Biochemistry of Mitochondrial Nitric-oxide Synthase*

Received for publication, May 28, 2002, and in revised form, July 24, 2002
Published, JBC Papers in Press, August 1, 2002, DOI 10.1074/jbc.M205256200

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We reported that the generation of nitric oxide by mitochondria is catalyzed by a constitutive, mitochondrial nitric-oxide synthase (mtNOS). Given that this production may establish the basis for a novel regulatory pathway of energy metabolism, oxygen consumption, and oxygen free radical production, it becomes imperative to identify unequivocally and characterize this enzyme to provide a basis for its regulation. The mitochondrial localization of mtNOS was supported by following the hepatic distribution of mtNOS, immunoblotting sub-mitochondrial fractions, and immunohistochemistry of liver tissues. mtNOS was identified as brain NOS by various methods (mass spectrometry of proteolytic fragments, amino acid analysis, molecular weight, pI, and analysis of PCR fragments), excluding the occurrence of a novel isoform or other splice variants. Distribution of mtNOS transcript indicated its occurrence in liver, brain, heart, muscle, kidney, lung, testis, and spleen. In contrast to brain NOS, mtNOS has two post-translational modifications: acylation with myristic acid and phosphorylation at the C terminus. The former modification could be linked to enzymatic regulation. These results are discussed in terms of the role that nitric oxide may have in cellular bioenergetics.

Our studies (1–5) and those of others (6–10) have provided evidence for the production of nitric oxide (NO) by intact, purified mitochondria and by mitochondria-rich fractions, respectively. In our laboratory the production of NO by mitochondria was demonstrated by using two spectroscopic techniques, i.e. oxidation of oxymyoglobin inhibitable by a nitric-oxide synthase (NOS) inhibitor and by electron paramagnetic resonance with a spin trapping technique (1–4). In other laboratories, the production of NO has been followed by the formation of \( \text{L-citrulline from radiolabeled L-arginine (8–10).} \)

These measurements along with the use of purified, intact mitochondria provided experimental support for production of NO by these organelles. Later, we isolated and purified a protein with NOS activity localized mainly at the mitochondrial membranes (mtNOS; 2). This mtNOS has characteristics similar to the mac isoform (antigenic cross-reactivity and tight binding of calmodulin). In contrast, mtNOS is constitutively expressed and membrane-bound (2).

The identification of mtNOS is a required step to assign this protein to one of the known isoforms (brain NOS (bNOS), endothelial NOS (eNOS), or inducible NOS (iNOS)) or to depict it as a novel isoform. The relevance of this assignment resides on understanding the expression and regulation of mtNOS, considering the biochemical differences of NOSs. For example, the constitutive forms, i.e. bNOS and eNOS, account for the rapid, transient, Ca\(^{2+}\)-dependent production of NO (11–13); the inducible form, i.e. macNOS, causes the slow onset of Ca\(^{2+}\)-independent NO synthesis in inflammatory cells (after stimulation by cytokines or lipopolysaccharides; 14, 15).

