

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: NO₂⁻ + NO₃⁻ 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 (μM heme per μM protein) of syNOS constructs expressed in the presence of excess hemin and δ -ala, or just δ -ala.

20 40 60 80 100
 syNOS 1 :MLVNDSRFTVEAHVLSVVRLVELCASGIPSNNEFKYKANVRVTCGTEQSNLQMLTRLQPSWLVLDIAHPSNCLFTVTFLFYRQGLGQFWHEAGSIKVTTA
 eNOS - :-----
 nNOS - :-----
 iNOS - :-----
 otNOS - :-----
 bsNOS - :-----

120 140 160 180 200
 syNOS 101 :DLFDKQRSVEISRFPVATWFAAPELMLNARFTCSDHTSQSGEAVLSLAGTRANASRRPTSLALVSDSIELPEAIPLTYSEAVIVKDVWNKLRWAKELQM
 eNOS - :-----
 nNOS 1 :MEENTFGVQCIQPNVISVRLFKRVGGLFVKVERVKPPVIIISDLIRGGAAEQSGLIQAGDIIILAVNDRFLVDLSYDALEVLRLGIASETHVVLILIRGP
 iNOS - :-----
 otNOS - :-----
 bsNOS - :-----

220 240 260 280 300
 syNOS 201 :ETFFKRLLEVPEDLYIFGEAFESIPDYFFEMFDCCVRELCPTENVVPEPMMGVPPERKGFADFTVADYGALFADIGMQPQHWRARQVMMWMLPQIPYL
 eNOS 1 :-----MGNLKSVAQEP-----GPPCGGLGLGLGLGCGKQ-----
 nNOS 101 :EGFTTHLETTFTGDTGPKTIRVTQPLGPPTKAVDLHSHQPSASKDQSLAVDRVTGLGNGPCHAGHGQAGSVSQANGVAIDPTMKSTRANLQDIGEHDDEL
 iNOS 1 :-----MACPWKFLFKTKFHQYAMNG-----EKDINNVEKAPCATS-----
 otNOS - :-----
 bsNOS - :-----

Globin Domain

320 340 360 380 400
 syNOS 301 :EEYDREDLAKGNKSALCKFFNTHVIGGMVAARDRYDLSALPPALVQKMDASWQYFAPRKNEMGVVEFYQTLFERYPQVLPVIFGRADMVLYSTHLFQSLFPIF
 eNOS 30 :-----GPATPAPEPS-----RAPASLL-----PPAP-
 nNOS 201 :LKEIEPVLISILNSGSKATNRGGPAKAEKMDTGIQVDRDLGKSHKAPPLGGDNDRVFNLDLWGDNDVPVILN-----NPFYSE
 iNOS 37 :-----SPVTQDDLQYHN-----LSKQNE-----SFQPL
 otNOS - :-----
 bsNOS - :-----

N-Terminal Hook

420 440 460 480 500
 syNOS 401 :LCLAEGETERLMKELRHLGRLHGNAGVPSFAYGAISEVMISMFKEYVPGFDEQLKEAWCVLIARVSNVIKPKLNEERLIKRERYTIVIAN-EQAWSES
 eNOS 51 :--EHSPPSSPLTQPPG----PKFPRVKNWEVGSITYDTLSAQACQDGPCTPRRCGLSLVFPKLCGRSPGPPAPEQLISCARDFNQYSSIKRSGSQ
 nNOS 277 :LKEQSPKSGKSPFKNGSPSRCPFLKVKNNWETDVVLTDDYHLKSTLETGCTEHCIMGSIPLPSQHTRKE-DVTRTKDQFPLAKEPFDQYSSIKRFGSK
 iNOS 61 :VETGKKSPELVKLDATPLSSPRHVIRKNWGSMTFQDILHKKAGILTCRSKSLGSIPTPKSLTRGPRDKPTPPDELPOPIEFVFNQYSGSFKEAKIE
 otNOS 1 :-----MASVGSATDDDGVDVPSRCPFAHGTVAIDPYPGYVHGKPRVCP-----RGCVPFPSPKPTESAESALRREAEEYIRLQKHEGWDDDE
 bsNOS 1 :-----GSHMETIWNREKAFIAECQELGK--EE

