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Site directed mutagenesis of subunit 8 of yeast mitochondrial ATP synthase

Functional and import properties of a series of C-terminally truncated forms

Debra Nero*, Sue M. Ekkel, Lin-Fa Wang, David G. Grasso and Phillip Nagley

Department of Biochemistry and Centre for Molecular Biology and Medicine, Monash University, Clayton, Vict. 3168, Australia

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The function of the positively charged C-terminal region of mitochondrially encoded subunit 8 of yeast mitochondrial ATP synthase was investigated using derivatives truncated at each of the 3 positively charged residues (Arg^{37}, Arg^{42} and Lys^{47}). Each construct, allotopically expressed in the nucleus, was tested for its ability to import and assemble functionally into ATP synthase in yeast cells unable to synthesize mitochondrial subunit 8. The efficiency of import of each construct into isolated wild-type yeast mitochondria was also determined. One construct truncated at the penultimate residue of subunit 8 (Lys^{47}) functions in vivo and shows efficient import in vitro. Thus subunit 8 can function with only two positively charged residues. The remainder of the subunit 8 variants failed to rescue in vivo. Since they all show greatly reduced or undetectable import in vitro, presumably because of the increased hydrophobic character of the subunit 8 moiety in the chimaeric precursors, the status of these variants as regards assembly and function is not clear.

Mitochondrial ATPase complex; Allotopic expression; Site directed mutagenesis; Protein import; *Saccharomyces cerevisiae*

1. INTRODUCTION

Subunit 8 (Y8) is one of the 3 hydrophobic subunits in the membrane-embedded F_o sector of the yeast mitochondrial proton-translocating ATP synthase (mtATPase). This subunit is characterized by a relatively short length of 48 residues. It contains a central nonpolar region representing a single transmembrane stem and a polar C-terminus with 3 positively charged residues [1,2]. This polar tail probably lies on the matrix side of the inner membrane [3]. Intensive investigations in *Saccharomyces cerevisiae* have shown that Y8, a product of the mitochondrial *aap1* gene, is critical for the assembly of a functional F_o sector [4,5]. The other two hydrophobic components of the F_o sector, subunits 6 and 9, contain transmembrane helices with charged amino acids thought to be directly involved in proton conductivity across the membrane [1].

The approach we have adopted for studying the structure and function of mitochondrially encoded proteins is denoted allotopic expression [6]. This entails relocating an organellar gene to the nucleus and redirecting the encoded protein back to the organelle. In the case of Y8, an artificial gene has been made which encodes a chimaeric protein consisting of the N-terminal leader peptide of 66 amino acids (N9L) from mtATPase subunit 9 of *Neurospora crassa*, the first 5 amino acids of mature *N. crassa* subunit 9, 2 additional serine residues resulting from the DNA sequence at the fusion point, and the 48 amino acids of Y8 (Fig. 1A). This chimaeric protein, denoted N9L/Y8-1 [7], is imported into yeast mitochondria both in vivo [1] and in vitro [8], where it is processed at the natural cleavage point of *N. crassa* subunit 9 [9]. The imported, processed Y8 is assembled functionally into mtATPase in vivo; this is observed when N9L/Y8-1 is allotopically expressed in *aap1 mit*$^-$ cells which are incapable of synthesizing Y8 inside mitochondria [1]. A derivative construct, denoted N9L/Y8-2 (Fig. 1B), in which the supernumerary 7 amino acids interposed between the N9L leader and the mature Y8 moiety have been removed by site directed mutagenesis [7], can be imported into mitochondrial in vitro [7] and functions in vivo much like its N9L/Y8-1 parent [2].