Considering the relevant role that NO may have in modulating \( \text{O}_2 \) consumption (3) and \( \text{O}_2 \) free radical formation (5, 16) by mitochondria, it is important to gain insight into the biochemical characteristics of this protein. The goals of this study are the following: first, to identify mtNOS; second, to study the molecular expression of mtNOS in different tissues; and third, to investigate putative post-translational modifications that may target this protein to mitochondria or may affect enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Isolation of Rat Liver Mitochondria and Submitochondrial Fractions**—Liver mitochondria were isolated from adult Wistar rats (180–200 g) by differential centrifugation (1, 17). Purified mitochondria were obtained by Percoll gradient centrifugation (1, 18) and subsequently washed with 150 mM KCl to dissociate arginase (1, 19) and other nonspecifically bound proteins. The preparations contained a contamination of non-mitochondrial origin of 1–4%, assessed by evaluating enzymatic markers of liver fractions (20 and references therein). The respiratory control ratio of these mitochondria was 8 ± 1, and the ADP/O ratio was 1.8 ± 0.2 with succinate. Mitoplasts were obtained from Percoll-purified mitochondria subjected to a controlled digitonin treatment. Mitoplasts, separated from outer membrane and inner membrane space by centrifugation, were sonicated, and this suspension was separated into two fractions by centrifugation: soluble (matrix) and particulate (inner membrane and contact sites). The inner membrane was purified using a sucrose gradient, sonicated in water, and washed twice with KCl to yield submitochondrial particles (21). The recovery of cytochrome oxidase, a marker for mitochondrial inner membrane, was 43.5%. The resultant submitochondrial particles contained less than 0.06% of the outer membrane marker, monooamine oxidase, and the inner membrane marker, adenylate kinase, and about 2% of the matrix-specific marker enzyme Mn-superoxide dismutase. The recoveries were based on the total activity of the mitochondrial markers in mitochondria and submitochondrial particles.

**Purification of mtNOS**—mtNOS was isolated from purified mitochondria from six to eight rat livers, obtained as described before (2).

**Measurement of NO Production**—The reaction medium used to follow NO production by purified mtNOS or crude fractions contained 1...
MM L-Arg, 1 mM magnesium acetate, 1 mM CaCl2, 0.1 mM NADPH, 20 μM tetrahydrobiopterin, 10 mM CHAPS, 1–5 μg/ml calmodulin, in 0.1 μM Hepes buffer, pH 7.5 (22), and 0.05–0.5 mg of protein. mtNOS activity was followed by absorption spectrophotometry by following the oxidation of oxymyoglobin. This assay is based on the oxidation of oxymyoglobin by NO (1, 23, 24) for reference and as a positive control. PCR products were electrophoresed on a 1.0% agarose gel using TAE buffer containing ethidium bromide and visualized on a MultiImageFC using FluorChem 2.0 software (Alpha-Innotech, San Leandro, CA). Nested PCRs contained 1.25 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), 1× PCR buffer II, 1.8 mM MgCl2, 200 mM dNTPs, 0.2 mM sense and antisense primers, and 1 μl of template cDNA in a final volume of 50 μl.

The samples were denatured at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 1 min, and extension at 68 °C for 2 min. The final extension was held at 68 °C for 7 min, then set at 4 °C until analyzed by gel electrophoresis as described above.

PCRs for Fig. 3 (A, lanes 6 and 7, and B and C) were performed using the Rat MTC™ panel 1 (Clontech, Palo Alto, CA) as the template and PCR Master Mix (Promega). The PCR conditions for the products obtained in Fig. 3A (lanes 6 and 7) were performed using 1× Master mix (1.25 units of Taq DNA polymerase, 200 mM dNTPs, 1.5 mM MgCl2, 0.5 mM sense and antisense primers, and 1 μg of template cDNA in a final volume of 50 μl). Initial denaturation was carried out at 94 °C for 1 min, followed by PCR. The PCR conditions were as follows: 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 55 °C, and 3 min elongation at 72 °C. The final extension was performed at 72 °C for 5 min and maintained at 4 °C until analyzed by gel electrophoresis. Nested PCRs were carried out with the same reaction components and cycling conditions as the parent reactions, except the cDNA template replaced 1 μl of the parent reaction, and different sense and antisense primers were used as indicated in the figure legend. Although the PCR products depicted in Fig. 3, B and C, were also performed with cDNA from MTC™ panel, the experimental conditions were slightly different than those in Fig. 3A with the following modifications. The parent PCRs contained 1.25 units of AmpliTaq Gold, 1× PCR buffer II, 1.8 mM MgCl2, 200 mM dNTPs, 0.2 mM sense and antisense primers, 1 μg of MTC™ panel cDNA in a final volume of 50 μl. The reactions were amplified under the following conditions: denaturation of 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 55 °C for 1 min, and extension at 68 °C for 2 min. The final extension was held at 68 °C for 7 min and maintained at 4 °C.

