Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: Leader peptides cleaved by two matrix proteases share a three-amino acid motif

(mitochondrial precursor sequences/protein transport/mitochondrial import)

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ABSTRACT We have compiled sequences of precursor proteins for 50 mitochondrial proteins for which the mature amino terminus has been determined by amino acid sequence analysis. Included in this set are 8 precursors that have leader peptides that are cleaved in two places by mitochondrial matrix proteases. When these eight leader peptides are aligned and compared, a highly conserved three-amino acid motif is identified as being common to this class of leader peptides. This motif includes an arginine at position -10 , a hydrophobic residue at position -8 , and serine, threonine, or glycine at position -5 relative to the mature amino terminus. The initial cleavage of these peptides by matrix processing protease occurs within the motif, between residues at -9 and -8 , such that arginine at position -10 is at position -2 relative to the cleaved bond. The rest of the motif is within the octapeptide removed by subsequent cleavage catalyzed by intermediate-specific protease. An additional 14 leader peptides in this collection (all of those that contain an arginine at -10) conform to this motif. Assuming that these 14 precursors are matured in two steps, we compared the internal cleavage sites at position -8 with the ends of the other 30 leader peptides in the collection. We find that 74% of matrix processing protease cleavage sites follow an arginine at position -2 relative to cleavage.

Evaluation of the predicted amino-terminal 40-amino acid residues of a collection of mitochondrial precursors has identified a few features that distinguish the amino termini of proteins destined for the mitochondrion: a nearly total absence of acidic amino acid residues; a preponderance of arginine, serine, and leucine residues relative to a sample of amino-terminal peptides of cytosolic proteins; and a segment with a large predicted helical hydrophobic moment (1). These features have led to the suggestion that mitochondrial targeting sequences may form positively charged amphiphilic α helices. It has been shown that almost any amino-terminal amphiphilic basic peptide will serve as a mitochondrial targeting signal (2, 3). While many different amino-terminal peptides will serve as targeting signals, only authentic mitochondrial leader peptides are recognized and removed by mitochondrial proteases. However, there is no obvious pattern in the amino acid sequences found at the junctions of mitochondrial leader peptides and their corresponding mature sequences. While investigators have noted that the cleavage of many leader peptides by mitochondrial matrix proteases fits the motif Arg-Xaa⁺Xaa (4, 5), in which Xaa is another amino acid residue, this pattern is neither universal nor of much predictive value, given the frequency of occurrence and the regular spacing of arginine residues in the amino termini of mitochondrial protein precursors. Several observations suggest that the proteolytic removal of mito-

chondrial leader peptides requires higher order protein structure rather than a specific primary sequence. First, mitochondrial proteases will not cleave denatured precursors (4). Second, experiments in which mitochondrial leader peptides have been altered by deletion of portions of the precursor molecule or by substitution of amino acid residues within it have revealed residues far from the cleavage site in the primary sequence that affect proteolytic processing (6, 7). Third, critical regions have been identified within the mature portion of some precursors (8).

It has been established (9, 10) that some leader peptides are removed in sequential steps by two distinct matrix proteases. These leader peptides are initially cleaved to an intermediate form by the general "matrix processing protease" (5). Formation of the mature amino terminus is then catalyzed by a second, intermediate-specific, matrix protease that most often removes eight amino acids from the amino terminus of the intermediate (5). In this communication we present an evaluation of mitochondrial leader peptide cleavage sites, taking into account the two-step cleavage of many mitochondrial proteins. This analysis reveals a motif in the primary amino acid sequence of a large subset of mitochondrial leader peptides that, we propose, distinguishes those precursors that are matured by a second cleavage catalyzed by intermediate-specific protease.