α

β

520 540 560 580 600
 syNOS 500 :DRERWQELKAEVQATTTTHTYBELAYGQCLAWRNWSPFCIRIOWSNVYVFRFHVTDDEDMQELPEHILRLGTNGCNIQIVMTVFRPKPKERWGP
 eNOS 145 :AHECRLOEVEAEVAATSTYQLRESELVGGARCAWRNAPRCVGRICWGRLOVEFARDCRCAQEMTYICNHHKYATKGNLRSATITVFPQRC-PGRGDFRI
 nNOS 376 :AHMRLDEEVNKEEESASTYQLKDDLELYGARHAWRNARCVGRICWGRLOVEFARDCTAHGCMNYICNHHKYATKGNLRSATITVFPQRC-PGRGDFRI
 iNOS 161 :EHLARVEAVTKETHTTGTQLTGELEIATKCAWRNAPRCIRIOWSNLOVEFARSCSAREMEHEICRHVRYSTINGNIRSATITVFPQRS-DGKHDFRV
 otNOS 86 :RVEMRVNEVLTSRETTGTAHTLDEIRGARVAWRNAPKCNKRYWSTLVIDARGATNEDMEAKKEHLHRGVEADVPLVMTVFRPOTPGTNDGPI
 bsNOS 27 :EVKRLDLSIKSEIDRTGSVHTKBELEHGARMWRNSNRCIRIOWSNVYVFRFHVTKEDVRDAEFHETATNGNIRSATITVFPQRC-PGRGDFRI

Oxygenase Domain

620 640 660 680 700
 syNOS 600 :WNEQLIRYAAWEMFDGSIINGDPAANETTHQIIEKMWQEPPEPSSYDILPLVLEVPVPRHE-FRLYSFAPEEILEVEHEHETIPDKTLELRWYVPAISNF
 eNOS 244 :WNSQLVRYAGYRQDQSGVGRDEANVEIHELICIQHG-WTEGNCR--FDLPLLLCAPDEP-PELELLEPELVLEVEHEHETLEWAMGLRWYALPAVSNM
 nNOS 475 :WNSQLIRYACYKQDQSGVGRDEANVEIHELICIQHG-WKAPGR--FDLPLLLCANGND-PELELLEPELVLEVEHEHETLEWAMGLRWYALPAVSNM
 iNOS 260 :WNAQLIRYACYQMDQSGVGRDEANVEIHELICIDLG-WKPKYGR--FDVPLVLCANGRD-PELELLEPELVLEVEHEHETLEWAMGLRWYALPAVSNM
 otNOS 186 :WNSQLMRYAHRDASGGVVDPAEIDFDVLLKHFQWAEKTKG-MFDLPLVVCINPTEPAMFLEDCFLVEHEHETIRGISQLKWKYCIAPVSNM
 bsNOS 126 :WNEQLIRYAGYE-SGDERIGDPAASRLTAACEQLG-WRGETRD--FDLPLDFRMRGDECVWVLELRSVLEVEHEHETLEWAMGLRWYALPAVSNM

720 740 760 780 800
 syNOS 699 :RNIIGGVVYACLPFNQWYMGTEIAR-DELEGGRYGMKAIENLGLNLSSEQLWRDRVALEMNNAVHSHFQKARVTMDDHOSACCELAHDLRFKFRAGR
 eNOS 340 :LEIIGGLEFFPAAPPSGWYMGTEIAR-DELEGGRYGMKAIENLGLNLSSEQLWRDRVALEMNNAVHSHFQKARVTMDDHOSACCELAHDLRFKFRAGR
 nNOS 571 :LEIIGGLEFFSACPPSGWYMGTEIAR-DELEGGRYGMKAIENLGLNLSSEQLWRDRVALEMNNAVHSHFQKARVTMDDHOSACCELAHDLRFKFRAGR
 iNOS 356 :LEIIGGLEFFPGPPNPGWYMGTEIAR-DELEGGRYGMKAIENLGLNLSSEQLWRDRVALEMNNAVHSHFQKARVTMDDHOSACCELAHDLRFKFRAGR
 otNOS 285 :INDLGGLEHTAAPPNPGWYMGTEIAR-DELEGGRYGMKAIENLGLNLSSEQLWRDRVALEMNNAVHSHFQKARVTMDDHOSACCELAHDLRFKFRAGR
 bsNOS 222 :RNEVGGLEHTAAPPNPGWYMGTEIAR-DELEGGRYGMKAIENLGLNLSSEQLWRDRVALEMNNAVHSHFQKARVTMDDHOSACCELAHDLRFKFRAGR

γ

β

Calmodulin Binding

820 840 860 880 900
 syNOS 798 :ECPADYGVVVPAGGACFVWE--HQRDEYLQFAHHAADRWAVEADIDLEQVQTT-----HESDHQRDRILIDGSETCTAGG
 eNOS 440 :GCPADWVIVVPPISGILTEVFE--CEVNNVFLSPARYPDPKGSAAKGTGITRKKT----FKEVANAVKISASLMGTVMMAKRVKATILYGETCRAGS
 nNOS 671 :GCPADWVIVVPPISGILTEVFE--CEVNNVFLSPARYPDPKGSAAKGTGITRKKT----FKEVANAVKISASLMGTVMMAKRVKATILYGETCRAGS
 iNOS 456 :GCPADWVIVVPPISGILTEVFE--CEVNNVFLSPARYPDPKGSAAKGTGITRKKT----FKEVANAVKISASLMGTVMMAKRVKATILYGETCRAGS
 otNOS 385 :YALGNWVIVVPPISGILTEVFE--CEVNNVFLSPARYPDPKGSAAKGTGITRKKT----FKEVANAVKISASLMGTVMMAKRVKATILYGETCRAGS
 bsNOS 322 :KLTCDWVIVVPPISGILTEVFE--RSDYLSIKRNFYQDKFEE-----AFSTVSIRVAIAAAKFRNKLVKGGVLYLVAADGCRSS