The allotopic expression strategy therefore provides a means of systematically analyzing structure-function aspects of Y8. This communication focuses on the charged hydrophilic tail towards the carboxy terminus of the protein. A series of mutants has been constructed in vitro, in which Y8 is prematurely terminated at each

Correspondence address: P. Nagley, Department of Biochemistry, Monash University, Clayton, Vict. 3168, Australia

**Present address:* Department of Entomology, Cornell University, Ithaca, NY 14853, USA

Abbreviations: mtATPase, mitochondrial proton-translocating ATPase (ATP synthase); N9L, cleavable N-terminal leader from the precursor of *Neurospora crassa* mtATPase subunit 9; Y8, subunit 8 of yeast mtATPase; PCR, polymerase chain reaction

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of the 3 positively charged amino acids found in this region. These variant constructs were examined for their ability to rescue an *aap1 mit*$^-$ host in vivo and to be imported into wild-type mitochondria in vitro. The data show that the most distal charged residue (Lys^{47}) is dispensable for the function of Y8. All other truncated constructs studied here failed to rescue *aap1 mit*$^-$ cells in vivo. With one exception, these non-rescuing variants were unable to be imported in vitro, so that detailed assessment of their function in vivo could not be made.

2. MATERIALS AND METHODS

2.1. *In vitro mutagenesis*

Most of the mutants were generated in recombinant bacteriophage M13mp18 using the BioRad MutaGene in vitro mutagenesis kit. Two of the mutants, N9L/Y8-1 (R37-STP) and N9L/Y8-2(R37-STP), were generated in the phagemid construct pMT17 [10]. The oligonucleotides used for each mutagenesis changed the codon encoding the relevant charged amino acid to TAA, and had 8 bases on either side of the mutation homologous to the artificial gene encoding Y8 [11]. In general, cassettes of mutaged genes, flanked by *Bam*HI sites, were transferred to pUC18 for propagation. For expression in vivo and in vitro, the mutant genes were transferred to vectors pLF1 [1] and pSP64T [12], respectively.

2.2. *DNA sequence confirmation*

The sequence of the mutant alleles following recloning in each of the vectors was confirmed by DNA sequencing following amplification by an asymmetric polymerase chain reaction (PRC) procedure. DNA of purified plasmids, or extracts of bacterial or yeast cells in which the plasmids are propagated, were diluted to give a sample of approximately 5 amol vector, which was then used to generate single-stranded sequencing template by asymmetric PCR [13]. The reaction used 50 pmol of a forward primer (5'CCACCAGTAGAGACATGGG3') for pLF1 or (5'GGCTGGTTTAGTGGTAAC3') for pSP64T, close to the 3' end of the Y8 sequence in the respective vector DNA, and 0.5 pmol of a reverse primer (5'GGATCCATGGCCTCCACTCGTGTCCT3'), within the 5' end of the N9L leader sequence. After purification by phenol:chloroform extraction (1:1) and ethanol precipitation in the presence of 2.5 M ammonium acetate, a sample of the asymmetric PCR products was directly sequenced with an internal sequencing primer (5'AGATGACCTCCATCGTCAAC3'), located in the N9L leader sequence near the junction with the Y8 sequence, using the T7 dideoxy sequencing kit of Pharmacia.

2.3. *Strains and genetic analyses*

Saccharomyces cerevisiae strain J69-1B [*rho*$^+$), the derived *aap1 mit*$^-$ strain M31, and growth media for genetic analyses are as described [1]. The yeast expression vector pLF1 was used to express variant N9L/Y8 constructs in the *mit*$^-$ strain M31. Where rescue was observed (i.e. acquisition by transformant M31 cells of the ability to grow on ethanol), confirmation was obtained concerning the plasmid dependence of the rescued phenotype by genetic analysis, as described [1].

2.4. *Import into isolated mitochondria*

Using ^{35}S-labelled variant constructs expressed by transcription and translation in vitro [14], import experiments were carried out in vitro with mitochondria isolated from yeast strain J69-1B, as described [15].

3. RESULTS

3.1. *Nature of subunit 8 truncation mutants*

The series of truncation mutants we have prepared is shown in Fig. 1. Two sets of variants were made, starting with constructs encoding either N9L/Y8-1 or N9L/Y8-2 [7], which differ in the junction between the N9L and Y8 sequences (Fig. 1). In each case a codon specifying one of the 3 positively charged residues in the C-terminal tail, Lys^{47}, Arg^{42} and Arg^{37}, was converted to the termination codon TAA. It is convenient to describe separately for each of two sets of constructs based on either N9L/Y8-1 or N9L/Y8-2, the results obtained in both in vivo functional tests and in vitro import assays.

