A nitric oxide synthase-like protein from *Synechococcus* produces NO/NO₃⁻ from L-arginine and NAPDH in a tetrahydrobiopterin- and Ca²⁺-dependent manner

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	nmol NO ₂ ⁻ + NO ₃ ⁻
syNOS	
+ Fe-MGD	1.0 ± 1.0
- Fe-MGD	35 ± 4
NOC - 7	78 ± 2

Table S1: $NO_2^- + NO_3^-$ production from syNOS measured by the Griess assay, in the presence or absence of the spin-trap Fe-MGD.

	Hemin & δ-ala	δ-ala
syNOS	1.00 ± 0.10	0.77 ± 0.10
C539A	0.72 ± 0.07	0.42 ± 0.02
H422A	0.39 ± 0.08	0.20 ± 0.03
H422A/C539A	0.10 ± 0.02	0.036 ± 0.009

Table S2: Heme incorporation (μ M heme per μ M protein) of syNOS constructs expressed in the presence of excess hemin and δ -ala, or just δ -ala.

syNOS	1	20 40 60 80 :MLVNDSRPTVEAHVLSVVRLVELCASGIPSNNEFKYKANVRVTCSGTEQSNTQLMTRLQPSWLVDIAHPSNCLFTVTLFYRQGGLG	100 QPWHEAGSIKVTTA
eNOS	_	:	
iNOS	-	:	
otNOS bsNOS	_		
syNOS	101	120 140 160 180 :DLFDKQRSVEISRPVATWPAAPELMLNARFTCSDHTSQSGEAVSLSLAGTRANASRRPTSLALVSDDSIELPEAIPLTYSEAVIVK	200 DVWNKLRAWKELOM
eNOS	-	:	TASETHVVLTLRGP
iNOS	-	:	
otnos bsNOS	_	;	
syNOS eNOS nNOS iNOS otNOS	201 1 101 1 -	220 240 260 280 :ETFFKRLLLEVPELDYIFGEAFESIPDYFFEMFDCCVRELCPHTENVVWEPMMGVPPEKGDAFDTVADYGALFADIGMQPQHWLRA :	300 RQVWMWMLPQIPYL TKANLQDIGEHDEL
DSNOS			
		Globin Domain	
syNOS eNOS nNOS iNOS otNOS bsNOS	301 30 201 37 -	320 340 360 380 :EEYDREDLAKGNKSALCKFFNTHVIGGNVAARDRYDSALPPALVQKMADSWQYFAPRKNEMGVEFYQTLFERYPQVLPIFGRADMD :	400 YLSTHLFQSLEFIF NPYSE SPQPL
		N-Terminal Hook	
syNOS eNOS nNOS iNOS otNOS bsNOS	401 51 277 61 1	420 440 460 480 :LCLAEGSTERLMKELHLGRLHGNAGVPSFAYGAISEVM SMFEKYVPGFDEQLKEAWOVLIARVSNVIKLPKLNEER IKKPREY :-EHSPPSSPITOPPEGPKFPRVKNWEVGSITYDT SACAQCCCPCTPRCLGSIVFPRKLOGRPSPCPPAPEOILSORED :KEQSPTSGKQSPTKNGSPSRCPRFLKVKNWETDVVIDT HLKSTLETGCTEHICMGSIMLPSOHTKRE-DVRTKDC :VETGKKSPESLVKLDATPLSSPRHVRIKNWGSGMTFQDT HHKAKGILTCRSKSCLGSIMTPKSLTRCPRDKPTPPDEILPQIPE :MASVGSGATDDDGVDVPVSRCPFAHGTVT DPYPGYVHGKNPRVCP	500 DVIAN-EQAWEES INGYYSSIKRSGSO DGY SSIKRFGSK VNGY GSFKEAKIE YIRI-GKEHGWDDE AECYQELGKEE