The analysis of mitochondrial cleavage has been carried out on a sample of 51 sites in 50 precursor molecules [the two sites of matrix processing protease cleavage of *Neurospora crassa* proteolipid subunit 9 of the proton-translocating ATPase (F_1/F_0 -ATPase; ATP phosphohydrolase, proton-translocating, EC 3.6.1.34) have been counted separately] (11). These precursors come from vertebrates, *Neurospora crassa*, and *Saccharomyces cerevisiae*. Represented in the collection are nuclear-encoded precursors for protein subunits that are found in three mitochondrial subcompartments: enzymes of the matrix space, integral proteins of the mitochondrial inner membrane, and inner membrane proteins exposed to the intermembrane space. We have included only those precursors for which the mature amino terminus has been determined by amino acid sequence analysis. In the collection are nine precursors for which two cleavages by matrix proteases have been identified (5, 9–15). Those proteins of the intermembrane space which are matured by a second cleavage in the intermembrane space (for example, cytochrome b_2) have not been included.

RESULTS

In Fig. 1, the 50 mitochondrial protein precursor sequences are presented, aligned according to the site of cleavage by matrix processing protease as determined experimentally

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PROTEIN NAME / GENBANK ACCESSION NUMBER

REF

ARGININE -10: CONFIRMED TWO-STEP CLEAVAGE

HUM ornithine transcarbamylase* / K02100	MLFNRLRL	LNNAAFRN	GHNFMVRN	FRCGPQLQ	MKVQLKGR	16
MUS ornithine transcarbamylase*	MLSNRLRL	LNNAALRK	GHTSVVRH	FWCGKPVQ	SQVQLKGR	17
RAT ornithine transcarbamylase* / M1266	MLSNRLRL	LNKAALRK	AHTSMVRN	FRYGPVQV	SQVQLKGR	18
MUS malate dehydrogenase* / M16229		MLSALARP	VGAALRRS	FSTSAQNN	AKVAVLGA	19
RAT malate dehydrogenase		MLSALARP	AGAALRRS	FSTSAQNN	AKVAVLGA	20
NEU ubiquinol-cytochrome c reductase iron-sulfur subunit* / X02472	MAPVSIYS	RAAMRAA	APARAVRA	LTTSTALQ	GSSSSTFE	21
NEU cyclosporin-binding protein	MFGP	RHFSVLKT	TGSLVSST	FSSSLKPT	ATFSCARA	22
YSC cytochrome oxidase subunit 4 / Y00152		MLSRQSI	FFKPATRT	LCSSRYLL	QQKPVVKT	23

ARGININE -10: TWO STEP CLEAVAGE NOT CONFIRMED

HUM pyruvate dehydrogenase beta subunit	MAAVSG	LVAETPSE	VSGLLKRR	FHMTAPAA	VQVTVRDA	24
YSC pyruvate dehydrogenase E3 subunit		MLRIR	SLLNNKRA	FSSTVRTL	TINKSHOV	25
RAT ornithine aminotransferase		M	LSKLASLO	TVAALRRG	LRTSVASA	26, 37
HUM ornithine aminotransferase		M	FSKLAHLQ	RPAVLSRG	VHSSVASA	27
BOV cytochrome oxidase subunit 4 / K02064			MLATRV	FSLIGRAT	ISTSVCYR	28
HUM cytochrome oxidase subunit 4			MLATRV	FSLVGKRA	ISTSVCYR	29
BOV F1-ATPase alpha subunit / M19680	MLS	VRIAAAVA	RALPRATG	LVSKNALG	SSFVGTNR	30
HUM 5-aminolevulinate synthase / Y00451	MDSVVR	CPFLSRVP	QAFLOKAG	KSLLFYAO	NCPKMEV	31
RAT 5-aminolevulinate synthase / J03190	METVVR	CPFLSRVP	QAFLOKAG	KSLLFYAO	NCPKMEV	32
CHK 5-aminolevulinate synthase / X03517	MEAVVR	CPFLARVS	QAFLOKAG	PSLLFYAO	HCPKMEA	33
BOV adrenodoxin / M11746	MA	ARLLRVAS	AALGDTAG	RWRLLVRP	RAGAGGLR	34
HUM adrenodoxin / J03548	MAAA	GGARLLRA	ASAVLGGP	AGRWLHHA	GSRGSSG	35
BOV F0-ATPase proteolipid 1 / X05218	MYTC	AKFVSTPS	LIRRTSTV	LSRSLSAV	VYRRPETL	36
BOV F0-ATPase proteolipid 2 / X05219	MQTTG	ALLISPAL	IRSCTRGL	IRPVSASF	LSRPEIQS	38

