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Calreticulin Transacetylase Mediates the Acetylation of Nitric Oxide Synthase by Polyphenolic Acetate

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Abstract Our earlier investigations identified acetoxy drug: protein transacetylase (TAase), a unique enzyme in the endoplasmic reticulum (ER) catalyzing the transfer of acetyl groups from polyphenolic acetates (PA) to certain functional proteins. Recently we have established the identity of TAase with ER protein calreticulin (CR) and subsequently transacetylase function of CR was termed calreticulin transacetylase (CRTAase). CRTAase was purified and characterized from human placenta. CRTAase catalyzed the acetylation of a receptor protein nNOS, by a model PA 7, 8-diacetoxy-4-methylcoumarin (DAMC), which was visually confirmed by using antiacetyl lysine. The aim of this report was to provide tacit proof by providing mass spectrometry evidence for CRTAase catalyzed acetylation of purified nNOS by DAMC. For this purpose, purified nNOS was incubated with DAMC and CRTAase, the modified nNOS was analyzed by nanoscale LC-MS/MS, which recorded 11 distinct peptides with a significant score as acetylated on lysine residues. The distribution was in order: lysines-24, -33, -38, -131, and -229 of the PDZ domain, Lys-245 of the oxygenase domain, Lys-754 and -856 of FMN binding domain, Lys-989 of connecting domain and Lys-1300, -1321, and -1371 of the NADPH-binding domain were acetylated. The results documented in this paper highlighted for the first time modification of nNOS by way of acetylation. Our earlier work recorded the profound activation of platelet NADPH cytochrome P-450 reductase and the acetylation of the reductase protein by DAMC, which also remarkably enhanced intracellular levels of nitric oxide. The results reported here coupled with the

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aforementioned previous observations strongly implicate the possible role of the acetylation of the reductase domain of nitric oxide synthase (NOS) in the NOS activation. In addition, the acetylation of nNOS can be expected to potentiate the interaction with CR, eventually leading to the augmented catalytic activity of NOS and expression of the related biological effects.

Keywords Calreticulin · Transacetylase · Polyphenolic acetates · Protein acetylation · Nitric oxide synthase

Introduction

Nitric oxide (NO) is an important regulator of a variety of physiological and pathological conditions. NO is produced in cells by the oxidation of arginine catalyzed by nitric oxide synthases (NOSs) utilizing NADPH and molecular oxygen. NOSs are multicomponent enzymes comprised of heme- and flavin-linked enzymes, which contribute to the overall regulation of NOS isoforms, namely, nNOS, iNOS, and eNOS [1]. These three quite distinct isoforms of NOS have been identified as products of different genes, with different localization, regulation, catalytic properties, and inhibitor sensitivity with 51–57% homology among the isoforms. Recent investigations have highlighted the role of intrinsic and extrinsic factors in the control of NOS catalytic activity [19]. A combination factors involving the heme environment, dimerization state, autoinhibitory domain, calmodulin-binding, and the C-terminal tail region contribute to the intrinsic factors governing the activity of NOS. The posttranslational modifications of NOS such as phosphorylation, myristoylation, and palmitoylation were shown to be extrinsic factors that would modulate the catalytic activity of NOS [12].

However, not much seem to be documented about one of the prominent protein modification, the acetylation on the activity of NOS. We have in our earlier communications [16] documented the existence of a unique enzyme termed acetoxy drug: protein transacetylase (TAase) in liver microsomes that catalyses the transfer of acetyl group from polyphenolic acetates (PA) to certain functional proteins. TAase purified from buffalo liver microsomes was shown to mediate the acetylation of Lys-51, -82, -124, -191, and -210 of glutathione *S*-transferase (GST) 3–3 by 7,8-diacetoxy-4-methylcoumarin (DAMC), a model PA based on the MALDI-TOF and LC-MS/MS analysis [5, 6]. Our recent studies [14, 20] identified rat liver and human placenta microsomal TAase as the calreticulin (CR), a prominent Ca^{2+}-binding protein of the endoplasmic reticulum (ER). The transacetylase function of calreticulin was designated as calreticulin transacetylase (CRTAase) [14, 20]. In our recent publication, we have demonstrated for the first time the role of TAase (CRTAase) in the remarkable activation of NOS in platelet by various PA [4]. In this paper we have furnished the results of mass spectrometry for the CRTAase catalyzed acetylation of nNOS by DAMC.