syNOS eNOS nNOS iNOS otNOS bsNOS	500 145 376 161 86 27	520 540 560 580 : DRERRWOEIKAEVQATCTYTHTYEEIAYGAUAWRNTSKCIGRIONSMYWRIRRHVTDPDEMEOEIEEHIRLGINGONUOIVMTV : AHEORIOEVAATGTYOLRESEIVFAATGAWRNAFRCVGRIOWGRIOFIARDCRAOEMITYICHHIKVATRKONIRSATTV : AHHORIEEVNREIESTSTOLKDIEIIYGAKHAWRNASRCVGRIOWGRIOHFARDCRAOEMITYICHHIKVATRKONIRSATTV : EHLARVEAVTREIETTGTYOLTGELIFATGAWRNAFRCIGRIOWGRIOWFARDCRIARGUNHUCHHKYATKKONIRSATTV : EVLRIVEVITSIETTGTYOLTGELIFATGAWRNAFRCIGRIOWGRIOWFARDCRIARGUNHUCHHKYATKKONIGAIT : EVLRIVEVITSIETTGTYOLTGELIFATGAWRNAFRCIGRIGNEWSTILUIDARGATINEDMISEAWIEHICHWRYSTNONIRSATTV : EVLRIVEVITSIETTGTYOLTGELIFATGAWRNAFRCIGRIGNEWSTILUIDARGATINEDMISEAWIEHICHWRYSTNONIRSATTV : EVLRIVEVITSIETTGTYOLTGELIFATGAWRNAFRCIGRIGNEWSTILUIDARGATINEDMIEAWIEHICHWRYSTNONIRSATTV : EVLRIVEVITSIETGTYONYTKEELEHGARWAWRNSNRCIGRIFMNSINNIERDVRIKELWRDAFFHHIETATNNEREPSITI : EVLRIVISINGTYONYTKEELEHGARWAWRNSNRCIGRIFMNSINNIERDVRIKELWRDAFFHHIETATNNEREPSITI : EVLRIVISINGTYSTY	600 BRPKLPKERWGPRI PORC-PGRGDFRI PORS-DGKHDFRV BPORS-DGKHDFRV BROTPGTNDGFRI BPPEE-KGEKQVEI
		Oxygenase Domain	
syNOS eNOS nNOS iNOS otNOS bsNOS	600 244 475 260 186 126	620 640 660 680 WN FOLIRYAA HEMP CSINGDAAN TELHHQITEKMCWOPPERSPYD IDVIS VPRHE-ERINSFAPEEILEVEIEHTIPD KT WN SOLIRYAA HEMP CSINGDAN VEIHELCICHG-WTEONGRERVIPLICAPEEP-ERISII PELVLEVEHEHTIPD KT WN SOLIRYAA HKOP CSINGDAN VEIHELCICHG-WTEONGRERVIPLICAPEP-ERISII PELVLEVEHEHTIEW AA WN SOLIRYAA HKOP CSINGDAN VEIHELCICHG-WKAPRGRERVIPLICANGND-FELSCIPELVLEVEHEHTIEW AA WN SOLIRYAA HKORGSIRGDAN VEIHELCICHG-WKAPRGRERVIPLICANGND-FELSCIPELVLEVEHEHTIEW AA WN SOLIRYAA HKORGSIRGDAN VEIHELCICHG-WKRYGR-ERVVOID ANGRD-ERIS TI DOCSEVEHEHTIRTAGISO HN SOLIRYAA HKORGSIRGDAN VEITURA HKAPRA WN HOLIRYAA HKORGSIRGDAN SESIMAACECIG-WRGERTDERIPLICHEFTEAN STI DOCSEVEHEHTIRGISO WN HOLIRYAA ERIGDA SRSINAACECIG-WRGERTDERIPLICHEFTEAN STI DOCSEVEHENTHEDIEA SD	700 Iclewyavpaisk Iclewyalpavsnm Iclewyclpavsnm Islewyalpav <mark>a</mark> nm Iclewyclpavsni Iclewycvp <mark>i</mark> isdm