Release and Identification of mtNOS-bovine Fatty Acid—Purified mitochondria (1 mg of protein) obtained after the isolation procedure, was precipitated with 5 μl of cold acetone and kept for 30–20 minutes. The solution was centrifuged in a Dupont® centrifuge in a SS-34 rotor at 10,000 × g for 15 min at 4 °C. The pellet was dissolved in 0.5 ml of 2% SDS w/v and precipitated again with 5 μl of cold acetone. The solution was kept at −20 °C for 30 min and centrifuged at 10,000 × g for 10 min at 4 °C. This latter procedure (SDS treatment, precipitation and centrifugation) was repeated twice. The pellet was extracted with 2 ml of Cl3CH-methanol (2/1, v/v), washed with 2 ml of diethyl ether, and dried under a stream of N2.

Decaylation of Protein—The diglypilated protein pellet was treated with 2 ml of 0.1% KOH and anhydrous methanol (less than 0.01% H2O), vortexed, and incubated at 30 °C for 30 min. The solution was centrifuged at 3,000 × g for 5 min, and the protein pellet was saved for acid hydrolysis (see below). The supernatant was acidified with 6 N HCl and extracted three times with hexane. These extractions were combined and dried under N2. The residue, containing the methyl esters of fatty acids originally bound to the protein by oxy- or thioster linkages, was subjected to mass spectrometric analysis. The protein pellet procured after the centrifugation at 3,000 × g was washed three times with ethanol and dried under N2. This pellet was resuspended in 1 ml of 3% HCl in anhydrous methanol and incubated at 105 °C for 20 h in sealed tubes (earlier purged with a N2 stream) under N2 to release amide-linked fatty acids from the protein as the corresponding methyl esters. The tubes were cooled and extracted three times with hexane. The extracts were combined and dried under a stream of N2. The residue was subjected to mass spectrometric analysis as described above.

Immunohistochemistry—NOS was immunolocalized in paraformaldehdye-fixed rat liver slices, essentially following the procedure described in Ref. 31 with the following modifications. After fixation, the slices were permeabilized by incubating in ice-cold acetone for 5 min, then washed three times with 0.1% Triton X-100 in phosphate-buffered saline (PBS-T). The slices were blocked with 10% normal goat serum, 1% bovine serum albumin in PBS-T for 30 min at room temperature.
Primary antibody incubations were performed using a 1:500 dilution of monoclonal antibodies to bNOS (Transduction Laboratories) and to subunit I of cytochrome oxidase (Molecular Probes, Eugene, OR) in blocking solution. The bNOS antibody was labeled with Alexa Fluor® 594 according to the manufacturer’s instructions (Molecular Probes). After labeling the antibody to bNOS, the specificity of these was confirmed by immunoblotting samples of rat liver and isolated mitochondria, using positive and negative controls. The antibody to cytochrome oxidase was commercially available as a conjugate with Alexa Fluor® 488. Overnight incubation of the slices with primary antibodies was performed in the dark in moist chambers, at 4°C. The slides were washed three times for 15 min each with PBS-T. In some experiments, instead of the antibody to cytochrome oxidase, mitochondria were labeled with MitoTracker Green FM (Molecular Probes) according to the manufacturer’s conditions. The slides were washed with PBS-T, the nuclei labeled with 4,6-diamidino-2-phenylindole according to the manufacturer’s conditions (Molecular Probes), air dried, and protected from light. The slides were mounted using ProLong Antifade medium (Manufacturer) and photographed using a digital camera (Spot II). The digital images were acquired using Metamorph software. Brightness, contrast, and background (the last defined as areas without cells or with cells without antibody addition) were adjusted and the images were overlaid using Adobe Photoshop (Mountain View, CA).