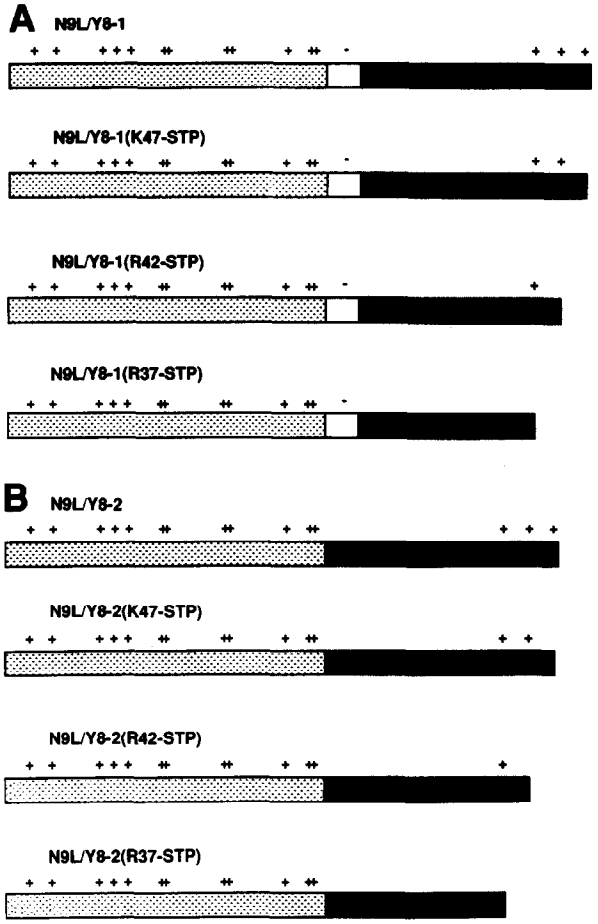

Fig. 1. Chimaeric fusion proteins and truncated derivatives used for allotopic expression. The truncated sets are based on the parent constructs N9L/Y8-1 (A) and N9L/Y8-2 (B) detailed in the text [7]. The nomenclature K47-STP, R42-STP and R37-STP, indicates that the codon for the designated amino acid has been converted to the TAA stop codon. The distribution of charged amino acids throughout each chimaeric polypeptide is indicated. Hatched blocks, N9L; solid blocks, Y8. In N9L/Y8-1 a 7 residue block is interposed [8] between N9L and Y8 (see text).

3.2. *The N9L/Y8-1 series*

The N9L/Y8-1(K47-STP) chimaeric protein does restore function to the mtATPase in vivo (Table I), as its allotopic expression in strain M31 confers on transformed cells the ability to grow on ethanol. The most distal positive charge (Lys^{47} in Y8 is thus dispen-

Table I

Properties of allotopically expressed variants of yeast mitochondrial ATP synthase subunit 8

Mutation	N9L/Y8-1		N9L/Y8-2	
	Rescue	Import	Rescue	Import
None	Yes	Yes	Yes	Yes
K47-STP	Yes	Yes	No	Weak
R42-STP	No	No	No	No
R37-STP	No	No	No	No

Behaviour of Y8 variants based on parent constructs N9L/Y8-1 and N9L/Y8-2 (Fig. 1) is indicated for rescue experiments (restoration of growth on ethanol following in vivo expression of construct in *aap1 mit*$^-$ strain M31) and import experiments (ability of precursor to be imported and processed in isolated mitochondria; Fig. 2).

sable in terms of the assembly and function of the yeast mtATPase. Whilst transformed M31 cells expressing N9L/Y8-1(K47-STP) show slower growth rates in liquid medium containing ethanol compared to those of J69-1B (data not shown), it is clear that Y8 can function with only the two positively charged residues Arg^{42} and Arg^{37} at its C-terminus.

