REVIEW ARTICLE

N-Acetylglutamate and its changing role through evolution

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N-Acetylglutamate (NAG) fulfils distinct biological roles in lower and higher organisms. In prokaryotes, lower eukaryotes and plants it is the first intermediate in the biosynthesis of arginine, whereas in ureotelic (excreting nitrogen mostly in the form of urea) vertebrates, it is an essential allosteric cofactor for carbamyl phosphate synthetase I (CPSI), the first enzyme of the urea cycle. The pathway that leads from glutamate to arginine in lower organisms employs eight steps, starting with the acetylation of glutamate to form NAG. In these species, NAG can be produced by two enzymic reactions: one catalysed by NAG synthase (NAGS) and the other by ornithine acetyltransferase (OAT). In ureotelic species, NAG is produced exclusively by NAGS. In lower organisms, NAGS is feedback-inhibited by L-arginine, whereas mammalian NAGS activity is significantly

enhanced by this amino acid. The NAGS genes of bacteria, fungi and mammals are more diverse than other arginine-biosynthesis and urea-cycle genes. The evolutionary relationship between the distinctly different roles of NAG and its metabolism in lower and higher organisms remains to be determined. In humans, inherited NAGS deficiency is an autosomal recessive disorder causing hyperammonaemia and a phenotype similar to CPSI deficiency. Several mutations have been recently identified in the NAGS genes of families affected with this disorder.

Key words: arginine biosynthesis, urea cycle, carbamylphosphate synthetase I, hyperammonaemia, *N*-acetylglutamate synthase, ornithine acetyltransferase.

HISTORICAL OVERVIEW

In the early 1950s, *N*-acetylglutamate (NAG) was identified as an intermediate in the arginine-biosynthesis pathway of *Escherichia coli* [1,2], and the enzymic activity that acetylates L-glutamate using acetyl-CoA (AcCoA) was found in this organism [3]. At that time it was already known that ornithine was an intermediate of arginine biosynthesis [4] (Scheme 1A). Biosynthesis of ornithine from glutamate proceeds through several *N*-acetylated intermediates [1], and the presumed role for *N*-acetylation is to prevent glutamate from being used in proline biosynthesis. It was established subsequently that, in most prokaryotes, NAG is produced by transacetylation of glutamate, using acetylornithine rather than AcCoA, thus recycling the acetyl group in a catalytic cycle of ornithine production (Scheme 1B) [5,6].

During the same period, Grisolia and Cohen [7] discovered that a derivative of L-glutamic acid is required for the biosynthesis of L-citrulline in preparations of rat liver. They tested various synthetic candidates and found that that *N*-acetyl, *N*-chloroacetyl, *N*-carbamyl, *N*-propionyl and *N*-formyl derivatives of glutamate were active in producing citrulline, and that acetyl- and carbamyl-glutamate activate the biosynthesis of carbamyl phosphate [7]. The natural compound was isolated from bovine liver and from yeast extract and identified as NAG [8]. The same group subsequently determined that NAG is an essential allosteric cofactor of mitochondrial carbamylphosphate synthetase I (CPSI), the first enzyme of the urea cycle in ureotelic (excreting nitrogen mostly in the form of urea) animals [9,10] (Scheme 2).

Thus NAG fulfils two distinctly different functions in living organisms, and its evolutionary path appears to be connected to

the evolution of the urea cycle from the arginine-biosynthesis pathway.

NAG IN PROKARYOTES

NAG synthesis

In prokaryotes, NAG can be produced by two different enzymes: NAG synthase (NAGS; EC 2.3.1.1), encoded by the *argA* gene, and ornithine acetyltransferase (OAT; EC 2.3.1.35), encoded by the *argJ* gene [11]. The majority, but not all, prokaryotes have a 'cyclic' ornithine-production pathway (Scheme 1B) in which most of the NAG is produced by OAT from glutamate and acetylornithine, whereas NAGS fulfils an anaplerotic (replenishment of pathway intermediates) role to replenish NAG that is lost due to degradation or cell division [11]. These observations may explain the low abundance of the NAGS protein in organisms that have OAT activity.

Other prokaryotes, such as members of the Enterobacteriaceae [11] and Vibrionaceae [12], *Muxococcus xanthus* [13] and the archaeobacterium *Sulfolobus solfataricus* [14] have a stoichiometric ornithine production, where the acetyl group is not recycled and NAG is produced exclusively by NAGS (Scheme 1A). These organisms are considered less evolved, as the biosynthesis of ornithine by the stoichiometric pathway uses AcCoA, requiring more energy than a transacetylation reaction in the cyclic pathway. In organisms with the stoichiometric pathway, NAGS is the target for feedback inhibition by arginine [12–16], whereas in organisms that use the cyclic pathway, both NAGS and NAG kinase (NAGK) may show inhibition by arginine [17–23]. Unlike most organisms with a cyclic pathway, in *Thermus aquaticus* and *Thermotoga maritima*, NAGS and NAGK are not

Abbreviations used: AcCoA, acetyl-CoA; CPS(I), carbamylphosphate synthetase (I); MTS, mitochondrial targeting signal; NAG, *N*-acetylglutamate; NAGK, NAG kinase; NAGS, NAG synthase; NCLG, *N*-carbamyl-L-glutamic acid; OAT, ornithine acetyltransferase; OTC, ornithine transcarbamylase.

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Scheme 1 Arginine biosynthesis pathways in micro-organisms and plants

(A) Linear (stoichiometric) pathway. (B) Cyclic pathway. Further abbreviations used: NAGPR, *N*-acetylglutamylphosphate reductase; AOAmT, acetylornithine aminotransferase; AOD, acetylornithine deacetylase; ASS, argininosuccinate synthase; ASL, argininosuccinate lyase; CoASH, CoA.

feedback-inhibited by arginine [14]. In *T. aquaticus*, OAT is inhibited by arginine, and this organism is the only known example where this step is involved in feedback regulation of arginine biosynthesis [14].