syNOS eNOS nNOS iNOS otNOS bsNOS	699 340 571 356 285 222	740 760 780 REFIGEV TACLED SKARVEN VET AR - FLEGGRIGKNER KARLINLIGUN SSECTLER FRALEM TAVIHSSOKARVEN VEHOSAG : LEIGGEFFRADEGRYN STEIGTRILCIDEN YN THELWYN YN DLORTTSSLWKIK RAVEIN VAN HSSOKARVEN VEHOSAG : LEIGGEFFRADEGRYN STEIGTRILCIDEN YN THELWYN YN DLORTTSSLWKIC AV EIN YAN HSSOKARVEN VEHON : LEIGGEFFRADEGRYN GTEIGVR YC NSRYN THEEVARM OLDMRKTSSLWKIC AV EIN TAN HSSOKARVEN VEHON : LEIGGEFFRADEGRYN GTEIGVR YC NSRYN THEEVARM OLDMRKTSSLWKIC AV EIN TAN HSSOKAVTIN DH SATE : LEIGGEFFRADESGWYN GTEIGVR YC VCNYN THEEVARM OLDMRKTSSLWKIC AV EIN TAN HSSOKAVTIN DH SATE : LEIGGEFFRADESGWYN GTEIGVR YC VCNYN THEEVARM OLDMRKTSSLWKIC AV EIN TAN HSSOKAVTIN DH SATE : LEIGGEFFRADESGWYN GTEIGVR YC VCNYN THEEVARM OLDMRKTSSLWKIC AV EIN TAN HSSOKAVYN THEHOL : KEVRGGIFYNAAPSN WYN GTEIGARN LAI ERYY RYN YN YN FLAR WCNYN YN	800 SEAHDIRE KAGR SEMKHIENE CARG SEIKHMENE YE CRG SEMKYMQNE YE SRG AFADWYYELI TRG CEKRFEECEEEAGR
		Calmodulin Binding	
syNOS eNOS nNOS iNOS otNOS bsNOS	798 440 671 456 385 322	820 840 860 880 :cC:ADMGNVVPEAGESACWWHO:RD:YTEEANHHAADR.AVEADIDLEQFVQTTFKEVANAVKISASLMGTVMAKVK HEEANHHAADR.AVEADIDLEQFVQTTFKEVANAVKISASLMGTVMAKVK :CC:ADMANIVPEISGSIFFY OB:UNYFEISAERVOPDE.KGSAAKGTGITKKKTFKEVANAVKISASLMGTVMAKVK :CC:ADMANIVPEMSGSIFFY OB:UNYFEISEVQPDE.MTHVWKGTNGTFTKRRAIGFKKLAEAVKFSAKLMGQAMARVK :CC:ADMINUPEMSGSIFFY OB:UNYFEISEVQPDE.MTHVWKGTNGTFTKRRAIGFKKLAEAVKFSAKLMGQAMARVK :CC:ADMINUPEMSGSIFFY OB:UNYFEISEVQPDE.MTHVWKGTNGTFTKRRAIGFKKLAEAVKFSAKLMGQAMARVK :CC:ADMINUPEMSGSIFFY OB:UNYFEISEVQPDE.MTHVWKGTNGTFTKRRAIGFKKLAEAVKFSAKLMGQAMARVK :CC:ADMINUPEMSGSIFFY OB:UNYFEISEVQPDE.MTHVWCDEKRFKRR-EIPLKVUVKAVLFACMLMRKTMASRVE :CC:ADMINUPEMSGSIFFY OB:UNYFEISEVQPDE.MTHVWCDEKRFKRR-EIPLKVUVKAVLFACMLMRKTMASRVE :CC:ADMINUPEMSGSIFFTSIFFT :CC:ADMINUPEMSGSIFFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :GC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :GC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :GC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :GC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFTSIFFT :CC:ADMINUPEMSGIFT :GC:ADMINUPEMSGIFT :CC:ADMINUPEMSGIFT :CC:ADMINUPEMSGIFT :GC:ADMINUPEMSGIFT :CC:ADMINUPEMSGIFT :CC:ADMINUPEMSGIFT :GC:ADMINUPEMSGIFT :CC:ADMINUPEMSGIFT :CC:ADMINUPMINUPEMSGIFT :GC:ADMINUPEMSG	900 ILLEGSETGTALG ATILYGSETGRAS ATILYATETGKSA VTLEATETGKSA GIVLYASDGCRSS