ARGININE -2: PROBABLE MPP-1 ONLY CLEAVAGE

MUS aspartate aminotransferase / J02622	MALLH	SGRVLSGV	ASAFHPGL	AAAASARA	SSWTHVE	38
PIG aspartate aminotransferase	MALLH	SSRILSGM	AAAFHPGL	AAAASARA	SSWMAHE	39
CHK aspartate aminotransferase / M12105		MALLQS	RLLLSAPR	RAAATARA	SSWMSHVE	40
HUM methylmalonyl-CoA mutase*	MLRAKNQL	FLLSPHYL	RQVKESGG	SRLIQQLR	LHQQQPLH	41
RAT isovaleryl-CoA dehydrogenase	MATAVR	LLGRRVSS	WRLRPLPS	PLAVPQRA	HSMLPYDD	42
HUM pyruvate dehydrogenase E1 alpha subunit	MRKML	AAVSRLVS	GASQKPAS	RVLVASRN	FANDATFE	43
YSC manganese superoxide dismutase / X02156	MF	AKTAAANL	TKKGLSL	LSTTARRT	KVTLPLDK	44
YSC cytochrome oxidase subunit 5 / M11770		MLRN	TFRAGGL	SRLTSVRF	AQTHALFK	45
YSC cytochrome oxidase subunit 8 / J02634	MLS	QQMIRTTA	KRSSNIMT	RPIIMKRS	VHFQDGVY	46
RAT succinyl-CoA synthetase alpha subunit / J03621	MVS	GSSGLAAA	RLLSRTFL	LQONGIRH	GSYATSRK	47
YSC F1-F0 ATPase subunit 4	MSM	SMGVGRGLA	LRVSYSKL	FSQGVRCR	SMVIGARY	48
YSC F1-ATPase alpha subunit / J02603	MLA	RTAARISL	SRTLINST	KAARPAVA	ALASTRLR	49
NEU F0-ATPase proteolipid subunit - intermediate* / V00864	MAS	TRVLASRL	ASQMAASA	KVARPAAR	VAQVSKRT	
NEU F0-ATPase proteolipid subunit mature cleavage* / V00864			IQTGSPSL	QTLKRTQM	TSIVNATT	50
RAT carbamyl-phosphate synthetase I / M11710	MTIRLT	ACKVVKTL	KSGFGLAN	VTSKRQWD	FSPRGIRL	51

NO ARGININE -2 NO ARGININE -10

YSC F1-ATPase beta subunit' / M12082				MVL	PRLYTATS	RAAFKAOK.	QSAPLLST	52
RAT medium-chain acyl-CoA dehydrogenase				M	AAALRRGY	KVLRVSYS	FECRAQHT.	53
HUM medium-chain acyl-CoA dehydrogenase				M	AAGFGRC	RVLRSISR	FHWRSQHT.	54
PIG citrate synthase				MAL	LTAAARLF	GAKNASCL	VLAARHAS.	55
BOV adrenodoxin reductase / M17029				MAPRC	WRWMPWS	WTRTRLPP	SRSIQNFG.	56
HUM pyruvate dehydrogenase E3 subunit				MQS	WSRVYCSL	AKRGHFNR	ISHGLQGL	57
HUM cytochrome oxidase subunit 5b				MASRLR	GAGTLAAQ	ALRARGPS	GAAAMRSM.	58
HUM cytochrome oxidase subunit 5a				M	LGAALRRRC	AVAATTRA	DPRGLLHS	59
YSC cytochrome oxidase subunit 6 / M10138				M	LSRAIFRN	PVINRLL	RARPGAYH	60
BOV cytochrome P450 (side-chain cleaving) / K02130				MLARGL	PLRSALVK	ACPPILST	VGGGWHGH	61
BOV mitochondrial phosphate carrier / X05340				M	YSSVVHLA	RANPFNAP	HLQLVHDG	62
HUM F0-ATPase proteolipid subunit / M16453			MT	SLWGKTGC	KLFKFRVA	AAPASGAL	RRLTPSAA	63
BOV branched-chain ketoacid dehydrogenase E1 alpha subunit			MQGSAM	AMAVAVAV	ARVVRPSR	GLGRGTGL	LLRLGAR	64
RAT branched-chain ketoacid dehydrogenase E1 alpha subunit / J02827				SAAKIWRP	SRGLRQAA	LLLLGRPG	ARGLARFH	65