Any further truncation of the N9L/Y8-1 construct at positively charged residues in Y8 beyond Lys^{47} (Fig. 1) prevents the chimaeric protein from restoring the function of the mtATPase, since no rescue of strain M31 was observed (Table I). In principle, such behaviour could be due to an impediment at one of several levels, for example: import of the chimaeric protein into mitochondria; assembly of the imported protein into mtATPase; or function of the enzyme complex containing the assembled protein. When these chimaeric proteins are further examined for their ability to be imported into mitochondria in vitro (Fig. 2A), a superficial correlation is found between in vivo rescue and in vitro import competence. Efficient import is seen with N9L/Y8-1(K47-STP), as evidenced by the production of a processed product close to the size of Y8 (lane 4), as occurs with the parent construct N9L/Y8-1 (lane 2). Note that import does not occur for either of the other truncated proteins based on N9L/Y8-1 (lanes 6 and 8); the data are summarized in Table I. It is thus not possible to define, from these results, the behaviour of the truncated derivatives R42-STP or R37-STP in assembly or function of mtATPase.

3.3. *The N9L/Y8-2 series*

The N9L/Y8-2 set of truncated proteins (Fig. 1B) are constructed to parallel the N9L/Y8-1 set, except that in each case the N9L leader directly abuts the Y8 sequence. In vivo rescue occurs with full length N9L/Y8-2 (Table I). The less efficient in vitro import of N9L/Y8-2 (Fig. 2B, lane 2) compared to that of N9L/Y8-1 (Fig. 2A) has now been repeatedly observed [16]. This indicates that the previously published data on in vitro import of N9L/Y8-2, in which comparable efficiencies were reported [7], was not a fully representative example. This differential import efficiency suggests that the 7 amino acids interposed between N9L and Y8 in N9L/Y8-1, but missing from N9L/Y8-2 (Fig. 1), do play a role in facilitating the relatively efficient import of N9L/Y8-1. The first 5 of these amino acids represent the N-terminus of mature subunit 9 of *N. crassa*, and they may thus be important for the efficient import and

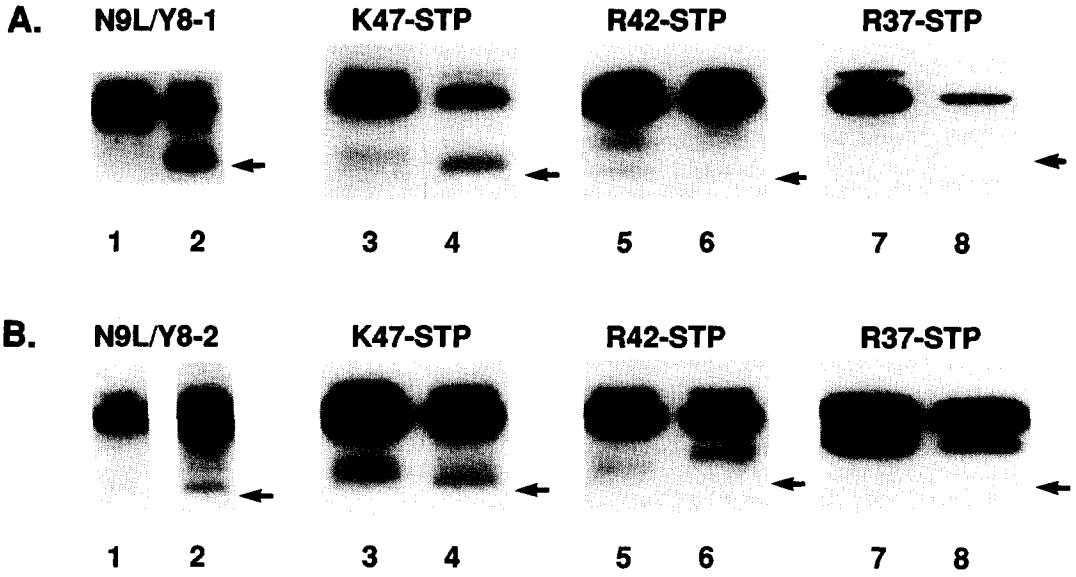

Fig. 2. In vitro import experiments on truncated derivatives of subunit 8. Radiolabelled fusion proteins (Fig. 1) based on parent constructs N9L/Y8-1 (A) and N9L/Y8-2 (B) were incubated with wild-type mitochondria under active import conditions [15]. Odd and even numbered lanes refer to samples of the import mix taken after 0 and 30 min, respectively, and represent autoradiograms of proteins separated by SDS-polyacrylamide gel electrophoresis. Arrows indicate the position of the Y8 standard for each pair of lanes.

processing of this hydrophogic protein directed by its natural leader (N9L). Their precise role is not understood; for example, it is not clear if the negatively charged glutamic acid in this short block of residues is important in precursor conformation.