Materials and Methods

Chemical and Reagents

Antiacetyl lysine polyclonal antibody was purchased from Cell Signaling Technology, Beverly, USA. Rabbit anti-rat HRP-conjugated secondary antibody was purchased from

Bangalore Genei, Bangalore (India), diaminobenzidine (DAB) were purchased from Sigma Chemical Co., St. Louis, MO (USA). All other chemicals used were of high purity and were obtained from local suppliers.

Chemistry

7,8-Dihydroxy-4-methylcoumarin (DHMC) was synthesized in our laboratory by the well-known Pechmann condensation of resorcinol with ethyl acetoacetate, its acetylated product DAMC was prepared by the acetylation of DHMC with acetic anhydride/pyridine. The structure of DAMC was confirmed from various spectral data reported by us earlier [2, 12].

Procurement of Placental Tissue

Human placenta was obtained from Gauri Nursing Home, Malka Ganj, Delhi, India after delivery from healthy mothers free of any infection as certified by the hospital.

Purification of human placental CRTAase

The purification of human placental CRTAase to homogeneity and characterized as described in our earlier communication [20].

Demonstration of Placental CRTAase Catalyzed Acetylation of Neuronal nNOS by DAMC Using Western Blot

The acetylated lysine residues were detected by using antiacetyl lysine polyclonal antibody. Purified human placental CRTAase (12 μg) was incubated with nNOS (50 μg), DAMC (100 μM), and 10 mM phosphate buffer (pH 7.2), and incubated for 30 min at 37°C in water bath. After the completion of reaction, sample buffer was added to the reaction mixture to stop the reaction. This reaction mixture was used to detect the acetylated protein using Western blot. Appropriate controls were taken to elucidate the role of CRTAase in catalyzing the acetylation of the receptor protein.

For Western blot, electrophoretically separated proteins were transblotted to PVDF membrane at 300 mA for 3 h at 4°C. Nonspecific sites on the PVDF-membrane were blocked with blocking reagent (5% Blotto). Primary antibody dilution (1:1,000) was prepared in TBST containing 1% bovine serum albumin and incubation was carried out at 4°C overnight with slight agitation. The PVDF membranes were extensively washed with TBST (TBS with 0.05% Tween). Goat anti-rabbit HRPO (horseradish peroxidase) conjugated secondary antibody, appropriately diluted in TBST was then added and an incubation of 1 h at room temperature was carried out. The sheets were washed extensively with TBST/TBS. Acetylated protein was immunodetected with rabbit polyclonal antiacetyl lysine (1:1,000) and developed with goat anti-rabbit IgG secondary antibody coupled to HRP.

Demonstration of Acetylation of nNOS by LC-MS/MS

The gel band containing the modified nNOS for mass spectrometric analysis was processed essentially according to Shevchenko et al. [21]. Sliced gel pieces were washed with 100 mM NH_4HCO_3 (BDH laboratory Supplies, Poole, England) and acetonitrile (Biosolve

B.V., The Netherlands) (1:1, v/v) (buffer A). Then, the protein was in-gel-reduced, and subsequently alkylated with iodoacetamide (Sigma). After a washing step with buffer A, the gel pieces were dried in a vacuum centrifuge, and then rehydrated at 4°C in digestion buffer (50 mM NH_4HCO_3, 5 mM $CaCl_2$) containing 25 ng/μl trypsin. After overnight incubation, the peptides were extracted from the gel using three separate washings with a mixture of acetonitrile/water/formic acid 70/25/5 (v/v/v). The extracts were combined and dried down in a vacuum centrifuge. The lyophilized digest was reconstituted in 20 μl of 4 M urea solution buffered at pH 8.0 with 25 mM Tris. Ten microliters of the solution was then injected for nanoscale LC-MS/MS analysis.

