectrometric analysis of trypsin-digested aldolase showed that Tyro363 is the most susceptible to nitration, with a modification of Tyr342 occurring only after nitration of Tyro363. These tyrosine nitrations likely result in altered interactions between the carboxyl-terminal region and enzyme substrate or reaction intermediates causing the decline in activity. The results suggest that tyrosine nitration of aldolase A can contribute to an impaired cellular glycolytic activity. Molecular & Cellular Proteomics 3: 548–557, 2004.

Covalent post-translational protein nitration occurs mainly at tyrosine residues in vivo as a result of oxidative stress (1). Proteomic studies support protein nitration as more than a tissue-specific footprint of free radical production in a variety of diseases and suggest that nitration may be directly involved in the onset and/or progression of diseases. Diseases associated with protein nitration include cardiovascular diseases (e.g. myocarditis, atherosclerosis, and heart failure) (2), inflammatory lung diseases (e.g. asthma) (3, 4), neurodegenerative diseases (e.g. Alzheimer) (5), inflammatory liver diseases (e.g. chronic viral hepatitis) (6), and diabetes (7). Protein nitration also occurs under other pathological conditions like ischemia-reperfusion (8).

Among the proteins that we recently identified as being nitrated in vivo are all three isoforms of mammalian fructose-1,6-bisphosphate aldolase [Fructose 1,6-bis-(phosphate):d-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13] (3, 9). Aldolases catalyze aldol cleavage/condensation reactions and can be classified into two groups that have strikingly different mechanisms (10). Class I aldolases are found in vertebrates and form a Schiff-base intermediate between the C-2 carbonyl group of the substrate and the e-amino group of a lysine residue. Class II aldolases, primarily occurring in bacteria and yeast, do not form covalent enzyme-substrate intermediates but require bivalent transition-metal ions such as zinc (11, 12). Class I aldolases of animals and higher plants are homotetrameric enzymes, comprising 363 amino acid residues, that are involved in glycolytic, gluconeogenic, and fructose metabolic pathways (13, 14). For example aldolases catalyze the reversible cleavage of fructose-1,6-bisphosphate (Fru-1,6-P$_2$) into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) as well as the cleavage of fructose-1-phosphate to DHAP and glyceraldehyde (14). Class I aldolases include three tissue-specific isoenzymes in vertebrate tissues: aldolase A predominates in skeletal muscle and red blood cells, aldolase B in liver, kidney, and small intestine, and aldolase C in neuronal tissues and smooth muscle (14). The aldolase isoforms have only 68% (human aldolase A to B (15, 16)) to 81% (human aldolase A to C (17)) sequence homology. This translates in differences in their preference for Fru-1,6-P$_2$ versus fructose-1-phosphate as substrate (18, 19), which is consistent with their different physiological roles and tissue-specific expression (13, 20). Aldolase B is believed to

1 The abbreviations used are: Fru-1,6-P$_2$, fructose-1,6-bisphosphate; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Q-TOF, quadrupole time-of-flight; G3P, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DHAP, dihydroxyacetone phosphate; $K_m$, Michaelis Menten constant; DTT, dithiothreitol; DMEM, Dulbecco’s modified Eagle medium; MS/MS, tandem mass spectrometry; LC, liquid chromatography; IPG, immobilized pH gradient.

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preferentially perform the gluconeogenic reaction, Fru-1,6-P₂ synthesis. Presumably, structural and electrostatic features have been conserved to enable each isoform to become a target for nitration. This raises questions about which tyrosine residues are nitrated and possible physiological consequences caused by this covalent modification. Alterations in tissue distribution of class I aldolases (21, 22) and/or catalytic deficiencies caused by mutations (23, 24) are involved in numerous diseases such as cancer, hereditary fructose intolerance, and glycogenosis type XII. Nevertheless, enzymatic consequences of nitration of class I aldolases have not been well characterized.