Weak import in vitro of N9L/Y8-2(K47-STP) is evident (Fig. 2B), but the more extensively truncated variants R37-STP and R42-STP show no detectable import. The significantly reduced import of N9L/Y8-2(K47-STP) (Fig. 2B, lane 4) in comparison to that of its N9L/Y8-1 counterpart (Fig. 2A, lane 4) is demonstrated by the relatively high ratio of precursor to processed product remaining after an import reaction incubation period of 30 min. This parallels the decreased efficiency of in vitro import noted for full-length N9L/Y8-2 compared to N9L/Y8-1 (see above), thus reemphasizing the facilitative role of the 7 amino acids interposed betwen N9L and Y8 in precursor import.

Tests of in vivo rescue of *aap1 mit*$^-$ cells show that none of the truncated N9L/Y8-2 derivatives is able to restore mtATPase activity. The failure of N9L/Y8-2(K47-STP) to rescue strain M31 may either be due to the non-assembly of the imported protein or, if assembled, its inability to function. It is also not yet possible to define assembly and functional properties of the more extensively truncated N9L/Y8-2 derivatives.

4. DISCUSSION

A series of truncated variants of Y8 expressed allotopically has been studied in order to define the role of the positively charged C-terminal tail of Y8. The data obtained show the dispensability of the most distal charged residue (Lys^{47}) for the function of Y8. Conclusions concerning the assembly and functional properties of the shorter Y8 derivatives are, however, difficult to make in view of the impaired import properties of these truncated variants. Detailed information on the in vivo import and assembly behaviour of the truncated variants is required to evaluate the impediment to restoration of function in each case.

Why do these C-terminally truncated variants of Y8 show such inefficient import in vitro? The positively charged amino acids in the tail of Y8 evidently make an important contribution to the overall conformation of the chimaeric precursor (N9L/Y8-1 or N9L/Y8-2) in relation to its presentation to the import apparatus. The special role of protein conformation in the importation of precursors containing hydrophobic domains (such as N9L/Y8-1) has been emphasized by recent studies in this laboratory [17]. Thus the import capability of N9L/Y8-1 is abolished after pretreatment with 4 M urea, which is thought to unfold this precursor. Moreover, after urea-pretreatment N9L/Y8-1 binds avidly to the mitochondria, presumably by hydrophobic interactions with the membrane [17]. Clearly there is a delicate balance between unfolding, productive binding and non-productive binding, that must be maintained by the import system in dealing with precursors containing very hydrophobic domains.

Against this background, we can consider possible explanations of why the removal of the positive charges from the C-terminus of N9L/Y8-1 or N9L/Y8-2 renders the precursor non-importable. As successive truncations are introduced, the passenger protein joined to the N9L leader becomes increasingly hydrophobic. One reason for the failure of these truncated Y8 variants to be imported could be that the now very hydrophobic passenger moiety embeds into the mitochondrial outer membrane, leading to a similar non-productive binding as is observed following urea-pretreatment of N9L/Y8-1 [17]. Another possibility is that the precursor may now be maintained in a tightly folded conformation in which the leader peptide is not available to interact with the import apparatus as a key step in the import process [18]. In the latter case, one could imagine that the C-terminal positive charges on the chimaeric precursors containing full-length Y8 are electrostatically repulsive to those on the N9L leader (Fig. 1), and so help the precursor maintain an 'open' configuration. As the charged residues in Y8 are removed by truncation, the increasingly hydrophobic passenger moiety could interact with the non-polar face of the amphipathic N9L leader sequence [19], thus 'closing up' the molecular configuration of the precursor and limiting import.

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