Genes and enzymes of NAG synthesis

The arginine-biosynthesis pathway is unique because two of its precursors, namely NAG and ornithine, can each be produced by two different enzymic reactions [11]. NAG can be produced either by NAGS encoded by *argA* or by OAT encoded by *argJ* [11]. Ornithine is produced either by *N*-acetylornithine deacetylase encoded by *argE* or by OAT [11]. There is little similarity between the amino acid sequences of NAGS and OAT (Figures 1 and 2), suggesting that these two proteins have different evolutionary paths [24]. Moreover, some organisms, such as *Vibrio* strains 2674 and 2693, have an unusual NAGS encoded by a composite *argH(A)* gene that can complement both *argH* or *argA* mutations in *E. coli* [12]. The *N*-terminal portion of the *argH(A)* protein has argininosuccinate lyase activity and an amino acid codon sequence similar to that of other *argH* genes. The C-terminal portion possesses NAGS activity and complements an *argA* mutation in *E. coli* [12].

In some bacteria, such as *Bacillus* species [20], *Nisseria gonorrhoeae* [25], *Thermotoga neapolitana* [26] and in yeast (*Saccharomyces cerevisiae*) [27], OAT can have a dual enzymic function. In these organisms, either *N*-acetylornithine or AcCoA can be used as acetyl donors for formation of NAG [24,26]. These *argJ* genes complement both *argE* and *argA* mutations in *E. coli*. However, organisms that have dual-function OAT proteins also possess separate NAGS enzymes encoded by *argA* genes [24,26,27]. The biological role of duplicate NAGS activity in these organisms remains to be elucidated.

NAG IN LOWER EUKARYOTES

The arginine-biosynthesis pathway in lower eukaryotes is virtually identical with that found in prokaryotes. Some enzymes that catalyse arginine biosynthesis in lower eukaryotes are localized in mitochondria, whereas others are in the cytosol [28–30]. As seen in prokaryotes, NAG is an intermediate of arginine biosynthesis in fungi, green algae and plants [19,30–33]. In these organisms, NAG is produced within mitochondria, either by NAGS or by OAT. The first NAGS gene to be cloned from a eukaryote was *arg-14* of *Neurospora crassa* [34]. Unlike other genes of the arginine-biosynthesis pathway, *arg-14* has little sequence homology with its bacterial counterpart (*argA*) [34,35]

Scheme 2 The mammalian urea cycle

The abbreviations are defined in the abbreviations footnote or in the legend to Scheme 1. The red arrow indicates CPSI activation by NAG.

(Figure 1). OAT, which is encoded by *ARG7* in *S. cerevisiae*, and *arg-7* in *N. crassa* produces most of the NAG, whereas NAGS has only an anaplerotic function (Figure 1B) [28,29,31]. In yeast and *Neurospora*, both NAGS and NAGK are targets for feedback inhibition by arginine [22,31,36].

BIOCHEMICAL PROPERTIES OF NAGS AND OAT IN PROKARYOTES AND LOWER EUKARYOTES

NAGS proteins from *Pseudomonas aeruginosa*, *E. coli* and *Salmonella typhimurium* have been studied in greater detail [15,17,37–40]. In all three, NAGS activity is inhibited by L-arginine as well as by NAG, but the respective sensitivities to inhibition varies [15,17,37,40]. NAGS from *E. coli* is a multimeric enzyme, with a trimer being the smallest active unit. The enzyme can transit between trimeric and hexameric states depending on its concentration and the presence of ligands [40]. In the presence of arginine and NAG, the enzyme becomes hexameric, suggesting that these two ligands inhibit activity by inducing protein oligomerization.

Fungal NAGS proteins have been characterized in *S. cerevisiae* and *N. crassa* [23,34,36]. In both organisms, NAGS is subject to feedback inhibition by arginine, and in yeast, arginine and NAG synergistically inhibit this enzyme [23,36]. NAGS from *Neurospora* has been characterized in more detail and its biochemical properties are summarized in Table 1. Fungal NAGS appears to form a functional complex with NAGK [35,36]. This probably explains the absence of NAGS activity in the *Neurospora arg-6* mutant, which lacks NAGK and NAG phosphate reductase [36]. Dependence of NAGS activity on protein–protein interactions between NAGS and NAGK has subsequently been demonstrated in yeast [35].

Detailed studies of OAT have been performed in three species of thermophilic bacteria [26,41] and in yeast [27,42,43]. All

four enzymes have similar amino acid sequences and share a conserved ATML (one-letter amino acid code) motif with other known OAT enzymes (Figure 3) [26,27,41–43]. OAT undergoes autoproteolytic cleavage between the alanine (A) and threonine (T) of the ATML motif to yield α - and β -subunits that assemble into a heterotetramer in prokaryotes [26,41] and heterodimer in yeast [43]. The conserved threonine is essential for autoproteolysis and catalysis in yeast and *B. stearrowthermophilus* [41,43]. OAT from thermophilic bacteria shares an identical mechanism of catalysis which involves formation of an acetyl-enzyme intermediate [26]. Enzymic activity of all four OAT proteins is inhibited by ornithine, while arginine inhibition is weak [26,27].

In the green alga *Chlorella vulgaris*, NAG appears to be produced by an OAT enzyme with dual function that can use either AcCoA or acetylornithine as the acetyl donor [30]. The NAGS and OAT enzymic activities could not be separated by purification from cell extracts, and only the activity using AcCoA (NAGS) was inhibited by arginine.

Unlike other enzymes of the arginine-biosynthesis pathway, there is little sequence similarity between NAGS genes from prokaryotes and fungi [34,35] (Figure 1). The amino acid sequence identity between prokaryotic and *N. crassa* NAGS is much lower than the 33% identity observed between *E. coli* and *P. aeruginosa* [34] and the 30–35% identity of other orthologous genes of the arginine-biosynthesis pathway between fungi and bacteria [44]. On the other hand, the amino acid sequence of OAT is similar in bacteria and yeast (32–43% identity; Figure 2), and the yeast *ARG7* gene encoding OAT complements both *E. coli argE* and *argA* mutations [27]. The unusual dissimilarity of NAGS genes between species would imply that either they evolved independently or their sequences diverged more rapidly than the sequences of the other arginine-biosynthesis genes.