To address these issues, we used peroxynitrite to nitrate mammalian aldolase A purified from rabbit muscle, which exhibits the unusually high sequence homology of 98.6% to human aldolase A (16). Peroxynitrite, a strong oxidant that is formed in biological systems by a diffusion-limited reaction between nitric oxide and the superoxide anion radical (25), was chosen because it is one of the favored candidates for physiological protein nitration. We determined the alterations in the enzymatic characteristics and used our proteomic methodology to identify the nitrated sites of purified aldolase A. We also identified nitrated aldolase A in human skin fibroblasts. The molecular mechanisms and possible physiological consequences of aldolase A nitration are discussed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human skin fibroblasts (GM05756A; American Type Culture Collection, Manassas, VA) were grown to 80% confluence in Dulbecco’s modified Eagle medium (DMEM; 4.5 g/liter glucose) supplemented with 10% fetal bovine serum, 0.58 g/literL-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under isobaric conditions (5% CO₂, 95% air) in humidified atmosphere. Then media was removed, cells were washed three times with phosphate-buffered saline and lysed by adding lysis buffer (7 M urea, 2 M thiourea, and 1% Triton X-100). For two-dimensional electrophoreoses 1% CHAPS, 1% DTT, and 1% immobilized pH gradient (IPG)-ampholytes (Bio-Lyte 3/10) were added.

**Aldolase Nitration**—Aldolase A purified from rabbit muscle (Calbiochem, La Jolla, CA) was diluted to 1 mg/ml in 100 mM Tris/HCl, pH 7.5 prepared with Milli-Q water with and without 25 mM sodium bicarbonate. Residual carbon dioxide in the buffer due to equilibration with carbon dioxide in the atmosphere was not removed. Nitration was done by treatment with 25–500 μM peroxynitrite (Calbiochem) for 30 s at 25 °C under vortexing. Then the enzyme solutions were stored on ice until the enzyme activity was measured. Aliquots were taken for enzyme activity measurement at 302 nm (A₃₀₂nm = A₀₂₄₀nm - A₂₄₀nmblank)/mg enzyme per ml reaction mixture). Immediately before use, the enzyme was diluted in buffer (100 mM Tris/HCl, pH 7.5 to 10 μg/ml, which is checked by absorbance at 280 nm (mg/ml = A₂₈₀nm × 1.1). To calculate V₅₀ and Kₘ, the activity was measured between 0.1 and 1.6 mM d-Fru-1,6-P₂.

**Carboxypeptidase Y Digestion**—Six milliliters of carboxypeptidase Y (Pierce Biotechnology, Rockford, IL) were added to 10 μg nitrated aldolase A (200 μM peroxynitrite, reacted as described above) in 0.1 M sodium citrate buffer and incubated for 1, 2.5, or 5 min at 25 °C. Digestion was stopped by adding phenylmethylsulfonyl fluoride at a final concentration of 5 mM. Loss of nitrotyrosine immunoreactivity was evaluated by Western analysis.

**One-Dimensional Gel Electrophoresis**—SDS-PAGE was performed essentially according to Laemmli with 1–2.5 μg aldolase A per lane (28). After SDS-PAGE, the acrylamide gels were soaked 20 min in transfer buffer (25 mM Tris/HCl, 192 mM glycine, 20% methanol) and then proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) using a semidy transfer apparatus according to the manufacturer. Gels were stained with colloidal Coomassie blue (GelCode blue stain; Pierce Biotechnology).

**Two-Dimensional Gel Electrophoresis**—Two-dimensional gel electrophoresis was performed with the isoelectric focusing/Criterion gel system (Bio-Rad). The first dimension used lysis buffer (above) and 11-cm nonlinear pH 3–10 IPG strips. IPG strips were rehydrated with sample at 50 V/14 h, and then isoelectric focusing performed by a linear increase to 250 V over 20 min followed by a linear increase to 8000 V over 170 min and then held at 8000 V until a total of 45 kVh is reached. For the second dimension, the IPG strips were equilibrated for 12 min in 50 mM Tris/HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT, and bromphenol blue, and then 15 min in 50 mM Tris/HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2% iodoacetamide, and brom phenol blue. The strips then were embedded in 1% (w/v) agarose on the top of 12.5% acrylamide gels containing 4% stacking gel (Criterion gel; Bio-Rad). The second dimension SDS/PAGE was performed essentially according to Laemmli. After completion acrylamide gels were soaked 20 min in transfer buffer (25 mM Tris/HCl, 192 mM glycine, pH 8.3, and 20% methanol) and then partially electro-transferred to PVDF membrane using a semidy transfer apparatus. The gels then were stained with colloidal Coomassie blue (GelCode blue stain).