Fig. 1. Compilation of mitochondrial leader peptides. Sequences are aligned according to the site of cleavage by matrix processing protease; asterisks identify proteins with demonstrated cleavage by matrix processing protease at the site of alignment. Leader peptides cleaved in two steps have eight or nine additional residues at the carboxyl terminus of the leader peptide. Sequences with accession numbers were obtained from GenBank, release 56.0 (66). The intermediate cleavage product of BOV adrenodoxin/M11746 has been seen but not characterized. BOV, *Bos taurus*; CHK, *Gallus gallus*; HUM, *Homo sapiens*; MUS, *Mus musculus*; NEU, *Neurospora crassa*; PIG, *Sus scrofa*; RAT, *Rattus rattus*; YSC, *Saccharomyces cerevisiae*.

(marked by asterisk), according to the amino acid sequence motif that is the subject of this report, or according to the mature amino terminus for those precursors for which neither the motif nor protease data were available.

The motif was discovered as follows: the 50 precursor sequences were first aligned according to the amino terminus of the mature protein, and the frequency at which each amino acid occurs at a particular position relative to the end of the leader peptide was determined. Initially, we found a significant peak of arginine at positions -2 (15 of 50) and -10 (22 of 50) and found a high frequency of serine at position -5 (25 of 50). We then considered whether any of these frequently occurring residues ever appear together in the same leader peptide. Highlighted in Fig. 2 are the features that become apparent when the precursors with arginine at position -10 are compared. Of these 22 leader peptides, 19 also have a serine or a threonine at position -5 relative to the mature amino terminus. In addition, the residue at position -8 is always a hydrophobic residue, usually phenylalanine. Remarkably, 8 of 9 precursors that have been demonstrated

be cleaved in two places are among the 22 that have an arginine at position -10 (5, 9-15). The amino-proximal cleavage of most of these precursors occurs precisely two peptide bonds to the carboxyl side of arginine-10. The exceptions are the precursors for rat and mouse malate dehydrogenase [(S)-malate:NAD⁺ oxidoreductase, EC 1.1.1.37], which nonetheless have arginine (arginine-11) at position -2 relative to the initial cleavage site. Sequential cleavage by two proteases has not yet been demonstrated for every precursor for which two cleavages have been identified, but the available evidence is consistent with the same kind of two-step processing for most of the fungal and yeast proteins that we have demonstrated for mammalian matrix enzyme subunits (13-15, 67). In support of this idea, we have recently been able to confirm that mammalian matrix fractions containing only processing protease activity cleave the *Neurospora crassa* iron-sulfur protein subunit of ubiquinol: cytochrome c oxidoreductase (EC 1.10.2.2) to an intermediate form, while fractions containing also intermediate-specific protease produce mature subunit (J.P.H., unpublished

CONFIRMED TWO-STEP CLEAVAGE

R	Φ	S	----	
N F M V R N F R C G Q P L Q			N K V O L K G R	16 HumOTC
T S V V R H F W C G K P V Q			S Q V Q L K G R	17 MusOTC
T S M V R N F R Y G K P V Q			S Q V Q L K G R	18 RatOTC
A A L R R S F S T S A Q N N			A K V A V L G A	19 MusMDH
A A L R R S F S T S A Q N N			A K V A V L G A	20 RatMDH
A R A V R A L T T S T A L Q			G S S S S T F E	21 NeuFeS
F S C A R A F S Q T S S I M			S K V F F D L E	22 YscCOX4
K P A T R T L C S S R Y L L			Q Q K P V V K T	23 NeuCYCPH
-14 -12 -10 -8 -6 -4 -2			+1 +3 +5 +7	