Nanoscale LC-MS/MS

Nanoscale chromatography was performed on an Ultimate nano-LC system from LC Packings (Amsterdam, The Netherlands). Ten microliters of the peptide mixture was injected on a 300-μm ID X 5 mm Pepmap C_{18} trap column (LC Packings) and washed at 30 μl/min for 10 min with 0.05% (v/v) trifluoroacetic acid (TFA) in water before the RP trap was switched on-line in backflush mode to a 75 μm×150 mm Pepmap C_{18} nano-LC column. Gradient elution of peptides was achieved at 300 nl/min going from 95% mobile phase A (water: acetonitrile: formic acid 97.9/2/0.08 v/v/v), 5% mobile phase B (water: acetonitrile: formic acid 19.9/80/0.1 v/v/v) to 45% B in 35 min, then to 60% B in the following 10 min.

The nano-LC column was coupled to a hybrid linear trap/Fourier transform ion cyclotron resonance LTQ-FT Mass Spectrometer (Thermo Electron, San Jose, CA, USA) *via* a nanoelectrospray interface operating in positive ion mode. Electrospray was performed by applying 1.6 kV to the electrospray pico tip (20 μm ID, 10 μm tip ID, distal coated from New Objective, Cambridge, MA, USA). The instrument operated in data-dependent mode. High resolution FT scans from 400 to 1,600 *m/z* were acquired at 100,000 resolutions, after up to 1-s ion accumulation time. In parallel to the FT scan, the five highest-intensity peaks assigned by a short FT prescan were selected for MS/MS analysis on the linear trap. MS/MS analysis was limited to charge states 2–5. Collision energy was set to 35 U. A complete MS+5×MS/MS scan lasted for 4 s. Protein identification was performed by searching MS/MS spectra against the MSDB database (accessed on June 2005) using the Mascot search engine http://www.matrixscience.com). Search parameters used were as follows. Peptide mass tolerance: 10 ppm. MS/MS tolerance: 1 Da. Allowed missed cleavages: 2. Enzyme: trypsin. Taxonomy: rattus. Fixed modification: carbamidomethyl (C). Variable modifications: oxidation (M), acetylation (K).

Results

Purification of Human Placental CRTAase

CRTAase was isolated from human placental microsomes after solubilization. The solubilized dialysate was subjected to CM, DEAE, and Q Sepharose chromatography where the major part of CRTAase was eluted at 0.35 M NaCl concentration and exhibited a single peak. Protein was purified to 50-folds and was found to be homogenous as revealed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [20]. The molecular weight of CRTAase was found to be 60 kDa and had a specific activity of 16,200 U/mg protein [20].

CRTAase-mediated Acetylation of nNOS by DAMC

The Application of Antiacetyl Lysine

To establish the placental CRTAase catalyzed acetylation of protein by DAMC, nNOS generously provided by Prof. Bettie Masters, University of Texas, Health Science Centre, San Antonio, TX, USA, was used as receptor protein for acetylation. nNOS upon incubation with DAMC and purified placental TAase was found to intensely react with antiacetyl lysine antibody as evident from the Western blot (Fig. 1). This observation is indicative of the acetylation of nNOS.