**Western Analysis**—PVDF membranes were blocked for 90 min by using blocking buffer (20 mM Tris, 150 mM NaCl, pH 7.5, 0.2% Tween 20, and 1.5% bovine serum albumin). Membranes were then probed overnight at 4 °C with a monoclonal antibody against nitrotyrosine (1:5000; clone 1A6; Upstate Biotechnology, Lake Placid, NY) in blocking buffer. The membranes were then washed four times in washing buffer (20 mM Tris, 150 mM NaCl, pH 7.5, and 0.2% Tween 20), probed 60 min with a goat anti-mouse antibody (horseradish peroxidase conjugate, 1:8000; Sigma) and finally washed again four times in washing buffer. Immunopositive spots were visualized by chemiluminescence using ECL-Plus reagent (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturer. The results were verified by reduction of nitrotyrosine to aminotyrosine by reduction with sodium hydrosulfite followed by determination of remaining nitrotyrosine immunoreactivity using anti-nitrotyrosine antibody (3).

**Protein Identification by MALDI-TOF Mass Spectroscopy**—Immunopositive fibroblast proteins were identified by in-gel tryptic digestion of protein spots followed by matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) mass spectrometry as primarily described in detail (3, 9). Measured peptide masses were used to search the Swiss-Prot, TrEMBL, and NCBI sequence databases using either MS-Fit (prospector.ucsf.edu/ucsfhtm13l4/msfit.htm) or Mascot (www.matrixscience.com). All searches were performed with a mass tolerance of 0.005% error (50 ppm).

**Quantification of Total Nitrotyrosine**—Aliquots of 300 μg of control
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and peroxynitrite (100, 200, and 500 μM)-treated rabbit muscle aldolase A were analyzed for total protein-bound nitrotyrosine content using stable isotope dilution methods employing high-pressure liquid chromatography with on-line electrospray ionization tandem mass spectrometry (29).

Identification of Nitrated Sites by LC MS/MS—Liquid chromatography tandem mass spectrometry (LC MS/MS) was used to analyze aliquots of the same samples as used for quantification of total nitrotyrosine. Aliquots were mixed with an equal amount of 10 mM urea, reduced for 5 min with 5 mM DTT at room temperature, and alkylated for 60 min with 20 mM iodoacetamide at room temperature in the dark. Modified and unmodified aldolase was subjected to SDS-PAGE according to Laemmli, the resulting protein bands excised, treated with trypsin, and analyzed by LC MS/MS as described in detail elsewhere (9). Briefly, 10 μl of the digest was injected by autosampler onto a 0.3 × 1-mm trapping column (PepMap C18; Applied Biosystems, Foster City, CA) using a CapLC system (Micromass, Beverly, MA), a switching valve (Micromass), and a flow rate of 5 μl/min. Solvent pumps were set at 8 μl/min, and the flow rate was controlled with a splitter in front of the switching valve. Peptides were eluted at 250 nM/min and chromatographed on a 50-μm × 5-cm Biobasic C18 column (New Objective, Cambridge, MA) with a gradient of 5–40% acetonitrile over 20 min followed by 80% acetonitrile for 5 min. The eluent was direct into a quadrupole time-of-flight mass spectrometer (Q-TOF2; Micromass) and ionized immediately using an electrospray designed in-house. The mass spectrometer was operated in standard MS/MS switching mode with the three most intense ions in each survey scan subjected to MS/MS analysis. In addition, the "include function" of the instrument operating software was used to program MS/MS analysis of precursor ions of all possible nitrotyrosine-containing trypic peptides based on the aldolase A structure (calculated as doubly and triply charged ions). MS/MS data analysis utilized Micromass software Protein-Lynx™ Global Server, MassLynx™ Version 3.5., and Mascot. The MS/MS spectra of possible nitrated peptides were examined manually to determine sites of modification.