FMN Domain	FM	IN.	Domain
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		FMN Domain
syNOS eNOS nNOS iNOS otNOS bsNOS	877 534 769 553 482 -	920 940 960 980 1000 :FrRAARQLS-AYHPKVMALDDYNVNT DEEKLUVVTSTFCNCEVEGNAQCFTQWLKQQPSDTLNG
syNOS eNOS nNOS iNOS otNOS bsNOS	943 630 862 618 554 -	1020 1040 1060 1060 1080 1100 :
syNOS eNOS nNOS iNOS otNOS bsNOS	1011 728 962 702 636 -	1120 1140 1160 1180 1200 TT PTTSKIK TYLADSESHALINLEAHSHSRVPVITNOELIKAVTPGSRSTRIJ FDTAKTELAVET DHVSVH HNPEDVJRVCRISLSPDTAFSA RSWKRORYRI SAQAEGLQLIPGLIHVHRKMFQAT RSVENLOSSKSTRATILVRI DTGGOEGLO OP CHIG VO PNRPGLVEALLSRVEDPPAPTEPV RSWKRNKFRI TYVAEAPDLTGCLSNVHKKKVSAAR LSRQNLOSPFSRSTIFVRI HTNGNGELO OP CHIG VF CNHEDLVNALIFRIEDAPPANHVV VTWDPHHYRI VQDSOPLDLSKALSSMHAKNVFTMRI KSRQNLOSPTSSRATILVEI SCEDCGGLN 1PC EHIG VC CNQPALVQCILERVVDGPTPHCTV GVALEPAFT KVRRQDVNGRIHLGPTVQHGTAAELIDRAITGNGARMNTVWLKFRI HAREPVQYLKPCHVAVWEOTSEARARYFAAHFGLTFRDVLEL
syNOS eNOS nNOS iNOS otNOS bsNOS	1111 828 1062 802 736 -	1220 1240 1260 1260 1280 1300 :KYVLPDGRQLEDEPPIAVPTTVGGALTEDLDIAFKEPFGEILNVLHQ-AENTEEKIRLETWLEILALEDGHEENAALRKMLRDNFMSVADLFD :AVEQLE-KGSPGGPPFGWVRDPRLPPCTTRGALTFFLDITSPSPCITRLESTLEEPRECGELEALSQDPRVEEWKWFRCCTLLEVLE :KVEMLEERNTALGVISNWKDESKLPPCTTFOOFKYYLDITTPFPTLCLQCGASLFTMEEKCRCLUVLSKGLQEVEEWKWFKCKPTMVEVLE :RLEDLDESGSYWVSDKRLPPCSISGALTYSFDITTPPTQLLICKLACV-TEEPERCRLEALSQ-PSEYSKWKFTNSPTFLEVLE :VVFKSDALLVKKSIDPAIPNVVSVEHLFTRVLDINGEASAALISALAHYTFNEDARQDWAEMTGARIFSIFEHFFTLSTLHRGD
		FAD/NADPH Domain
syNOS eNOS nNOS iNOS otNOS bsNOS	1204 917 1152 885 819 -	1320 1340 1360 1360 1400 :EFPSACTILEMLIEVIPREKER LYSISSCPOLOPGKIOTTVGVLQIOTDAGKTROCICSNYLAGISEGDL RIETHTS-DERE ND SALUMVOPGT :QFPSVAIPAPLLITOIPLLOPRYSVSSAPSTHGE HLTVAVLAYRTODGLGPLHYGVCSTWISGIKPGDPUPCFIRGAPSERIEPD SLIC :EFPSIGN PATILITOISLLOPRYSISSSPDMYPDEVHLTVAIVSYHTRDGEGPVHHCVCSSWINRIQADDVVPCFVRGAPSEHLERN QVFCILVGPGT :EFPSIGN SAGFLISGIPILKER FYSISSSRDHTTETHLTVAVVTYHTGDGGCHLHEVCSTWINSIKFODPUPCFVRAASAEHLED SHICTLIGPGT :KAVGLDURDIILK-IPKIRPRYSVSSSPCHAGNNYFALTVGRVTYKSGAGARMHLGFCSDF1ATUPIGAN, TVEFRPAPSERIERS QASI :
syNCS eNOS nNOS iNOS otNOS bsNOS	1301 1017 1252 985 918 -	1420 1440 1460 1460 1500 IGIS FLIASIL HR-EYLNSGGIPLGKATIYTGCSN-HD FLYEDCLRVWLEGGTITDLGVAFSR-LTAG VYVONLYODN-ARSIWQQISHSQG YYVGD IGIAFFRG-W CRLHDIESKGLQPTPMTIVFGCRCSQL HLYRDSVQNAQQRCVFGRVLTAFSREPDNP, TYVQDIIRTELAAEVHRVICLERGHMFVCGD IGIAFFRS-W CRCFDIQHKGMNFCPMVIVFGCRCSKI HIYRDSTLQAKNKCVFRELITAYSREPDRF, KYVQDVIQCLAESVYKAIKEQGE IYVCGD IGIVFRS-W CRLHDSCHKGVRGGRMTIVFCCRRPDE HIYRDSMLEMACKCVLHAVHTAYSRLPGKP, VYVQDIIRQQLAESVKAIKEQGE IYVCGD IGIAFFRG-VEHFATLNPGERGEAWIIACCS-OD QLYATSFNEAVHICHLTKYLVGFSROPGVFFTYVDTVIREHADDLVDISRGAEVYVCGD ISI
syNOS eNOS nNOS iNOS otNOS bsNOS	1397 1117 1352 1085 1012 -	1520 1540 1560 1580 :AK ADN FEVENCIARTEGGITHEAVDEFNR KERKESTDVØGVI NEKORKOVEKDNYARAEKWLANL