RESULTS AND DISCUSSION

Distribution of NOS Activity in Liver—The recovery of rat liver NOS activity (followed by either the oxidation of oxymyoglobin or 1-[14C]citrulline, inhabitable by N6-monomethyl-L-arginine) was 70% in the mitochondrial fraction and 45–50% in Percoll-purified mitochondria, supporting the mitochondrial localization of this enzyme. The sub mitochondrial distribution of mtNOS indicated that inner membrane and contact sites were the only fractions that exhibited the presence of mtNOS by Western blotting. The almost complete recovery (60–70%) of mtNOS activity from mitochondria isolated from purified rat hepatocytes (>98% purity and >98% viability by trypsin blue exclusion) indicated that this enzyme had originated from parenchymal cells, excluding contributions from other cell types. These observations were confirmed further by (i) immunoprob ing formalin-fixed liver slices for cytochrome oxidase and NOS, resulting in the colocalization of these proteins in parenchymal mitochondria (Fig. 1; similar results were obtained by using Mitotracker Green instead of antibodies to cytochrome oxidase) and (ii) considering the high contribution of hepatocytes to liver cellular composition (92.5%; 33) and mitochondrial volume (98.4%; 33).

Identification of mtNOS—As reported before, isolated rat liver mtNOS shared certain properties with macNOS (antigenic cross-reactivity, \( V_{\text{max}} \) of the purified form), whereas the constitutive expression of mtNOS, requirement of cofactors, and the main membrane localization suggested the presence of either a novel isoform or one of the well characterized isoforms with concomitant post-translational modifications. To gain insight into the protein sequence, microsequencing of purified mtNOS from blots was attempted; however, the finding of a blocked N-terminal residue precluded a direct Edman degradation because the chemistry requires a free NH2 group (or imino for Pro; Ref. 34). Thus, mtNOS was separated by two-dimensional electrophoresis, followed by in-gel digestion with either trypsin or endoproteinase V8. MALDI-TOF and/or Q-TOF analyses were performed on the eluted fragments. The resulting peptide masses and sequences were blasted against in silico trypsin- or V8-digested proteins from the PDB to permit the identification of mtNOS based on the digest profile (MALDI-TOF) and amino acid sequences (Q-TOF). The peptidic fragments from both treatments (i.e. trypsin and endoproteinase) matched sequences of constitutive rat bNOS (Fig. 2). All fragments had a 100% homology to bNOS and only between 21 and 78% to macNOS and eNOS, precluding these isozymes as mtNOS. A minor percentage of fragments (5–10%) matched those from mature carbamoyl-phosphate synthetase2 and iNOS.

The amino acid composition of mtNOS, obtained after acid hydrolysis of the protein, closely matched that of bNOS (Table I). The amino acid analysis combined with \( pI \) (7.0 ± 0.5) and molecular weight (130,000 ± 26,000) extracted from two-dimensional gels, in addition to the peptide mass fingerprinting and amino acid sequences, were used to perform a protein identification search using the Multident protein identification on the ExPaSy server. The best integrated score in terms of amino acid composition, \( pI \), molecular weight, and best-matching set of peptide masses for the species Rattus resulted in bNOS (Swiss-Prot P29476).

mtNOS and Splice Variants of bNOS—From these results it could be surmised that mtNOS is the brain isoform. However, given that four splice variants of full-length bNOS (nNOSα) have been identified (nNOSβ, nNOSγ, nNOSμ, and nNOS-2; 38–40), the question remained whether mtNOS was one of these products or represented a novel alternative splicing product. Fragments from amino acids 238 to 242 and from 278 to 290 excluded NOSγ, whereas fragments from amino acids 512 to 516 and from 548 to 555 precluded NOS-2. Given that the other fragments were present in all three remaining isozymes (NOSα, β, and μ), the identification was not possible by using data from MALDI or Q-TOF.