RESULTS

Protein Identification in Human Skin Fibroblasts—Two-dimensional Western analysis of protein extracts from human skin fibroblasts grown in DMEM containing 4.5 g/liter (25 mM) glucose, a concentration that has been associated with endogenous peroxynitrite formation (30), revealed a limited number of nitrotyrosine-immunoreactive proteins (Fig. 1). The accuracy of these immunoreactive spots was verified by excluding nonspecific nitrotyrosine immunoreactivity through reduction of nitrotyrosine to aminotyrosine (data not shown). Many of the nitrotyrosine immunoreactive gel spots contained proteins involved in glycolysis/gluconeogenesis, including aldolase A.

Aldolase Activity and Nitrotyrosine Immunoreactivity—In a separate analysis characterization of nitrated and non-nitrated purified rabbit muscle aldolase A was evaluated for changes in enzyme activity. Peroxynitrite added to a solution of aldolase A fully decayed in less than 4 s (measured at 302 nm, data not shown), which correlates with published decay rates in the presence of tyrosine (31) or human serum albumin (32). Thus after 30 s, no peroxynitrite was left. The very short exposure of aldolase A to peroxynitrite caused a peroxynitrite concentration-dependent decrease in Fru-1,6-P₂ cleavage activity that was closely correlated with an increase in nitrotyrosine immunoreactivity (Fig. 2). This decrease in enzyme activity and increase in nitrotyrosine immunoreactivity was quasilinear between peroxynitrite concentrations of 25–150 μM. As the reaction of peroxynitrite with carbon dioxide or bicarbonate enhances tyrosine nitrations reactions by nitrosodioxycarboxylate formation (33), which reduces hydroxylation and oxidation reactions at the same time, we tested for the effects of 25 mM sodium bicarbonate on aldolase nitrification and inhibition. In the presence of bicarbonate no significant alterations in the inhibition of aldolase A activity or nitrotyrosine immunoreactivity occurred within the used concentration range of peroxynitrite compared with the buffer with residual carbon dioxide. Lower tyrosine nitrification was observed when the buffer was sparged with nitrogen for 10 min prior to the addition of peroxynitrite, as measured by nitrotyrosine-specific absorption at 423 nm (34) (data not shown). This data is consistent with previous reports that suggest that sparged (argon, helium, or nitrogen) or boiled buffers are required to eliminate carbon dioxide mediated catalysis (29, 31, 35). Kinetic Properties—Kinetic alterations in aldolase A caused by peroxynitrite treatment were determined for the aldol cleavage reaction. Rabbit muscle aldolase A was treated with
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200 μM peroxynitrite under the conditions described under "Experimental Procedures." Following protein nitration, $V_{\text{max}}$ decreased by 49.3% ($p < 0.01$) whereas the $K_m$ for Fru-1,6-P$_2$ exhibited a statistically insignificant decrease (Table II).

Carboxypeptidase Y Digestion—The C terminus of aldolase A was of special interest because it contains a solvent-exposed tyrosine as the final residue, Tyr$^{363}$, that is critical for enzyme function (36). As a step toward identifying nitrotyrosine residues in aldolase A following treatment with 200 μM peroxynitrite, partial C-terminal (carboxyl-terminal) carboxypeptidase Y digestion of aldolase A was performed. Carboxypeptidase Y cleaves all amino acids, including aromatic amino acids like tyrosine, from the carboxyl terminus (C terminus) of a peptide or protein. Western analysis revealed a rapid decrease in the nitrotyrosine immunoreactivity of aldolase A during digestion (Fig. 3). After 5 min of digestion, the immunoreactivity was almost completely diminished. Incubation of nitrated aldolase A in citrate buffer without carboxypeptidase Y caused no change in nitrotyrosine immunoreactivity (data not shown). Following digestion, Coomassie staining revealed no change in protein mass, band intensity, or additional bands of smaller molecular mass (Fig. 3). These results suggest that the major fraction of nitrotyrosine is located at or near the C terminus of aldolase A.