Figure S1: Protein sequence alignment of syNOS to other NOS proteins. The alignment was performed using ClustalX2 and visualized with GeneDoc; proximal heme binding residues (a), pterin binding residues (β), arginine coordinating glutamate (γ), Homo sapien endothelial NOS (eNOS), Rattus norvegicus neuronal NOS (nNOS), Homo sapien inducible NOS (iNOS), Ostreococcus tauri NOS (otNOS), Bacillus subtilis (bsNOS).



Figure S2: Protein sequence alignment of $syNOS_g$ to other globins and flavohemoglobins of known structures (*Saccharomyces cerevisiae* 4G1V, *Vitreoscilla stercoraria* 1VHB, *Escherichia coli* 1GVH, *Methylokorus infernorum* 3UBC). The alignment was performed using ClustalX2 and visualized with GeneDoc; proximal heme binding residue (α).



Figure S3: Michaelis-Menten plots for syNOS activity as a function of L-arg concentration (A) and calcium concentration (B). K_M values for arginine and calcium were calculated to be $101 \pm 12 \mu$ M and $228 \pm 9 \mu$ M, respectively.



Figure S4: UV-vis spectra of the heme double mutant H422A/C539A compared to the syNOS reductase domain reveals minimal heme is bound when the proximal heme ligands are mutated.