The Application of LC-MS/MS

nNOS was incubated with DAMC and CRTAase for 30 min as described under "Materials and Methods." The reaction mixture was subjected to SDS-PAGE for the separation of nNOS (modified) from CRTAase. The modified nNOS so obtained was devoid of contaminating proteins and was considered suitable for MS analysis. To confirm acetylation of nNOS and to locate the possible acetylation sites, the protein was in-gel digested and the resulting peptide mixture was subjected to nanoscale LC-MS/MS analysis. NCBI-nr database search by Mascot search engine identified NOS1 (neuronal NOS) of *Rattus* sp. with a Mascot score of 3,000 and successfully assigned 89 tryptic peptides, which covered 43% of the protein sequence entry contained in the database. Eleven distinct peptides with a significant score were indicated by Mascot as acetylated on lysine residues, which are listed in Table 1. MS/MS spectra of two of the acetylated peptides are shown in Figs. 2 and 3. The acetylated lysines of nNOS are highlighted in the amino acid sequence (Fig. 4).

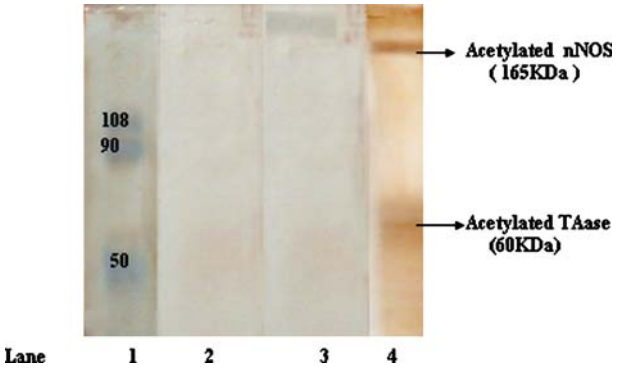

Fig. 1 CRTAase catalized acetylation of nNOS by DAMC. Western blot using polyclonal antibody against acetyl lysine. Lane 1, prestained molecular weight makers (kDA); lane 2 (control 1), purified nNOS was incubated with DAMC, lane 3 (control 2), same as for the lane 2 except for that DAMC was replaced by DMSO; lane 4, purified nNOS was incubated with CR TAase an d DAMC. All the samples separately preincubated at 37°C followed by SDS PAGE and transblotted on to PVDF membrane, which was treated with antiacetyl antibody and goat ant rabbit IgG conjugated with the HRP to detect the presence of acetylated nNOS using DA B as the substrate for color development. Details are given in "Materials and Methods"

Table 1 Acetylated peptides identified by nanoscale LC-MS/MS and Mascot database search.

Observed *m/z*	Observed mass	Calculated mass	Ion score	Peptide sequence
537.27	1,072.52	1,072.51	26	KSSGDGPDLR
693.91	1,385.81	1,385.80	44	KVGGLGFLVKER
703.35	1,404.68	1,404.67	37	SHKAPPLGGDNDR
710.84	1,419.68	1,419.67	33	AEMKDTGIQVDR
718.91	1,435.80	1,435.79	36	VKATILYATETGK
739.96	1,477.90	1,477.89	30	VSKPPVIISDLIR
950.51	1,899.01	1,898.98	28	IDHIYREETLQAKNK
1,055.55	2,109.09	2,109.07	60	KYVQDVLQEQLAESVYR
1,084.04	2,166.06	2,166.06	55	IMTQQGKLSEEDAGVFISR
1,113.58	2,225.15	2,225.17	46	LTYVAEAPDLTQGLSNVHKK
1,150.62	2,299.23	2,299.22	50	VTQPLGPPTKAVDLSHQPSASK

Eleven distinct peptides with a significant score were indicated by mascot as acetylated on a lysine residue. Eleven peptides fragments bearing 12 acetylated lysines with observed, calculated mass and ion score are listed in this table. Acetylated lysines and oxidized methionine are underlined.