TWO-STEP CLEAVAGE NOT CONFIRMED

R	Φ	S	----	
A V A T R T L S V S G R A Q			S S S E D K I T	24 BovADD
A E A S R S L S V S A R A R			S S S E D K I T	25 HumADD
F V G T R N L H A S N T R L			Q K T G T A E M	26 BovATPA
L T P S R S F Q T S A I S R			D I D T A A K F	27 BovATP1
Q V A R R E F Q T S V V S R			D I D T A A K F	28 BovATP2
L I G R R A I S T S V C V R			A H G S V V K S	29 BovCOX4
L V G K R A I S T S V C V R			A H E S V V K S	30 HumCOX4
K Q P S R I V H C S S T L P Q			D Q E T P P A S	31 Hum5ALAS
K P A P R T V S T S A A Q C Q			Q V K E T P P A	32 Rat5ALAS
P A A A R G L A T S A A R G Q			Q V E E T P A A	33 Chk5ALAS
G L L K R R F H W T A P A A			V Q V T V R D A	34 HumPDHB
L N N K R A F S S T V R T L			T I N K S H D V	35 YscPDHE3
A A L R R G L R T S V A S A			T S V A T K K T	36 RatOAT
A V L S R G V H S S V A S A			T S V A T K K T	37 HumOAT
-14 -12 -10 -8 -6 -4 -2			+1 +3 +5 +7	

FIG. 2. Comparison of residues surrounding arginine-10 in precursors carrying an arginine residue at this position and a hydrophobic residue (Φ) at position -8. Initial cleavage of precursors 16-23 occurs between positions -9 and -8 (-10 and -9 in precursors 19 and 20). Table entries are in the same order and are identified by reference number as in Fig. 1; refer to Fig. 1 for the complete name and GenBank accession number.

observation). The one precursor that we know of that is cleaved twice by matrix proteases and does not have the intermediate signature motif at the internal cleavage site is the proteolipid subunit 9 of *Neurospora crassa* F₁/F₀-ATPase. This precursor has been shown to be processed twice by matrix processing protease alone, both cleavage sites having an arginine at position -2 (11, 67).

We consider it likely that most, if not all, of the 22 precursors with arginine residues at -10 are cleaved twice by matrix proteases. Of the 14 for which an internal cleavage site has not been identified, 13 have not been subjected to the kind of scrutiny that would reveal intermediates; 1, the precursor to bovine adrenodoxin, has been incubated with a rat heart mitochondrial matrix fraction with processing protease activity but deficient in intermediate-specific protease [as evidenced by production of only the intermediate form of ornithine transcarbamylase (carbamoyl-phosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3) in a parallel gel track] (68). Under these conditions this precursor is cleaved to an intermediate-size species. Thus, in every case in which an arginine-10-containing precursor has been subjected to a test capable of detecting a cleavage intermediate, such an intermediate has been detected. We stress that, in some cases, a discriminatory test to detect two-step processing is not simple: some intermediates (the intermediate form of human ornithine transcarbamylase, for example) do not accumulate to detectable levels unless intermediate-specific protease is removed from the protease fraction or inhibited; other intermediates (adrenodoxin, for example) are difficult to resolve from the mature subunits on polyacrylamide gel electrophoresis.

Alignment of these 22 leader peptides according to their signature motif places arginine-10 at position -2 relative to the processing protease cleavage site. The number of matrix processing protease cleavage sites having an arginine at position -2 is more than doubled by this procedure, from 17 to 37. Thus, if all of the peptides containing the intermediate motif were cleaved internally by matrix processing protease, 74% of all matrix processing protease sites would have an

arginine at position -2. Does the existence of 14 precursors with *neither* arginine at -2 nor arginine at -10 indicate that these precursors are cleaved by yet a third mitochondrial protease? Perhaps, but at least one of these precursors, the precursor to the β subunit of *Saccharomyces cerevisiae* F₁/F₀-ATPase, is cleaved to its mature size by a 2000-fold purified matrix processing protease fraction (J.P.H., unpublished observations). Furthermore, mutation of arginine-10 in the human ornithine transcarbamylase precursor or rat malate dehydrogenase precursor to alanine has little effect on formation of mature subunits, indicating that precursors without arginine at position -10 can still be substrates for two-step cleavage (69, 70).