Quantification of Tyrosine and Nitrotyrosine—Tandem mass spectrometric quantitation of the total nitrotyrosine content in native (control) aldolase A revealed insignificant amounts of nitrotyrosine (Table III). Upon exposure of aldolase A to peroxynitrite, an increasing content of nitrotyrosine was observed in a dose-dependent manner. When aldolase A was treated with 500 μM peroxynitrite, about four tyrosine residues out of 12 per aldolase A subunit became nitrated.

MS/MS—LC MS/MS analysis was performed to determine which aldolase A residues were actually nitrated after exposure to 100, 200, and 500 μM peroxynitrite (Fig. 4). At 100 and 200 μM peroxynitrite, we found Tyr$^{243}$, Tyr$^{342}$, and Tyr$^{363}$ to be

### Table I

#### Protein identification in fibroblasts

<table>
<thead>
<tr>
<th>Gel spots</th>
<th>Protein</th>
<th>Sequence coverage (%)</th>
<th>Peptide matches</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Aldolase A</td>
<td>36</td>
<td>9</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>4, 6, 7</td>
<td>Annexin A2</td>
<td>51</td>
<td>16</td>
<td>Membrane fusion, plasminogen activator receptor</td>
</tr>
<tr>
<td>13, 14</td>
<td>Enolase 1</td>
<td>51</td>
<td>16</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>1</td>
<td>Eukaryotic translation elongation factor 1α</td>
<td>20</td>
<td>5</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>2, 3, 8, 9</td>
<td>GAPDH</td>
<td>25</td>
<td>5</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>5</td>
<td>G protein β polypeptide 2-like 1</td>
<td>30</td>
<td>8</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>11, 12</td>
<td>Pyruvate kinase</td>
<td>41</td>
<td>17</td>
<td>Glycolysis</td>
</tr>
</tbody>
</table>

*All identifications were obtained from the two-dimensional gels in Fig. 1.*

### Table II

#### Kinetic properties

<table>
<thead>
<tr>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>161.3 ± 28.7</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>110.8 ± 33.6</td>
</tr>
</tbody>
</table>

*Statistically significant alterations ($p < 0.01$) determined by t test ($n = 3$).

Fig. 2. *Aldolase activity versus intensity of nitrotyrosine immunoreactivity.* Enzyme activity of rabbit aldolase A (U/mg protein, solid circles) treated with 0–500 μM peroxynitrite (initial concentrations) drawn against nitrotyrosine immunoreactivity measured as chemiluminescent signal intensity (MultiImage light cabinet; Alpha Innotech Corporation, San Leandro, CA) and shown as arbitrary units (AU). Background nitrotyrosine immunoreactivity was set as 1 AU, as unmodified aldolase A shows no immunoreactivity.

TABLE I

Human skin fibroblast proteins in nitrotyrosine-immunoreactive gel spots. Values for sequence coverage and peptide matches originate from Mascot search using MALDI-TOF mass spectrometry peptide map data.

TABLE II

Michaelis Menton constant ($K_m$) and $V_{\text{max}}$ of aldolase A. Values ± S.D. of rabbit aldolase A treated with 200 μM peroxynitrite for 30 s.
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FIG. 3. Carboxypeptidase Y digestion. One-dimensional PAGE analysis of peroxynitrite (200 μM)-treated rabbit aldolase A digested with 6 mU carboxypeptidase Y per μg aldolase A for 0–5 min. Western blot shows anti-nitrotyrosine immunoreactivity.