Figure 1 Alignment of bacterial (A) and fungal (B) NAGS protein sequences

Only characterized bacterial proteins were included in the alignment. Conserved amino acids are in **bold type**.

Table 1 Biochemical properties of NAGS

Source	M* (kDa)	pH optimum	K _m (mM)		K _i (mM)		[Arg] _‡ (mM)	[Arg] _§ (mM)	Reference(s)	
			Glutamate	AcCoA	NAG	CoA				
<i>Neurospora</i>	na†	8.8–9.0	6.3	1.6	na	na	1	0.16	[36]	
Rat liver	160 000	8–8.5	3.7	0.8	0.07	na	0.02	0.005	[88,89]	
Human liver	190 000	8.5	8.1	4.4	0.5	4.7	0.5	0.03	[87]	
* Estimated molecular mass of the trimer.										
† Not available.										
‡ Arginine concentration at which maximal NAGS inhibition (<i>Neurospora</i>)/activation (rat and human) was observed.										
§ Arginine concentration at which half-maximal NAGS inhibition (<i>Neurospora</i>)/activation (rat and human) was observed.										
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<i>B. stearothermophilus</i>	1				50	51			100	
<i>B. subtilis</i>	-----M	IQLSEDIQIVK	VT.GDVSSPK	G FQAKGVHCG	LRYS.KKDLG	VIIS...ETP	AVSAAVYTQS	HFC AAPIKVT	QDSL.KHGPT	.L K AVIVNSA
<i>T. neapolitana</i>	-----MT	ITKQTGQVTA	VADGTVVTPE	G FQAAGVNAG	LRYS.KNDLG	VILC...DVP	ASAAAVYTQS	HFC AAPLKVT	QASL.AVEQK	.L C AVIVNRP
<i>T. thermophilus</i>	-----	-----	-----MFVPR	G FSYAGVHCR	IKRK.RKDLG	IIFS...EVP	CTAAGVFTTN	VVK AAPVIYD	MEIL.GKNPS	GIR A ITVNSG
<i>M. jannashii</i>	-----	-----	-----MAVRLPR	G FRAGATRAG	IKPSGRPDLA	LLVS...GLP	AAWAYAATON	R .AAAPSIHR	GRAL.YASGK	PL R AVVVNAG
<i>S. cerevisiae</i>	-----MR	VIDGGVTAPK	G FKANGYK...	...EGKFGVA	IIIS...EKD	AVGAGTFTTN	KVV AHPVLS	RE.L.IKNRD	KFR A IVANS	
<i>N. gonorrhoeae</i>	MRISSTLLQR	SKQLIDKYAL	YVPKTSFPP	G FVEVGYTASG	VKKNGSLDLG	VILNTNKSRL	STAAAVFTTN	KFK AAPVLTS	KKVLETARGK	NIN A IVVNSG
<i>C. glutamicum</i>	-----	-----MAVNLT	KTAEQLPDID	G IALYTAQAG	VKKPGHTDIT	LIAVAAG...	STVGAVFTTN	RFC AAPVHIA	KSHLF...DED	GVR A LVINTG
Consensus	-----	-----	MAEKGITAPK	G FVASATTAG	IKASGNPDMA	LVVNQGPPEF	.SAAAVFTRN	RVF AAPVKVS	RENV...DG	QIR A VLYNAG
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<i>B. stearothermophilus</i>	101				150	151			200	
<i>B. subtilis</i>	IANACTGEQG	LKDAYTMRES	FASQLGIEPE	LVAVS STGVI	GEHLDMEKIH	AGIELLK...	E...TPAGSG	DFEE A ILTTD	TVIKQTCYEL	AI.GGKTVTI
<i>T. neapolitana</i>	CANACTGAQG	LKDAYEMREL	CAKQFGLALH	HVAVA STGVI	GEYLPMEKIR	AGIKQLV...	PGVTMADAE	AFQ T AILTTD	TVMKRACYQT	TI.DGKTIVT
<i>T. thermophilus</i>	VANACTGEQG	MINARRMAEK	TAKELNIPVE	SVLVF STGVI	GVQLPMEKVE	SGIEEAV...	KNLS.KDPV	PFAE A IMTTD	TKIKHSKKV	TI.EGKEITV
<i>M. jannashii</i>	NANCATGERG	YEDDRMAEL	AALRLGLSPE	EVLTA STGVI	GVPLPVEKIA	KGLPEI...	.GLTPF.AE	AFAE A ILTTD	TRPKVA...EA	EV.AGARIV
<i>S. cerevisiae</i>	NANCF.T.KDG	MEDAKEMQRL	VAELFNINED	EVLVA STGVI	GRKMDMNIK	DRINKVY...	NLIKEGNSSI	NAKA A IMTTD	TKPKETAVEF	EV.NGKTVRV
<i>N. gonorrhoeae</i>	CANSVTGDGL	MKDAQVMIDL	VNDKIG.QKN	STLVM STGVI	GQRLQMDKIS	TGINKIFGEE	KFGSDFNSWL	NVAK S ICTTD	TFFKLVTSRF	KLP S GTeyTL