Although carbamoyl-phosphate synthetase I is present in the mitochondrial matrix (35), contamination with this protein is not unexpected given its high concentration (estimated to represent between 13 and 17% of total mitochondrial protein, 0.4–0.5 mU (36) or 1–1.5 mU (37)). Assuming that contamination with this enzyme resulted from its entrapment in membrane vesicles during the sonication procedure, the latter procedure was performed in water, followed by washes with 0.15 M KCl. The resulting preparation resulted in undetectable activity of carbamoyl-phosphate synthetase, without affecting the MS profile of bNOS.
To gather more information on what type of isoform is expressed in liver, RT-PCR experiments were performed on mRNA using primers based on either Q-TOF sequences (outlined sequences in Fig. 3 and underlined sequences in Table II) or gene-specific (Table II). To decrease the degeneracy of the primers derived from protein sequences, they were matched to the corresponding coding oligonucleotide sequences in rat brain NOS sequence (GenBank X59949). The other primers used were gene-specific, based on the same cDNA, designed using the software Primer3 and selected from five alternative sets to best suit PCR conditions, specificity, and discrimination among the various isoforms.

RT-PCR was performed on an enriched poly(A)/H11001 mRNA from rat liver, treated previously with DNase to avoid genomic DNA contamination. PCR products were separated by gel electrophoresis and visualized by using ethidium bromide (Fig. 3A).

Control experiments included performing RT-PCR excluding primers from the reaction mixture, followed by PCR using PS4 (Fig. 3A, lane 2) or PS5 (Fig. 3A, lane 3). The lack of DNA bands when using these primers (and all the others, not shown) indicates the lack of genomic DNA contamination, which would have led to false positives. Positive controls included the amplification of a C-terminal region present in all NOS isoforms, which resulted in a product of 515 bp (Fig. 3A, lane 5). RT-PCR of rat liver poly(A)+ mRNA using PS1 followed by a nested PCR using PS4 resulted in product size similar to that expected for NOSα, NOS-2, and NOSβ (Fig. 3A, lane 4; 788 bp). This result excluded the possibility of the occurrence of the transcripts for either NOSγ or NOSβ (Table III). By performing two consecutive PCRs using PS3 and PS6 on rat liver cDNA the resulting product (206 bp) excluded the occurrence of the transcript for NOS-2 (Fig. 3A, lane 6). The lack of NOS-2 transcript was confirmed further by performing an RT-PCR using PS1 followed by two sequential nested PCRs using PS9 and PS8, resulting in a fragment size of 676 bp not present in NOS-2 (not shown). Combining these results, it was concluded that the transcript amplified from rat liver was either from NOSα/H9251 or NOSβ/H9262. PCR experiments using PS3 and PS7 performed on rat liver cDNA allowed us to obtain a fragment of 81 bp not present in NOSβ (Table III and Fig. 3A, lane 7). Therefore, our results indicated the presence of the transcript for NOSα in rat liver, suggesting that the synthesis of NOSα from this transcript is feasible.

Confirmation of the identity of these PCR products was obtained by MALDI-TOF analysis of trypsin-digested mtNOS. Outline fragments correspond to those sequenced by Q-TOF. Bold sequences represent sequenced PCR products translated into protein sequences. The boxed fragment indicates the phosphorylated sequence.

![Fig. 2. Primary sequence of rat mtNOS. Underlined fragments correspond to MALDI-TOF analysis of endoproteinase-digested mtNOS. Outline fragments correspond to those sequenced by Q-TOF. Bold sequences represent sequenced PCR products translated into protein sequences. The boxed fragment indicates the phosphorylated sequence.](http://www.jbc.org/content/38082/7/38082/F2.large.jpg)
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NOSα transcript. PCRs were performed on rat cDNA from various tissues using PS1 followed by a nested PCR with PS4. All of the tissues exhibited only one band of 788 bp, thus excluding the presence of a transcript for NOSγ or NOSβ. Performing sequential PCRs using PS3 followed by a nested PCR using either PS7 (Fig. 3B, lanes 2–9) or PS6 (Fig. 3C, lanes 11–18) resulted in the formation of two products (81 bp and 206 bp) consistent with the presence of NOSα transcript in all these tissues. The only tissue that exhibited another transcript for NOS, in addition to NOSα, was skeletal muscle (Fig. 3C, lane 5) resulting in a 183-bp product consistent with the simultaneous occurrence of NOSμ.