Discussion

Protein acetylation is known to bring about alteration in several functions of the protein such as catalytic activity, flexibility, DNA binding, protein interaction and peptide receptor recognition. Several studies have documented the alterations in pattern of protein acetylation in aging and in several pathological conditions [17, 23]. Protein acetylation is catalyzed by a wide range of acetyl transferases that transfer acetyl groups from acetyl CoA to largely the ε-amino group of lysine residues located at different position. The enzymatic

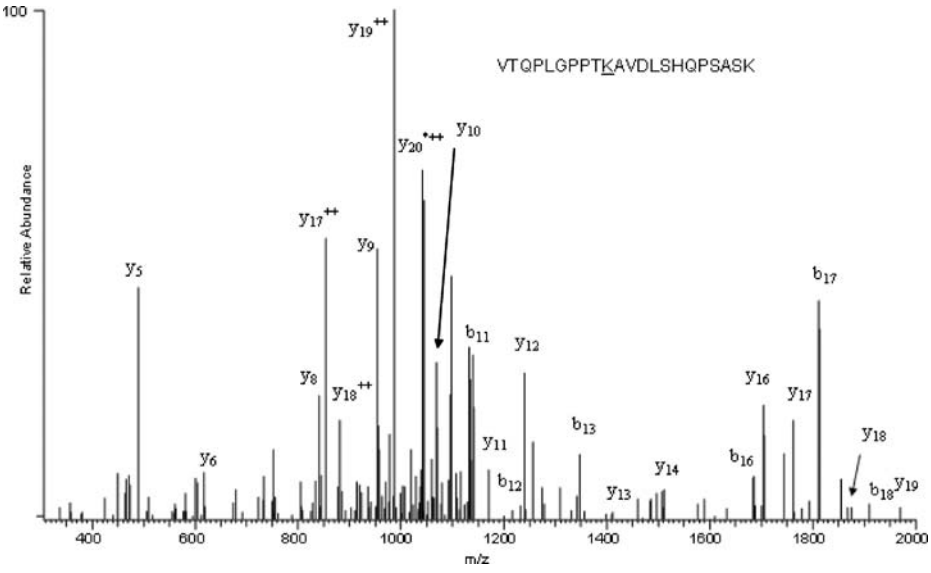

Fig. 2 MS/MS spectra of one of the acetylated peptides. Assignment of the site of acetylation was confirmed by ions y_{12} and y_{13}. nNOS (50 μM) was incubated with CR Aase (12 μM) and DAMC (100 μM), the modified protein separated on SDS PAGE was in gel digested and processed for nanoscale LC MS/MS as described under "Materials and Methods"

protein acetylation independent of acetyl CoA was established for the first time by our identification of a unique enzyme termed TAase in the ER of animal tissues [17] utilizing PA as the acetyl group donors [5, 18]. Our earlier work provided mass spectrometric evidence for the acetylation of GST 3–3 by DAMC, a model PA mediated by TAase purified from buffalo liver. Our earlier investigations strongly indicated the TAase catalyzed acetylation of NADPH cytochrome P-450 reductase by DAMC culminating in the remarkable hyperbolic activation of the reductase [17]. Several classes of PA were also found effective in causing the activation of the reductase activity as described above [15, 22]. These observations prompted us to examine whether NOS, which bears the domain of reductase could similarly be activated by DAMC leading to enhancement of intracellular levels of NO [4]. The extensive investigations carried out by us deciphered the identity of TAase with CR, a resident protein of the ER [9] and consequently the TAase is termed as CRTAase. An effort was made in the first instance to examine the CRTAase mediated acetylation of purified NOS by DAMC. For this purpose we have purified and characterized CRTAase from human placenta [20]. The purified placental CRTAase was incubated with DAMC and nNOS, the modified NOS avidly reacted with antiacetyl lysine antibody (Fig. 1), strongly suggesting the possibility of acetylation of lysines of NOS. Furthermore, the modified NOS was in gel digested and analyzed by mass spectrometry. The results highlighted 11 peptide residues bearing 12 acetylated lysines: Lys-24, -33, -38, -131, -229, -245, -754, -856, -989, -1300, -1321, and -1371. It is clear from these data that five lysine residues (Lys-24, -33, -38, -131, and -229) of the PDZ domain of nNOS were found to be acetylated whereas Lys-245 was the lone lysine of the oxygenase domain, which was acetylated. The other six lysines (Lys-754, -856, -989, -1300, -1321, and -1371) that were acetylated belonged to the reductase domain of nNOS. The distribution of lysines that were acetylated in the reductase domain of nNOS was in the order: FMN binding domain