### TABLE III
Quantitation of tyrosine and nitrotyrosine

<table>
<thead>
<tr>
<th>Peroxynitrite (μM)</th>
<th>NO$_2$-Y (mmol)/total Y (mol)</th>
<th>NO$_2$-Y residues/subunit</th>
<th>Enzyme activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.01 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>20.7 ± 0.7</td>
</tr>
<tr>
<td>100</td>
<td>95.66 ± 1.90</td>
<td>1.15 ± 0.02</td>
<td>16.6 ± 2.6</td>
</tr>
<tr>
<td>200</td>
<td>159.88 ± 12.18</td>
<td>1.92 ± 0.15</td>
<td>12.1 ± 2.1</td>
</tr>
<tr>
<td>500</td>
<td>299.62 ± 45.79</td>
<td>3.60 ± 0.55</td>
<td>5.7 ± 2.2</td>
</tr>
</tbody>
</table>

nitrated (Table IV). Increasing the peroxynitrite concentration to 500 μM resulted in the nitration of one additional tyrosine residue, Tyr$^{222}$ (Table IV), and a total of four nitrotyrosine residues out of 12 tyrosine residues in rabbit aldolase A. The C-terminal peptide, which contains tyrosine residues Tyr$^{342}$ and Tyr$^{363}$, was found with either one or both tyrosine residues nitrated. The single-nitrated form always contained only Tyr$^{363}$ as the nitrated residue. With increasing peroxynitrite concentrations, the double-nitrated C-terminal peptide appeared more frequently.

### DISCUSSION

Our results show that peroxynitrite causes tyrosine nitrination in mammalian aldolase A in vitro. The fast decay rate of peroxynitrite suggests that the actual amount of peroxynitrite available to react with aldolase A is lower than the initial peroxynitrite concentrations (25–500 μM) and that the modified tyrosine residues are highly reactive. Nevertheless, these concentrations are high compared with the mathematically estimated nanomolar range of cellular steady-state levels of peroxynitrite in vivo (37, 38). However, a direct measurement of a cellular peroxynitrite concentration has not been technically feasible and may be misleading as the formed peroxynitrite is thought to be confined to relatively small regions surrounding the site of its formation inside a cell (37). A prolonged localized production of peroxynitrite in specific cell compartments such as mitochondria and the cytosol adjacent to them would likely lead to an accumulation of nitrated proteins, as the range of half-life times for nitrated mitochondrial proteins lays within 0.5–4 h (39).

The changes in the enzymes catalytic profile in response to nitrination exhibited a tight correlation between the increase in tyrosine nitrination and the decrease of its Fru-1,6-P$_2$ cleavage activity as well as $V_{\text{max}}$ and $K_m$ for Fru-1,6-P$_2$. Carboxypeptidase Y digestion demonstrated that the C-terminal region of aldolase A contained the majority of nitrated tyrosine. In aldolase A, two tyrosine residues are located in this region, Tyr$^{342}$ and Tyr$^{363}$. In the lower range of the applied peroxynitrite concentrations (100–200 μM), MS/MS revealed single-modified peptides with nitrated Tyr$^{363}$ or Tyr$^{343}$ together with double-modified peptides with both Tyr$^{363}$ and Tyr$^{342}$, but no single-modified peptides with nitrated Tyr$^{342}$. These results, together with nitrotyrosine quantitation revealing statistically only one (100 μM) to two (200 μM) nitrated tyrosine residues per aldolase subunit, suggest that Tyr$^{363}$ is the residue most susceptible to nitration and the most intensely modified. Carboxypeptidase Y digestion also demonstrated that above 100 μM peroxynitrite, rising peroxynitrite concentrations increase the double nitration of the C terminus at Tyr$^{363}$ and Tyr$^{342}$, whereas nitrated Tyr$^{342}$ remains a minor component. At 500 μM peroxynitrite, an escalation of tyrosine nitration occurred with one additionally nitrated tyrosine residue, Tyr$^{222}$. Tyr$^{222}$ together with Tyr$^{363}$, Tyr$^{342}$, and Tyr$^{343}$ account for the statistically 3.6 nitrated tyrosine residues per aldolase subunit, implying a significant contribution of Tyr$^{343}$ nitration beside highly nitrated Tyr$^{363}$ and Tyr$^{342}$. Thus, four of the 12 tyrosine residues in rabbit aldolase A are nitrated to different extents. Tyr$^{222}$, Tyr$^{342}$, and Tyr$^{363}$, are highly conserved and found not only in rabbit aldolase A but also in human aldolase A, B, and C as well aldolase A from Drosophila melanogaster (15, 16). Tyr$^{343}$ is not found in aldolase A from humans or D. melanogaster, therefore its nitration may be of less physiological significance.