Lastly, our results indicated that the transcript of NOSα was present in liver, brain, heart, muscle, kidney, lung, testis, and spleen, suggesting that these tissues have the capability to express NOSα. Because our previous results showed that mt-NOS is bNOSα, it could be surmised that these tissues have the potential of synthesizing mtNOS. However, these experiments did not provide information on the steady-state concentration of NOSα nor on the fraction of NOSα present in mitochondria. To this end, we evaluated the activity of mtNOS in isolated, purified rat heart mitochondria. This activity was 2–3 times higher than that found in liver, but because the content of the enzyme (obtained by immunoprecipitation of mtNOS from mitochondria) was also lower (about half), the specific activity of the enzyme remained the same. The results obtained with liver and heart indicated that the content of active mtNOS followed that of the transcripts, with the understanding that this association between activity and content of transcript might not be applicable to other tissues.

Acylation of mtNOS.—Comparison of the activities of permeabilized mitochondria and submitochondrial particles indicated that most NOS activity (30–40%) was detected in the latter fraction, suggesting a localization of the enzyme at the inner mitochondrial membrane (1–2). The association of mt-NOS with membranes was further supported using a mild detergent to solubilize the enzyme during the purification procedure (2). The main particulate distribution of mtNOS resembled that of eNOS (41). The specific distribution of eNOS has been attributed to the presence of an N-myristoylated terminal and palmitoylated Cys residue (42, 43), which may allow a protein-membrane association. The presence of a blocked N-terminal group in mtNOS, aside from its membrane distribution, led us to investigate whether mtNOS was acylated.

To this end, samples of purified mtNOS were precipitated with aceton, washed with organic solvents (to remove any unspecifically bound lipids), and finally subjected to alkaline methanolysis to release methyl ester derivatives of the fatty acids. The derivatives were extracted with hexane, and the fatty acids were identified by mass spectrometry upon comparison with standards. About 80–100% of the fatty acids were recovered as the methyl ester derivative of myristic acid (Fig. 4) after alkaline methanolysis, indicating that this fatty acid was linked through an oxy- or thioester. The starting material had 0.56 nmol of enzyme, and 0.7 nmol of fatty acid was recovered in this procedure, indicating that the ratio of lipid to protein was near unity. The present finding, that mtNOS is acylated with myristic acid via an ester bond, is consistent with a reversible and post-translational process, probably catalyzed by acyltransferases (44–47).

The acylation of mtNOS might explain the discrepancy between the molecular mass of NOSα (MW = 160,000) and that obtained from SDS-PAGE and immunoblotting, which indicated that mtNOS migrated at a slightly faster rate than nNOS. Although this observation has not been studied for most acylated proteins (e.g. 48–50), an analogous phenomenon has been reported for acylreductase (51), acyl carrier protein (52), and some subunits of enzymes involved in the mitochondrial β-oxidation pathway (53). The difference in the migration of these latter proteins compared with unacylated ones (12–20%; calculated from 48–50) was found to be comparable with that observed with mt- and n-NOS (15–19%).