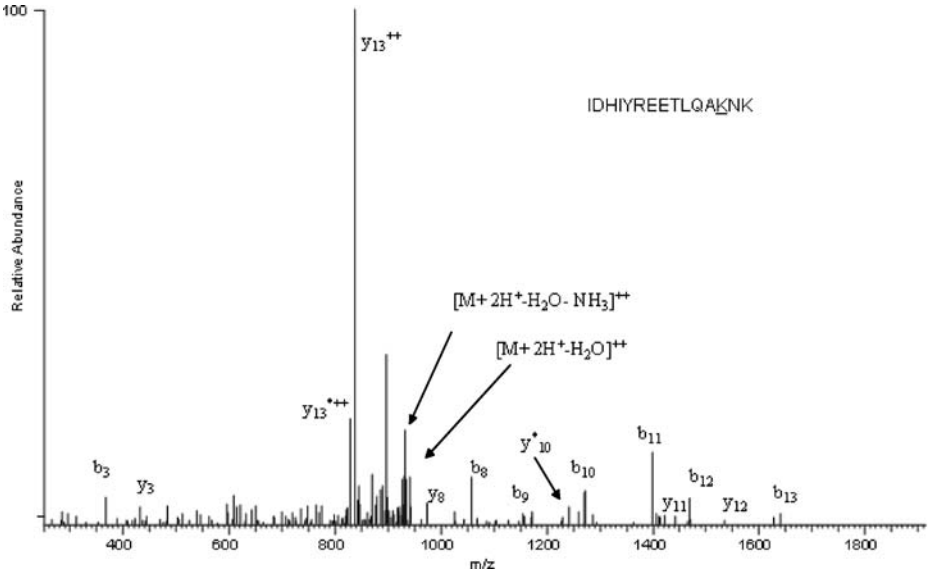

Fig. 3 MS/MS spectra of one of the acetylated peptydes. Assignment of the site of acetylation was confirmed by ions b_{12} and b_{13}. nNOS (50 μM) was incubated with TAase (12 μM) and DAMC (100 μM), the modified protein separate on SDS-PAGE was in gel digested and processed for nanoscale LC-MS/MS as described under "Materials and Methods"

(Lys-754 and -856); connecting domain (Lys-989) and NADPH binding domain (Lys-1300, -1321, and -1371). Furthermore, visual inspection of MS/MS data and assignment of ions y_{12} and y_{13} (Fig. 2) and b_{12} and b_{13} (Fig. 3) confirmed the site of acetylation on the indicated lysines in all cases (Fig. 4), except for peptide KYVQDVLQEQLAESVYR, for which the y-ion series was detected up to ion y_{15}, pointing the site of acetylation at either the peptides in N-terminal lysine or at the adjacent tyrosine. Although the posttranslational modification of NOS is documented, mass attention is focused on the role of phosphorylation, especially of Ser-1412 in the regulation of nNOS activity [25]. Practically nothing is reported on the role of lysines of NOS in the control of NOS activity. We have in this paper elaborated for the first time the enzymatic acetylation of NOS. The role of acetylation of lysines in the activation of NADPH cytochrome P-450 reductase has long been contemplated by Nadler and Strobel [11] who observed that the acetylation of specific lysine residues by acetic anhydride resulted in the significant activation of the reductase activity. NADPH cytochrome P-450 reductase was found to under go acetylation by DAMC mediated by CRTAase [14]. DAMC and other classes of PA caused profound activation of the catalytic activity of the reductase [15, 17, 22]. These observations strongly indicated the possible role of the acetylation of the reductase domain in the activation of NOS. In addition several reports [24]