<i>C. glutamicum</i>	NANAGTGAQG	RIDALAVCAA	AARQIGCKPN	QVMPF STGVI	LEPLPADKII	AALPKMQPAF	WN	EAA R AIMTTD	TVPKAASREG	KVG D QHTVRA
Consensus	NANACNLQGG	EKDARESUSH	LAQNLGLEDS	DIGVC STGLI	GELLPMCKLN	AGIDQLTAE	PL...GDNGA	AAKA A IMTTD	TV D KE....	TVV F ADGWTV
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<i>B. stearothermophilus</i>	201				250	251			300	
<i>B. subtilis</i>	GGAKGSGMI	HPNM...ATML	GFVITDAEIE	EKALQKALRE	TTDVSFNQIT	VDGET STNDM	VLVMANACAE	NECL.TEDHP	DWPVFKKALL	LTCED L AKEI
<i>T. neapolitana</i>	GGAAGKSGMI	HPNM...ATML	AFITTDANVS	SPVLHAALRS	ITDVSFNQIT	VDGDT STNDM	VVVMASGLAG	NDEL.TPDHP	DWFNFYEALR	KTCED L AQKI
<i>T. thermophilus</i>	LGIAGKSGMI	HPNM...ATML	SFITTDANVS	EDALKLLKI	SVDDSYNNID	VDGDT STNDM	VIIILANGLAG	NAPI.QEETD	GFWKLYEAVH	EVNQVL A EKI
<i>M. jannashii</i>	.GLAKGSGMI	HPNM...ATML	AFLVTDALVP	QEALRAGWRR	VVERTFNQVT	VDGDT STNDL	ALLMANGAYG	EVPL.R...	...AFFEGVE	AEAQEL A MI
<i>S. cerevisiae</i>	GGIAGKAGMI	APNMLHATML	CFITTDIEID	KESLTNLIQK	VVDKTFNNIS	VDGDT STNDT	VFVLNGLSG	...V.NYEEC	G.EEFENALL	YVCRE L AKMI
<i>N. gonorrhoeae</i>	TGMAKAGMI	CPNM...ATIL	GFIIVTDLPIE	SKALQKMLTF	ATTRSFNCIS	VDGDM STNDT	ICMLANGAID	TKEI.NEDSK	DFEQVKLQVT	EFAQRL A QLV
<i>C. glutamicum</i>	TGIAGKSGMI	HPNM...ATML	GFIATDAKVS	QVVLQMLTQE	IADETFTNIT	VDGDT STNDS	FVLIATGKNS	QSEIDNIADP	RYAQLKELLC	SLALE I AQAI
Consensus	GGMGKGVMGM	APSL...ATML	VCLITDASVT	QEMAQIALAN	ATAVTFDTLD	IDGST STNDT	VFLASGASG	ITPTQDE...	...LNDAVY	AACSD I AAKL
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<i>B. stearothermophilus</i>	301				350	351			400	
<i>B. subtilis</i>	ARDGEGATKL	IEAQVQAKN	NLDANVIAKK	IVGSNLVKTA	VYGTDAANGR	IIGAIGHSAA	Q...VTAAE	VEVYL....	...GGQCLFK	NNEPQPFSES
<i>T. neapolitana</i>	AKDGEGATKL	IEVRVRGAKT	DEEAKKIAKQ	IVGSNLVKTA	VYGADANAGR	IIGAIGYSDA	E...VNPDN	VDVAI....	...GFMVMLK	GSEFPQPFSE
<i>T. thermophilus</i>	VEDGEGATKV	IEVEVRNAPD	RNSARLIARA	IVSSNLVKPA	IYGEDANAGR	VIAAAGYSGA	Q...FDPDR	LDLFF....	E.SAAGRIKVAE	NGQGVDFDED
<i>M. jannashii</i>	ARDGEGATKL	MVVRVVGAT	EEGARARA	LAGSALWKA	LYGNDPNAGR	ILAAIGNSGA	R...FDPDR	VRVL....	Q.GIP....	LY.EGAVLPFDRE
<i>S. cerevisiae</i>	VKDGEATKF	MEVVVKGAKT	EEDAVKASKA	IVNSLLVKTA	VFGDPNAGR	IVAAGVYSGA	D...FNPEV	VDVIL....	S.NYKDEVYLVK	DGIPLADEGT
<i>N. gonorrhoeae</i>	VRDGEATKF	VTNVKNAH	FEDAKIAES	ISNSMLVKTA	LYGQDANAGR	ILCAIGYAKL	NDLKSLDVNK	INVSFIATDN	SEPRELKLVA	NGVPQ L ...
<i>C. glutamicum</i>	QADAEGVTRK	ITAVTVGGTTN	NEQAQNAAT	VARDNLFKCA	MFGSDPNAGR	VLAAGVMADA	D...MEPEK	ISVFF....	...	NGQAVCLDST
Consensus	--D-EG-TK-	-----V-----	-----A-----	-----L-K-A-----	-----D-N-G-----	-----A-G-----	-----	-----	-----	-----
<hr/>										
<i>B. stearothermophilus</i>	401				449					
<i>B. subtilis</i>	E....AKEYL	EGDEITIVIK	MAE.GDGNR	AWGCDLTYDY	IKINASYRT					
<i>T. neapolitana</i>	E....AAAYL	QOETVVIEVD	LHI.GDGVG	AWGCDLTYDY	VKINASYRT					
<i>T. thermophilus</i>	T....AKKIL	SEKRVKIILD	MKQ.GKELAR	AWGCDLTKY	VEINGRYRT					
<i>M. jannashii</i>	E....AGQAM	RAEEVEVLVD	LRE.GQGAGE	AYGCDLTEDY	VRINALYTT					
<i>S. cerevisiae</i>	EELKKAEEIM	KSDEIKIVVD	LKM.GEFENV	CYGCDSLEYE	VRINAEYTT					