Phosphorylation of mtNOS.—Agonists promote the phosphorylation of eNOS facilitating its solubilization (54) probably by decreasing the positive charge of a region that contributed electrostatically to the binding of the protein to lipid. Other studies provided evidence that all three NOS isoforms are phosphorylated when immunoprecipitated from host cells (54, 55). It has been shown that kinase- and phosphatase-depend-
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TABLE II

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Nucleotide no.</th>
<th>Nucleotide sequence</th>
<th>Nucleotide no.</th>
<th>Nucleotide sequence</th>
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<td>GAA GAG CGT TTC CTT TG Ag</td>
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<td>3787–3807</td>
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<td>6</td>
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<td>9</td>
<td>1522–1541</td>
<td>AGG ACC TAC CAG CTC AAG GA</td>
<td>2227–2246</td>
<td>ATC TCC ACC AGT GCT TGG TC</td>
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</table>

TABLE III

Expected sizes of PCR products from alternatively spliced bNOS

A fragment from amino acid 504 to 608 is missing in NOS-2, and NOSβµ has an insertion at amino acid 839 with the sequence KYPEPLRFPPRKPGLSHVDSQHSLVAARDGSQHR.

<table>
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<tr>
<th>Primer set</th>
<th>NOSα</th>
<th>NOS-2</th>
<th>NOSβ</th>
<th>NOSγ</th>
<th>NOSββ</th>
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</tbody>
</table>

*Boldface numbers indicate the size of fragments experimentally obtained.*

FIG. 4. Mass spectra of mtNOS acylation products. Samples of purified mtNOS were precipitated with acetone, washed with organic solvents to remove any unspecifically bound lipids, and finally subjected to alkaline methanolysis to release the fatty acid methyl esters. The derivatives were extracted with hexane, and the fatty acids were identified by mass spectrometry upon comparison with standards. Generally, the molecular ion peak of methyl ester of a straight chain aliphatic acid is weak; however, the most characteristic peak is the result of the McLafferty rearrangement (m/z = 74). Other assignments were based on typical cleavage of aliphatic esters and those obtained with a standard (methyl ester of myristic acid). By comparing the obtained ions with those expected theoretically, a spectrum match index of 0.9761 was obtained (analysis performed Mass Spec Calculator Pro™ software, ChemSW).

TABLE IV

MALDI-TOF analyses of phosphorylated peptides

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<tr>
<th>Fragment</th>
<th>Expected m/z</th>
<th>Experimental m/z</th>
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<tr>
<td>After phosphatase treatment</td>
<td></td>
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<tr>
<td>SSESIAFIESK</td>
<td>1367.70</td>
<td>1367.48</td>
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<tr>
<td>LRESSEIAFIESK</td>
<td>1508.76</td>
<td></td>
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<tr>
<td>Before phosphatase treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSESIAFIESK</td>
<td>1447.67</td>
<td>1447.30</td>
</tr>
<tr>
<td>LRESSEIAFIESK</td>
<td>1588.76</td>
<td>1588.52</td>
</tr>
</tbody>
</table>

*Expected m/z was calculated by adding (before phosphatase) 80 units to the in silico tryptic digest of bNOS (after phosphatase).  
NF, not found.*

Ser found in bovine (Ser-1179) or human eNOS (Ser-1177) is homologous to rat bNOS Ser-1412.

The phosphorylation of this fragment (in addition to the
acylation) might add to the faster migration of mtNOS compared with nNOS, and this provides support for the lower affinity to monoclonal antibodies to the C-terminal region of nNOS and the cross-reactivity to nNOS earlier observed (1–5).

CONCLUSIONS

Our results unequivocally indicate that mitochondria are endowed with a NOS, extending and confining our previous results (1–5, 16, 32) and those of others (6–10). mtNOS was identified as bNOS with post-translational modifications. This conclusion is supported by the amino acid analysis, pI, molecular weight, tryptic- and endoproteinase-peptidic maps, and peptide sequences. The identification of mtNOS with bNOS has also been suggested through functional studies performed on isolated cardiac mitochondria of bNOS wild-type and knockout mice (61). Given the important role that endogenous NO has in regulating the O2 consumption and ATP production of mitochondria (3–5), it is not surprising that the enzyme is localized close to its target site, cytochrome oxidase. We have showed (3, 4) that the production of NO by mitochondria modulated the O2 consumption of the organelle by competitive inhibition of cytochrome oxidase, and, as a consequence, the O2 free radical production (5). In this regard, the broader implications of our work can help to redefine the way we view regulation of O2 consumption in vivo. Based on our initial findings, it can be proposed that mitochondrial production of NO helps average O2 utilization among cells at different distances from capillaries. The basic concept is that NO will slow O2 consumption by cells closest to blood vessels, allowing O2 to penetrate to cells at the boundary of becoming hypoxic. In addition, NO might help dilate blood vessels and potentially increase O2 delivery to borderline hypoxic cells.3