1 meentfgvqq iqpnvisvrl fkrkvgglgf lvkervskpp viisdlirgg aaeqsgliqa
61 gdiilavndr plvdlsydsa levlrgiase thvvlilrgp egftthlett ftgdgtpkti
121 rvtqplgppt kavdlshqps askdqslavd rvtglgngpq haqghgqgag svsqangvai
181 dptmkstkan lqdigehdel lkeiepvlsi lnsgskatnr ggpakaemkd tgiqvdrdld
241 gkshkapplg gdndrvfndl wgkdnvpvvl nnpysekeqs ptsgkqsptk ngspsrcprf
301 lkvknwetdv vltdtlhlks tletgctehi cmgsimlpsq htrkpedvrt kdqlfplake
361 fldqyyssik rfgskahmdr leevnkeies tstyqlkdte liygakhawr nasrcvgriq
421 wsklqvfdar dcttahgmfn yicnhvkyat nkgnlrsait ifpqrtdgkh dfrvwnsqli
481 ryagykqpdg stlgdpanvq fteiciqqgw kaprgrfdvl plllqangnd pelfqippel
541 vlevpirhpk fdwfkdlglk wyglpavsnm lleigglefs acpfsgwymg teigvrdycd
601 nsrynileev akkmdldmrk tsslwkdqal veiniavlys fqsdkvtivd hhsatesfik
661 hmeneyrcrg gcpadwvwiv ppmsgsitpv fhqemlnyrl tpsfeyqpdp wnthvwkgtn
721 gtptkrraig fkklaeavkf saklmgqama krvkatilya tetgksqaya ktlceifkha
781 fdakamsmee ydivhlehea lvlvvtstfg ngdppengek fgcalmemrh pnsvqeerks
841 ykvrfnsvss ysdsrkssgd gpdlrdnfes tgplanvrfs vfglgsrayp hfcafghavd
901 tlleelgger ilkmregdel cgqeeafrtw akkvfkaacd vfcvgddvni ekannslisn
961 drswkrnkfr ltyvaeapdl tqglsnvhkk rvsaarllsr qnlqspkssr stifvrlhtn
1021 gnqelqyqpg dhlgvfpgnh edlvnalier ledappanhv vkvemleern talgvisnwk
1081 desrlppcti fqafkyyldi ttpptplqlq qfaslatnek ekqrllvlsk glqeyeewkw
1141 gknptmvevl eefpsiqmpa tllltqlsll qpryysisss pdmypdevhl tvaivsyhtr
1201 dgegpvhhgv csswlnriqa ddvvpcfvrg apsfhlprnp qvpcilvgpg tgiapfrsfw
1261 qqrqfdiqhk gmnpcpmvlv fgcrqskidh iyreetlqak nkgvfrelyt aysrepdrpk
1321 kyvqdvlqeq laesvyralk eqgghiyvcg dvtmaadvlk aiqrimtqqg klseedagvf
1381 isrlrddnry hedifgvtlr tyevtnrlrs esiafieesk kdadevfss

Fig. 4 Sequence coverage in nNOS. Twelve identified acetylated lysines (Lys-24, -33, -38, -131, -224, -245, -754, - 856, -989, -1300, -1321, and -1371 in PDZ, oxygenase and reductase domains of nNOS are highlighted

have provided evidence for the enhanced protein–protein interaction because of the acetylation of lysine residues of one of the interacting proteins. Patel et al. [13] have demonstrated the interaction of eNOS with CR resulting in the possible calmodulin mediated upregulation of eNOS in lung endothelial cells. The isoforms of NOS are known to share extensive amino acid sequence similarity reflecting their common catalytic function and cofactor requirements [8, 24] and hence, it is possible to conclude that nNOS could similarly interact with CR as highlighted in the present work. The acetylation of nNOS can be expected to potentiate the interaction with CR, eventually leading to the expression to the enhanced catalytic activity of NOS.

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