<i>N. gonorrhoeae</i>	IDETRASEIL	ALNDLEVSVD	LGT.GDQAAQ	FWTCDLSHEY	VTINGDYRS					
<i>C. glutamicum</i>	YTEAQGGAVM	SKDEITVRIK	LHR.GQAAAT	VYTCDSLHGY	VSINADYRS					
Consensus	GAPGAREVDL	SGADIDVRID	LGTSGEGQAT	VRTTDLFSFY	VEINSAYSS					
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hNAGS	1	MATALMAVVL	RAAAVAPRLR	GRGGTGGARK	LSCGARRRAA	RGTSPGRRRLS	TAWSQPQPPP	EEYAGADDVS	QSPVAE.EPS	WVPSRPPVP	HESPEPPSGR	100
mNAGS		MATAWVATAL	RSAAAARRLR	SPGGPGGSRR	LSGSARRRGA	KSASPGRRRLS	TA...RAHA	EDAEGAKGRV	QSPAVDPRVR	WTPL...PTP	LESAPPAGR	
fNAGS		-----	-----MAK	LNSGSPGCRA	VALAGKYLSS	PSLPALTRGS	GQRQRMAAP	RRCMMSSDAS	GDAGKSPLLL	HQQDSLGLCS	PADRQAPTNR	
Consensus		-----	-----R-G-R-	-----	-----	-----R-S-	-----	-----	-----	-----	-----P--R	
hNAGS	101	SLVQRDIQAF	LNQCGASPG	ARHWLTQFQT	CHHSADKPFA	VIEVDEEVK	CQQGVSSLAF	ALAFLLQMDM	KPLVVLGLP.	.APTAPSGCL	SFWEAKAQLA	200
mNAGS		SLVQRDIQAF	LNQCGASPG	ARHWLTQFQT	CYHSVDKPF	VMEVDEEVIR	CPQAVSRLLAF	ALAFLLQMDM	KPLVVLGLP.	.TPTAPSGCL	SFWEAKAQLA	
fNAGS		NLIYRDVRAF	LSEVGGDPRE	ARYWLTQFQR	ATATQSPFA	VLEVDSSVFE	SREMVQRLLAF	GLSFLQMDM	KPVVIMGWSE	HEDLSPGEPI	AGSQCTKGLV	
Consensus		-L--RD--AF	L--G--P-E	AR-WLTQFQ-	-----FA	V-EVD--V--	-----V--LAF	-L-FLQMDM	KP-V--G--	-----P--	-----L-	
hNAGS	201	KSCKVLVDAL	RHNAAAVFF	FGGGSVLRAA	EPAPHASYGG	IVSVETDLLQ	WCLESGSIPI	LCPIGETAAR	RSVLLDSLEV	TASLAKALRP	TKIIFLNNTG	300
mNAGS		QSCCKVLVDEL	RHNAATAVFF	FGGGSVLSAA	EPAPHASYGG	IVAVETDLLQ	WCLESNSIPI	LCPIGETAAR	RSVLLDSLEV	TASLAKALQP	TKIIFLNNSG	
fNAGS		KRSKQLTEAL	QQHSAVLP	FSAESFLLQ	ETQTGSSAPP	SISVDSSLLQ	WSLGCGTIPL	VCPVGRDRG	CSVILDSTEV	TAASRVLRKP	YKVVFLNTSG	
Consensus		---K-L---L	---A---P-	F---S-L---	E---S-S-	---V---LLQ	W-L---IP-	-CP-G---	-SV-LDS-EV	TA-----L-P	-K--FLN--G	
hNAGS	301	GLRDSSHVKL	SNVNLPAADL	LVCNAEWSVT	KERQQMRLIV	DVLSRLPHHS	SAVITAASLT	LTELFSNKG	GTLFKNAERM	LRVSLDKLD	QGRLVDLVNA	400
mNAGS		GLRNNSSQKIL	SNVNLPAADL	LVTNAEWLSI	KERQQIRLIV	DVLSRLPHYS	SAVITAASLT	LTELFSNKG	GTLFKNAERM	LRVRNLDSD	QGRLVNLVNA	
fNAGS		GLRSQEHKVL	ETVLLPSNLL	ILSTAANLSK	AQRCRVATIA	RLNLQLPES	SAVITSADAL	LTELFSHRGS	GTLFKNGDPI	.HRSLAGID	VERLLALINK	
Consensus		GLR---K-L	--V-LP--L-	----A-W-S-	--R-----I-	--L-LP--S	SAVIT-A--L	LTELFS--G-	GTLFKN----	---R-L---D	--RL--L-N-	
hNAGS	401	SFGKKLRDDY	LASLRPLHS	IYVSEGYNAA	AILTMEPVLG	GTPYLDKFFV	SSSRQGGSG	QMLWECLRRD	LQTLFWRSRV	TNPINPWYFK	HSDGSSFNKQ	500
mNAGS		SFGKKLRDDY	LESRLPLHS	IYVSEGYNAA	AILTMEPVLG	GTPYLDKFFV	SSSRQGGSG	QMLWECLRRD	LQTLFWRSRV	TNPINPWYFK	HSDGSSFNKQ	
fNAGS		SFDKTLRRDY	IDSLQGRVHS	IYVSEGYNAA	AILTMEPVNN	GTPYLDKFFV	SSSKQGGTS	HLLWECIRQD	LGKLFWRSSRA	TNRINPWYFK	HSDGSEVNGA	
Consensus		SF-K-LR-DY	--SL--R-HS	IY-SEGYNAA	AI-T-EPV--	GTPYLDKFFV	SSS-QGG--	--LWEC-R-D	L--LFWRSR-	TN-INPWYFK	HSDGSE-N--	
hNAGS	501	WIFFWFLGLAD	IRDSYELVNH	AKGLPDSF.H	KPASDPGS--	-----	-----	-----	-----	-----	-----	547
mNAGS		WIFFWFLGLAD	IRDSYELVNH	AKGLPDSF.C	KPASDPGS--	-----	-----	-----	-----	-----	-----	
fNAGS		WTVFWFLGTD	IRDSYELVEY	ARNLPESFQA	SPLGTPEQPL	TAAAKS*	-----	-----	-----	-----	-----	
Consensus		W--FWFLG-D	IRDSYELV--	A--LP-SF--	-P--P--P--	-----	-----	-----	-----	-----	-----	