DISCUSSION

The apparent lack of sequence specificity of mitochondrial matrix protease has long been a dilemma for those studying the targeting of precursor proteins to mitochondria. One of the paradoxes is the lack of primary sequence similarity at the end of leader peptides. Another is the occurrence of alternative cleavages in particular leader peptides, depending on the source of protease or on the nature of attached passenger protein (14). Yet a third is the striking effect of changing certain amino acids within the leader peptide on cleavage (69). These paradoxes are resolved by our two-step model for proteolytic processing of mitochondrial precursor proteins. First, inappropriate comparison of once-cleaved with twice-cleaved precursors overestimates the amount of sequence diversity at cleavage sites. Second, the detection of apparently novel cleavage sites within leader peptides by construction of chimeric precursors reflects matrix processing protease sites revealed by inhibition of intermediate-specific processing of the chimera. Third, many (but not all) of the internal deletions and substitutions that inhibit proteolysis of precursors are close to or include residues adjacent to the initial cleavage site of twice-cleaved precursors.

We do not yet know the functional significance of two-step cleavage. No obvious functional correlation arises upon an examination of the putative twice-cleaved precursors in this collection. Proteins from three different mitochondrial sub-compartments carry the intermediate motif. Proteins from *Saccharomyces cerevisiae*, *Neurospora crassa*, birds, and mammals are represented in the category. There are subunits for heteromeric as well as homomeric enzymes included. While the arginine-10-containing leader peptides are on average longer (mean = 38 versus 27 for arginine-2-containing leaders), there are arginine-10-containing leader peptides of only 22 residues and arginine-2-containing peptides as long as 67 residues.

We propose that the intermediate octapeptides function predominantly to supply an optimized processing protease cleavage site in the precursor molecule. We speculate that the formation of the particular secondary structure required for matrix processing protease cleavage might be in competition with the formation of downstream structures important to the function of the mature "passenger" protein. The octapeptide might stabilize the correct structure for cleavage of the leader peptide by processing protease, while insulating this structure from the amino-terminal mature domain. Removal of the eight-amino acid joint by intermediate-specific protease would insure that the insulator need not conform to the structural requirements of either targeting peptide or mature amino terminus during protein evolution. Consistent with this idea are deletion-mutagenesis studies performed on yeast F₁-ATPase β subunit precursor and rat ornithine transcarbamylase precursor. Deletion of residues within the mature amino-terminal domain of ATPase β subunit precursor (which is cleaved once by matrix processing protease) interferes with processing protease cleavage, suggesting that

these residues are important in mediating protease recognition (8). Deletion of residues within the mature portion of rat ornithine transcarbamylase precursor (which is matured in two steps) has no effect, while deletions or substitutions within the last 10 amino acids of the leader peptide (the intermediate portion) block mature formation (9, 71).

Finally, can the motif we have identified be used to predict the mature amino termini of nuclear-encoded mitochondrial proteins, based on their predicted primary sequence? We looked within the amino-terminal 50 amino acids of all of the precursors in the collection for R-X-(F)-X-X-(S) sites, where R = arginine; X = other amino acid; (F) = phenylalanine, leucine, valine, or isoleucine (all hydrophobic residues); and (S) = serine, threonine, or glycine (see Fig. 2). There were 30 matches to the motif: the 19 found at the termini of leader peptides 19–37 and 12 which are not at the end of the leader peptide. Thus, the motif in its most general form is a poor discriminator of authentic amino termini. Nevertheless, recognition of the motif identifies the majority of exceptions to the current best “rule” that mitochondrial leader peptides end two residues after an arginine.

Note. Upon completion of this manuscript, we became aware of a similar survey compiled by von Heijne *et al.* (72). These authors also have noted the preponderance of arginine residues at positions –10 and –2 relative to the mitochondrial leader peptide cleavage site, and also suggest that this reflects two-step cleavage of a subset of mitochondrial precursors.

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