Regarding the covalent modifications found in mtNOS, i.e., acylation and phosphorylation, it could be speculated that they are important to target (as with other proteins, e.g., Ref. 62) and regulate mtNOS activity. Interest in covalent modifications of proteins has been strengthened by the observations that several viral transforming proteins have covalently attached lipid. The observations that mutants of the transforming proteins p21 and p60src, which lack acylation, are transformation-defective and no longer associated with the plasma membrane (63, 64) serve to underline the importance of the attachment of lipid to these proteins for full expression of their transforming potential. In the case of mtNOS, and considering that the acylation pattern of eNOS is required for localization to plasmalemmal caveolae of endothelial cells (43), it could be speculated that acylation is a required step to target the protein to mitochondria after its cytosolic synthesis, or its acylation (once inside mitochondria) facilitates its membrane localization closer to cytochrome oxidase.

The other covalent modification, phosphorylation of Ser, may indicate a more direct pathway to regulate mtNOS activity. In fact, phosphorylation of eNOS Ser-1179 enhances the ability of the enzyme to generate NO (59–60). Although the mechanism by which this phosphorylation increases NO production has not been elucidated, it has been proposed that the addition of a negative charge at this site may permit the binding of calmodulin at resting Ca2+ concentrations (60). If this is the case for mtNOS, then phosphorylation could redress the main path-
Mitochondrial Nitric-oxide Synthase

Additions and Corrections


Function and solution structure of huwentoxin-IV, a potent neuronal tetrodotoxin (TTX)-sensitive sodium channel antagonist from Chinese bird spider Selenocosmia huwena.

Kuan Peng, Qin Shu, Zhonghua Liu, and Songping Liang

Page 47567, Fig. 4 legend: The following sentence is missing from the figure legend.

“An asterisk indicates that the C-terminal carboxyl group is amidated.”

The figure with the corrected legend is shown below.

FIG. 4. Comparison of amino acid sequence of HWTX-IV with HWTX-I and previously known conotoxins (CTX) blocking at site I (O = 4-trans-L-hydroxyproline). The proposed key residues important for their functions are displayed in the frame boxes. Conotoxin GS (38, 39), μ-conotoxin PIIA (6), μ-conotoxin GIIB (7, 35, 36), and μ-conotoxin GIIIB (8, 37) block at site I. HWTX-I was suggested to be an N-type calcium channel inhibitor (33). Like HWTX-IV and conotoxin GS, it adopts a 1–4, 2–5, 3–6 disulfide pattern and cystine knot motif (32). An asterisk indicates that the C-terminal carboxyl group is amidated.


Biochemistry of mitochondrial nitric-oxide synthase.

Sarah Liv Elfering, Theresa Maria Sarkela, and Cecilia Giulivi

Page 38081, Fig. 1: Fig. 1 should have printed in color. The correct figure is shown below.

FIG. 1. Colocalization of mtNOS and cytochrome oxidase in liver slice. Formalin-fixed rat liver slices were probed with fluorescently tagged monoclonal antibodies to nNOS (A) and cytochrome oxidase (B). Nuclei were stained with 4,6-diamidino-2-phenylindole (C). The superimposition of images from A–C was performed with Adobe Photoshop (D). Other experimental details are described under “Experimental Procedures.”