The catalytic mechanism of aldolase A has been well established in terms of reaction intermediates (40), but with the exception of the Schiff base-forming Lys$^{229}$ that is located toward the center of an eight-stranded β-barrel (41), the exact role of other amino acid residues in the active site is more controversial. Nevertheless, recent studies suggest that Tyr$^{363}$ is important for the substrate specificity toward Fru-1,6-P$_2$ as well as for product binding and release (36, 42–45). In the past, Tyr$^{363}$ had also been suggested to be involved in a proton transfer during the C3-C4 cleavage reaction (42), but this idea has fallen out of favor (46). Site-directed mutagenesis of the Tyr$^{363}$ (Y363S) (43) as well as cleavage of Tyr$^{363}$ by carboxypeptidase treatment (44, 45) have shown effects on $V_{\text{max}}$ for the Fru-1,6-P$_2$ cleavage reaction and/or $K_m$ for Fru-1,6-P$_2$ that are comparable with the present results obtained by nitration of aldolase A. This further favors Tyr$^{363}$ as the primary modification site responsible for most of the observed alterations.

The C-terminal region of aldolase A (amino acid residues

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344 to 363), localized at the open end of a β-barrel, shows substantial conformational differences between the substrate/product-loaded versus -free forms of the enzyme (36). Two distinct folds have been observed. In a substrate/product-loaded subunit, the C terminus folds over a surface region including the active site, allowing conformational stabilization of Arg303, probably by Glu354. This stabilized conformation allows an Arg303 product interaction in the active site by hydrogen bond or ion pair interactions (Fig. 5, green subunit).

In contrast, the substrate/product-free subunit is characterized by a C terminus that is directed toward an intersubunit cleft, leaving the active site fully exposed and Arg303 in a conformation ready to capture substrate (Fig. 5, magenta subunit). Ion pair and hydrogen bond interactions of the Tyr363 hydroxyl and its main chain carboxylate moiety with Arg258, Lys199, and Lys12 of an adjacent subunit stabilize this conformation (36). Tyrosine nitration leads to a decrease in the pKₐ of its phenolic group (47), which can be followed by alter-

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**FIG. 4. Sites of tyrosine nitration in peroxynitrite treated aldolase A.** MS/MS spectra are shown identifying Tyr222, Tyr243, Tyr342, and Tyr363 as nitrated residues (nY) in peroxynitrite-modified rabbit aldolase A. A, aldolase residues 215–242 (Tyr222); B, aldolase residues 243–257 (Tyr243); C, aldolase residues 342–363 (Tyr363); and D, aldolase residues 342–363 (Tyr342 and Tyr363).

**TABLE IV**

MS/MS analysis

Nitrated peptides. Calculated nitrated peptide masses, LC MS/MS aldolase A sequence coverages, and sequences of nitrated peptides from rabbit aldolase A treated with 100, 200, or 500 μM peroxynitrite are shown. Nitrated tyrosine residues are highlighted.

<table>
<thead>
<tr>
<th>Peroxynitrite concentration (μM)</th>
<th>Sequence coverage (%)</th>
<th>Nitrated peptides</th>
<th>Mass</th>
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<tr>
<td>100</td>
<td>84</td>
<td>243–257</td>
<td>1735.82</td>
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<td>YTPSQGAGAAASELSFISHAY</td>
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Molecular & Cellular Proteomics 3.6