ββ

β

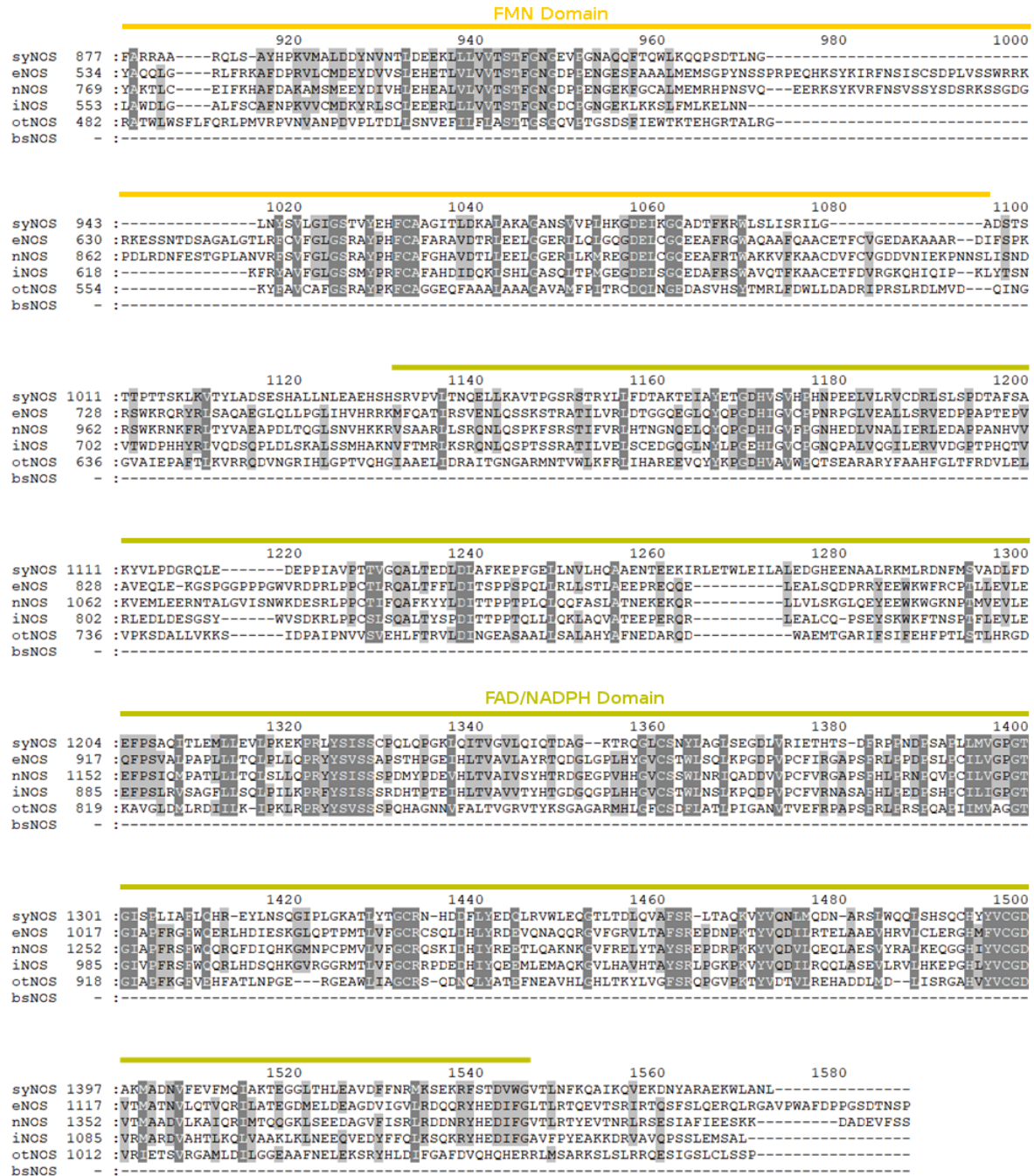


Figure S1: Protein sequence alignment of syNOS to other NOS proteins. The alignment was performed using ClustalX2 and visualized with GeneDoc; proximal heme binding residues (α), pterin binding residues (β), arginine coordinating glutamate (γ), *Homo sapien* endothelial NOS (eNOS), *Rattus norvegicus*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