Figure 3 Alignment of NAGS proteins from mouse (mNAGS), human (hNAGS) and puffer fish (fNAGS)

Conserved amino acids are in **bold** type. Amino acids highlighted in red are conserved between vertebrate and fungal NAGS proteins. Boxed sequences are stretches of four or more conserved amino acids that are likely to be important for NAGS function. (*) indicates a stop codon.

NAG IN VERTEBRATES

Little is known about the biology of NAG in vertebrates. Many vertebrates have a complete urea cycle, but the physiological role of ureagenesis varies in different species [50]. Amphibians, for example, produce urea as a means of ammonia detoxification and long-term adaptation to salinity [51–54]. Ureagenesis has been studied in the North American bullfrog (*Rana catesbeiana*), which has CPSI activity [55,56]. Since CPSI requires NAG for function, this and other amphibians are likely to have NAGS enzymes; however, no amphibian NAGS gene or gene product has been identified to date. In marine elasmobranchs (sharks, rays and skates), coelacanth and holocephalan fishes, urea plays a role in osmotic regulation [50]. Teleost fish are ammonotelic (excrete ammonia *per se* without further metabolism), and most of them do not have urea-cycle enzymes [50,57]. However, some teleosts produce urea [57–60], and, in them, ureagenesis seems to have a role in stress response. Urea-cycle enzymes are also expressed in rainbow-trout (*Oncorhynchus mykiss*) embryos [61,62], and it is possible that, in teleosts, urea synthesis has a yet to be determined role in embryogenesis.

Intermediates and enzymes of the urea cycle are identical in all vertebrates with the exception of mitochondrial CPS (CPSI and CPSIII) and arginase. Fish have glutamine-dependent CPSIII activated by NAG, and fish arginase is mitochondrial rather than cytosolic [50,63–65]. Since NAG enhances, but does not seem to be essential for, CPSIII activity [64,66], several fish species were examined for the presence of NAG. NAG was found in liver mitochondria of the spiny dogfish shark (*Squalus acanthias*), as well as in liver of largemouth bass (*Micropterus salmoides*), rainbow trout (*O. mykiss*) and gulf toadfish (*Opsanus beta*) [57]. It is also present in muscles of

rainbow trout and gulf toadfish [57]. In liver and muscle of the gulf toadfish, the amount of NAG increases along with CPSIII activity in response to stress and feeding [57]. Changes observed in tissue levels of NAG in response to various stimuli may suggest a regulatory role for NAG in ureagenesis. This subject is discussed in detail below (also see [67]). The detection of NAG in fish implies the presence NAGS. In fact, a candidate NAGS gene has been recently identified in the puffer fish (*Fugu rubripes*) on the basis of its similarity to the mammalian gene [68].

NAG IN MAMMALS

In mammals, arginine biosynthesis does not use NAG as a precursor and rather involves the enzymes of the urea cycle (Scheme 2). Here, arginine's precursor ornithine can also be produced from proline or glutamate via ornithine aminotransferase [69]. The only known function of NAGS in mammals is to supply mitochondrial CPSI with its essential cofactor. Mammalian tissues do not express the OAT seen in lower organisms as part of the arginine-biosynthesis pathway [70].

Within the liver cell, NAG is present mainly in mitochondria (56% according to one study), where the CPSI enzyme resides; the nuclear fraction contains about 24% and the rest is found in the cytosol [71]. Without NAG, the enzymic activity of CPSI is virtually undetectable [8], and the binding of NAG to CPSI alters the conformation and subunit structure of the enzyme [72]. Bacterial and fungal CPS, and a cytosolic CPSII involved in pyrimidine biosynthesis, are not dependent on NAG. CPSI exists in monomeric and dimeric forms and the interaction with NAG yields a catalytically active, but unstable, configuration that is mainly monomeric [73]. Following protease digestion

of CPSI, NAG was found to bind to a 20 kDa fragment of the C-terminus [74,75]. Labelling studies identified also N-terminal peptides near the binding site for NAG, raising the possibility that at least part of the glutamine-binding domain of ancestral CPS could have evolved into a portion of the NAG binding site of CPSI [76]. The K_a of NAG for CPSI was estimated to be 0.1–0.2 mM [77,78], and a similar *in vivo* liver mitochondrial concentration was calculated based on mitochondrial space of approx. 20% of cellular volume [71]. These observations may imply that concentrations of NAG within mitochondria control the activity of CPSI and as such regulate flux through the urea cycle. Liver and small intestine are the tissues with the highest concentration of NAG, a level corresponding to their expression of CPSI. In the intestine, NAG-dependent CPSI, along with ornithine transcarbamylase (OTC) produce citrulline, which is then converted into arginine in the kidney [79]. Smaller amounts of NAG were found in brain, where it is cytosolic rather than mitochondrial [80,81]. The localization NAG in brain (cytosolic), and the absence of NAGS expression, indicate that NAG is produced by a different enzyme in brain, and its physiological role in this organ remains to be determined. The half-life of NAG in liver was estimated to be between 20 min and several hours [82,83]. NAG can be degraded by a cytoplasmic liver deacylase yet to be characterized, but a similar NAG-degrading enzyme is not found in mitochondria [84]. According to one report, NAG does not enter the mitochondria [85], but another report documented unidirectional transfer out of the mitochondria into the cytosol, where NAG is presumably degraded [82,84].