Tyrosine Nitration in Aldolase A

ations in the local ionization state, hydrophobicity, and conformation within a protein. Thus it can be assumed that nitration of Tyr$^{363}$ likely interferes with its interactions in the substrate-free subunits, resulting in an altered ability to interchange between the mentioned conformations of the C terminus. This would interfere with substrate binding and processing. Because nitration of Tyr$^{342}$, which is located close to this C-terminal region, tightly followed the nitration of Tyr$^{363}$ and was never found without Tyr$^{363}$ nitration, we assume that the conformation favored by the Tyr$^{363}$ nitration enables subsequent Tyr$^{342}$ nitration. The altered ionization state and hydrophobicity of the nitrated Tyr$^{342}$ probably alters its interactions with residues in the α-helices that are close to it, further restricting the mobility of the C-terminal region.

Although Tyr$^{222}$, which is located at a subunit interface, contributes to the stabilization of the tetrameric structure of aldolase by the formation of a ionic bond to the adjacent subunit (48), the effect of its nitration may not be important at all because it only appears at high concentrations of peroxynitrite.

The physiological effects of aldolase A nitration are determined through the actual levels of inhibition in vivo. Thus the physiological effects will depend on the nitrated residues and the extent to which the aldolase A population is nitrated in vivo and therefore on conditions in the local environment like the distance to sources of peroxynitrite, carbon dioxide/bicarbonate concentration, and the presence of antioxidants. These conditions may be different from the conditions used for the in vitro nitration by peroxynitrite. A possible estimation of the level of the Fru-1,6-P$_2$ cleavage inhibition required to cause significant metabolic alterations in vivo can be obtained from aldolase A deficiencies. Different mutations are associated with aldolase activities that are 4–11% of the basal levels (49). One of these mutations (E206K) causes metabolic alterations like impaired glucose consumption (66% of controls), decreased contents of DHAP (74.7% of control) and G3P (80% of control), as well as Fru-1,6-P$_2$ accumulation (182.9% of control) in erythrocytes. However, the content of ATP (92.6% of controls) is not significantly altered (49).

Thus it may be assumed that if aldolase A is the only modified protein in a tissue, a lasting nitration at least at Tyr$^{363}$ and Tyr$^{342}$ of most of its population would have to occur to cause any significant metabolic impact. This is very improbable regardless of the nitration mechanisms and conditions in vivo. The situation likely changes when enzymes downstream of aldolase become nitrated at the same time. A small negative value for Gibbs energy ($\Delta G = -0.72$ to $-1.3$ kJ/mol) suggests that the forward reaction of aldolase is only weakly favored (50) and that the enzymes that remove its products DHAP and G3P must be capable of high activity to continue the reaction. When enzymes downstream in glycolysis like glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fig. 6)

**Fig. 5. Molecular modeling.** The nitrated tyrosine residues and interacting amino acid residues are shown. The locations of nitratated tyrosine residues were mapped to the quaternary structure of aldolase A (PDB accession code 1ADO) (36) using Sybyl (Tripos Inc., St. Louis, MO).
in a cell (37, 59), increasing the probability of nitration and inhibition.

In summary, several lines of evidence show that protein nitration is a specific process regarding the protein targets and the tyrosine residues that are modified within each target. In rabbit aldolase A, only four of 12 tyrosine residues were nitrated and this led to progressive inactivation of the enzyme. Most of the inactivation was likely caused by the nitration of two tyrosine residues, Tyr363 and Tyr342. Our knowledge of these alterations in aldolase A function creates a basis for further studies on the effects of tyrosine nitration on glucose metabolism in living systems, including studies on changed interactions between different glycolytic enzymes when more then one enzyme is modified. Finally, it should be noted that these molecular insights could only have been achieved through the combined use of modern proteomic methods like two-dimensional gel electrophoresis, Western analysis, and MALDI-TOF and Q-TOF mass spectroscopy together with kinetic analysis.

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REFERENCES
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nitric oxide in the initiation of the inflammatory response after postisch-emic injury. Shock 18, 169–176


Brownian dynamics simulates aldolase binding glyceraldehyde-3-phosphate dehydrogenase and the possibility of substrate channeling. *Biophys. J.* **80**, 2527–2535