		340		360		380		400
syNOS	325	:IGGMVAARDRYDSATPPALVQKMAISWQYFAPRKNE	NGVEFYQTL	ERYE	CVL	IFGRADMDYLS	THLFQSLEFI	FLCLAE
S._cerevis	1	:-----MIAEKTRSLIKATVPVIEQOGTV	TRTFYKNNL	TEHTE	LLNFNRTNQKVGAC	PNA	ATTVLA	AAKNI
V._stercor	1	:-----MLDQQTINIKATVPVIEKEHGV	TTTTFYKNNL	AKH	PEVRELEDMGRQESLE	CPKA	AMTVL	AAAQNI
E._coli	1	:-----MLDAQTIAIVKATIPLEIVETGPR	TAHFYDRM	ETHN	PEIKELFNMSNQRNGD	CREA	FNA	AAAYASNI
M._inferno	1	:-----IDQKEKELIRBSWKRTEPNKNE	GLLFYANL	KEE	TVSVLFQN	---	PISS	SRKMQVIGILVGGI

		420		440		460		480	
syNOS	406	:GSTERL	NKEERH	GRIGHNAGVPSFAYGATSEVM	ISMFEKYV	-PGFDE	QIKBAW	CVLIARVSNVIKLPKLN	NEERLLKKARE
S._cerevis	69	:DDISV	LDHVKCHGHK	HRALCKKPEHYPIVGEV	LLKATKEVI	GDAATPE	IINAWGEA	IQ	-----
V._stercor	69	:ENIPAL	IPAWKKA	VKHCQAGVAAAHYPIVGE	ELLGATKEVI	GDAATD	LLD	AWGKANG	-----
E._coli	69	:ENIPAL	IPAWKKA	VKHCQAGVAAAHYPIVGE	ELLGATD	MFSPGQ	-EYLL	AWGKANG	-----
M._inferno	65	:DNEGL	IPPTQD	GRNPKQYGVVDSHYPLV	EDLLKSHQ	EYLGQGF	TEAKP	AWTKV	IG

α

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).

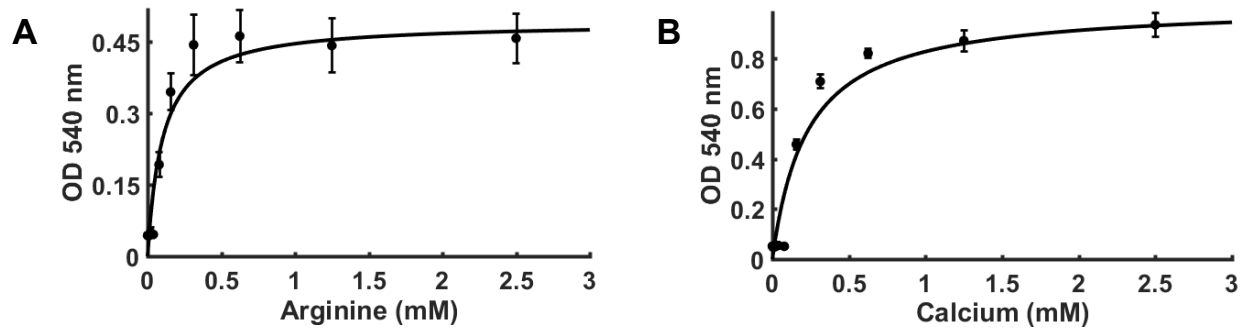


Figure S3: Michaelis-Menten plots for syNOS activity as a function of L-arg concentration (A) and calcium concentration (B). K_M values for arginine and calcium were calculated to be $101 \pm 12 \mu\text{M}$ and $228 \pm 9 \mu\text{M}$, respectively.

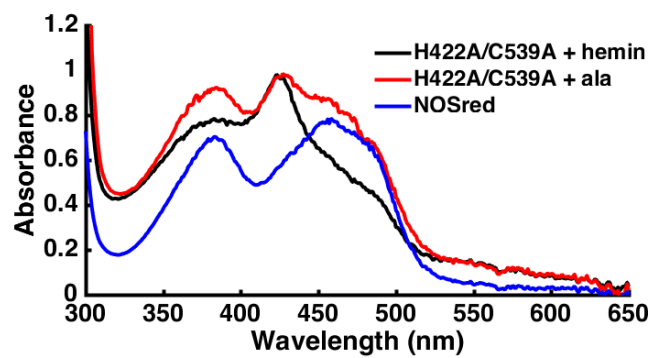


Figure S4: UV-vis spectra of the heme double mutant H422A/C539A compared to the syNOS reductase domain reveals minimal heme is bound when the proximal heme ligands are mutated.