Tatibana and colleagues [83] were the first to report on the biosynthesis of NAG in mammalian tissue. As in many lower organisms, NAG is synthesized from acetyl-CoA and glutamate by an enzymic reaction catalysed by NAGS [83]. The content of NAG in rat liver is in the order of 15–40 nmol/g, and the mitochondrial concentration was estimated to be 10^{-4} M – within the range of the activation constant for CPSI [71]. NAGS activity was detected in liver, intestine, testis, lung and submaxillary glands, but not in kidney and spleen [83]. The activity in rat fetus is about 5% of that in adults and increases slowly up to 10 weeks postnatally. Intestinal activity in the fetus is about one-third that of the adult and increases more rapidly after birth [86]. NAGS was purified to apparent homogeneity from rat and human livers [87–89]. The rat enzyme has a molecular mass of 160 000 Da and acetylates almost exclusively L-glutamate with low acetylation of glutamine, glycine, aminoadipate and aminopimelate, but not other amino acids [89]. The AcCoA substrate is also very specific and could be substituted by propionyl-CoA to a much lesser degree [89]. The molecular weight of the human holoenzyme was reported to be approx. 190 000, with affinity constants of 4.4 and 8.1 mM for AcCoA and L-glutamate respectively [87], whereas those for the rat enzyme were 0.7 mM and 1 mM respectively (Table 1 [89]). The 1000-fold discrepancy between the reported specific activities of the rat and human enzymes [87–89] could be resolved by characterizing their recombinant proteins. Rat NAGS from mitochondrial fractions of the small intestine has characteristics similar to the liver enzyme [90]. Rat liver NAGS is inhibited by its product NAG ($K_i \approx 0.07$ mM) and by other structural analogues, including N-acetylaminoadipate, N-propionylglutamate and N-acetylglutamine [88]. In the presence of saturating concentrations of substrates, the enzymic activity can be more than doubled by L-arginine, with half-maximal activation at 30–50 μ M *in vitro*, which is in the range of liver arginine concentrations [83,87–89,91]. Arginine activation was recently confirmed for recombinant mouse and human NAGS, showing a 4–5-fold increase in specific activity [68,92] and suggesting a possible role for arginine in the regulation of NAG

production and thus CPSI activity. Arginine does permeate the mitochondria, and therefore could activate NAGS [93]. L-Arginine seems to increase the enzyme velocity without an effect on affinity for either substrate [90]. However, according to one report, the activation of NAGS by arginine increases with increasing pH, while the affinity for glutamate decreases with increasing arginine concentration [94]. The activation of NAGS by arginine seems to be very specific, as other amino acids do not increase enzymic activity [89]. In addition to arginine, protamines and other cationic polypeptides (e.g. polyarginine and polylysine) also increase the activity of NAGS, but this increase is additive and not competitive with the effect of arginine, suggesting a different mechanism of activation [89]. At this stage, a merely stabilizing effect of arginine on this unstable enzyme cannot be ruled out; such an effect was suggested by experiments in crude liver samples and crude mitochondria [95,96] and needs to be studied in detail with the recombinant enzyme. Mammalian NAGS appears to be a trimer, but its oligomerization state may depend on the concentration of L-arginine [88].

NAG as a regulator of CPSI activity and urea synthesis

The hypothesis that NAG concentrations within liver mitochondria regulate CPSI activity and thus ureagenesis was entertained on the basis of the following observations:

- NAG is an essential cofactor for CPSI which could control flux through the urea cycle
- NAG concentrations in liver are similar to the K_a for CPSI
- Increased intake of protein and ammonia is associated with an increase in the activity of NAGS and in the hepatic content of NAG

Several reports have confirmed the association between hepatic NAG levels and dietary protein intake and amino acid or ammonia administration [97–104]. The level of NAG in rat liver was found to have diurnal variations associated with dietary intake. Increasing the protein in the diet of rats from 0 to 60% resulted in doubling NAG content in the liver [83]. A high-protein diet was associated with an increase of NAG content in rat liver mitochondria and in a higher rate of citrulline synthesis [105]. Arginine administration stimulates hepatic synthesis of NAG, and its effect is further augmented by combined administration with glutamine [88]. Another report showed that 1 mM arginine increased NAG content and citrulline synthesis by more than 5-fold in liver mitochondria isolated from protein-starved rats, suggesting that arginine may be a regulatory signal of protein intake [105]. One study in mice found that feeding was associated with increased sensitivity of NAGS to arginine activation and was later reproduced in isolated mouse liver mitochondria and in partially purified NAGS protein [91,106]. A more recent study found that rats given ornithine showed increased liver NAG, owing to a decrease in its degradation rather than increased production [107]. As NAG is degraded in the cytosol [84], ornithine may affect NAG transport to the cytosol. Ingestion of ammonium by rats increased NAG content of liver mitochondria without changing the activity of NAGS [98], suggesting that decreased efflux of NAG from the mitochondria was responsible. A similar observation made previously documented a prolongation in the half-life of NAG from 14 to 60 min in fasted versus fed animals respectively [108]. The authors concluded that the degradation of NAG is mediated by its efflux from the mitochondria to the cytosol. These observations could explain the finding that relatively low concentrations of NAG inhibit NAGS activity [88]. The concentrations of liver NAG seem also to respond to hormonal changes. For example, the level of NAG increases in

growth-hormone deficiency and decreases upon administration of this hormone [109]. NAG levels were also reported to increase in response to glucagon administration [110]. In hypothyroid rats, liver concentrations of NAG increased via enhanced synthesis and reduced degradation in correlation with increased urea production [111,112]. NAGS activity was also reported to increase in response to extramitochondrial free Ca^{2+} [113].

The physiological role of NAG in the regulation of ureagenesis was questioned by Lund and Wiggins [114]. They contended that the biological method previously used for determination of liver NAG (based on citrulline production) was inaccurate, and by using a more sensitive method, they estimated the concentration of liver NAG to be much higher (1 mM; 10-fold the K_a for CPSI) than was previously estimated. In addition, Van Dijk and Lund [115] found that NAG is present in fetal rat liver at 17 days of gestation, and its tissue content remains constant throughout fetal life, inconsistent with the very low capacity of fetal liver to generate urea before 20 days of gestation. The validity of the above arguments was contested by Meijer et al. [116]. There is, however, abundant evidence for the association between NAG levels and changes in nitrogen metabolism. It is believed that control of CPSI activity by NAG allows changes in flux through the urea cycle at relatively constant ammonium concentrations [117–119]. One has to wonder what was the evolutionary transition of NAG from being the first intermediary in arginine biosynthesis to being the essential cofactor for the first enzyme of ureagenesis? and what is the evolutionary advantage of having CPSI activity dependent on NAG? This enigma makes the hypothesis that NAG is a regulator of ureagenesis quite attractive. If this hypothesis is correct, mitochondrial concentrations of glutamate and/or arginine may reflect the need for nitrogen disposal through their effect on the production of NAG by NAGS, glutamate as a substrate and arginine as an effector. Indeed, a direct correlation was found between mitochondrial concentrations of glutamate and NAG [104]. The recent cloning of NAGS genes from various species, including mammals, will undoubtedly provide new tools for exploring the role of NAG in ureagenesis and the evolution of NAGS.

The NAGS gene in mammals

The mouse and human NAGS genes were recently cloned on the basis of similarity to the *N. crassa arg-14* (NAGS) gene [68,92]. Both genes have each seven exons and six introns and are localized to chromosomes 17 and q21.31 (human) and in a syntenic region of chromosome 11 in mouse. The open reading frames, which are 85% identical, encode 528-amino-acid (mouse) and 534-amino-acid (human) proteins. The NAGS mRNA is found almost exclusively in liver and small intestine. The deduced amino acid sequence contains an N-terminal putative mitochondrial targeting signal of approx. 50 amino acids (63% identity between mouse and human), followed by a 'variable domain' of 40–46 amino acids (35% identity) and a 'conserved domain' of 440 amino acids (92% identity). The addition of L-arginine increases the catalytic activity of the purified recombinant NAGS enzymes by approx. 4–5-fold.

NAG synthase deficiency

Being an indispensable cofactor for ureagenesis, it would be expected that liver NAG deficiency would cause a proximal block of the urea cycle at the level of CPSI with resulting hyperammonaemia. There is evidence that NAG deficiency

Figure 4 *N*-Carbamylglutamate is an active and biologically stable structural analogue of NAG

can be caused by inherited defects in the NAGS gene or by interference with the catalytic function of the NAGS enzyme. For example, pent-4-enoic acid is known to inhibit fatty acid oxidation and diminish the production of acetyl-CoA in the liver. This compound was shown to lower the level of NAG in rat liver cells [102,120], and, at concentrations of 0.1–1 mM, it strongly inhibits ureagenesis, concomitant with a decrease in NAG concentrations, without directly affecting NAGS catalytic ability [121]. NAGS activity of rat mitochondria can be inhibited by acyl-CoA species, probably due to competition with AcCoA for the enzyme's active site [122]. This seems to be the mechanism of interference with NAG production that occurs in propionic acidemia, an inherited disorder caused by propionyl-CoA carboxylase deficiency that leads to accumulation of propionyl-CoA in mitochondria [123]. This toxin acts as a competitive inhibitor of NAGS with an inhibition constant of 0.7 mM [122]. Therefore, inhibition of NAGS seems to be a likely explanation for the hyperammonaemia observed in patients with propionic acidemia [124]. A similar mechanism for hyperammonaemia is invoked by isovaleryl-CoA, which accumulates in isovaleric acidemia [125]. When purified NAGS was studied, propionyl-CoA was the only CoA derivative among those tested, in addition to acetyl-CoA to be used as a substrate by NAGS, albeit with a lower affinity [88,89]. Similarly, the hyperammonaemia seen in some patients who are treated with valproic acid [126] may result from inhibition of NAGS activity by the drug and/or its metabolites [127,128]. An identical pathogenic mechanism, or alternatively, CoA trapping in the form of acyl-CoA molecules, may be responsible for the mild hyperammonaemia seen in β -oxidation defects. Another possible mechanism for mitochondrial injury by short-chain fatty acids may be the increase in permeability of the mitochondrial inner membrane [129].

Inherited NAGS deficiency has been reported to date in a dozen patients [130–142], but is likely to be under-diagnosed, owing to the lack of specific biochemical markers and the low abundance of NAGS. This autosomal recessive disorder shows a phenotype similar to that of CPSI deficiency. It is characterized by hyperammonaemia in the newborn period or later within the first 5 years of life and could be fatal or lead to severe mental retardation and developmental disabilities. Plasma amino acids generally show increased levels of glutamine and reduced or absent citrulline. The urine orotic acid level is normal. Analysis of NAGS activity in the livers of patients showed various extents of deficiency from undetectable to normal residual activity that is unresponsive to arginine. Following the cloning of the human NAGS gene, molecular confirmation of NAGS deficiency was recently reported in three families [143,144].

N-Carbamyl-L-glutamic acid (NCLG) (Figure 4) is a structural analogue of NAG and could substitute for it in the activation of CPSI [8]. Although its affinity for CPSI is lower than that of NAG (K_m 2 and 0.15 mM respectively [145]), it is much more resistant to degradation by aminoacylase [85] and hence its

attractiveness for therapy of NAGS deficiency. Moreover, NCLG crosses the mitochondrial membrane and enters the mitochondria [85,146,147]. In mice injected with radiolabelled NAG and NCLG, only labelled NCLG was detected in liver mitochondria [145]. It was also demonstrated that NCLG could reduce blood ammonia, increase blood urea and protect animals administered a lethal dose of ammonia [85,146,148,149]. Several patients with NAGS deficiency have been reported to respond clinically to treatment with NCLG presumably through the alleviation of NAG deficiency by supplying its functional analogue [136–138,141].

CONCLUSIONS

The progress made in the research of NAGS and NAG and the cloning of the NAGS genes from various organisms should help to elucidate the evolution of this interesting system and provide tools for exploring its role in urea cycle regulation and human disease. Genetically modified cells or animal models (either transgenic animals or NAGS knock-out and/or knock-in mice) should prove useful for studies on the role of NAG and NAGS in the urea cycle particularly as carbamylglutamate could be used to replace NAG and to modulate CPSI activity in these model.

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