Volume 325, number 1,2, 70–75 FEBS 12436 June 1993

Minireview

NADH-cytochrome b_5 reductase and cytochrome b_5 isoforms as models for the study of post-translational targeting to the endoplasmic reticulum

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Received 14 March 1993; revised version received 24 March 1993

Cytochrome b_5 and NADH-cytochrome b_5 reductase are integral membrane proteins with cytosolic active domains and short membrane anchors, which are inserted post-translationally into their target membranes. Both are produced as different isoforms, with different localizations, in mammalian cells. In the rat, the reductase gene generates two transcripts by an alternative promoter mechanism: a ubiquitous mRNA coding for the myristylated membrane-bound form, and an erythroid mRNA which generates both the soluble form and a nonmyristylated membrane-binding form. The available evidence indicates that the ubiquitous myristylated form binds to the cytosolic face of both outer mitochondrial membranes and ER. In contrast, two genes code for two homologous forms of cytochrome b_5, one of which is found on outer mitochondrial membranes, the other on the ER. The gene specifying the ER form probably also generates an erythroid-specific mRNA by alternative splicing, which codes for soluble cytochrome b_5. Possible molecular mechanisms responsible for the observed localizations of these different enzyme isoforms are discussed.

Endoplasmic reticulum; Outer mitochondrial membrane; Protein myristylation; Alternative promoter

1. INTRODUCTION

A large number of endoplasmic reticulum (ER) enzymes, many of which are involved in lipid and drug metabolism, have a cytosolically exposed active site and only a small lumenal domain (or possibly no lumenal amino acid residues at all) so that large portions of their polypeptide chain must not be translocated across the ER membrane. The biosynthesis of this class of proteins, their mechanism of targeting to the ER and of correct insertion into the phospholipid bilayer, represents an important aspect of ER biogenesis.

Studies on the biosynthesis of ER enzymes with cytosolically exposed active domains have shown that different pathways of insertion are followed by different proteins. As summarized in Table I, of the 11 proteins studied to date, five (all lacking a cleavable signal sequence), were shown to share the co-translational or Signal Recognition Particle (SRP)-dependent pathway mechanism with translocated proteins (for review see [11]), while the other 6 are inserted by an alternative pathway. In Table I, we have indicated this alternative pathway as 'post-translational', based mainly on the finding that the corresponding mRNA is recovered with free polysomes in cell fractionation experiments, in contrast to the situation for the co-translational, SRP-dependent proteins, the mRNA of which is always found associated with tightly bound polysomes. If the list of Table I will turn out to be representative, over half of the ER proteins with cytosolically located active domains might use such alternative mechanism(s) for membrane insertion.

We have chosen cytochrome b_5 (cyt b_5) and NADH-cytochrome b_5 reductase (reductase) as models for the study of post-translational insertion of ER membrane proteins, because they are well characterized integral membrane proteins, with opposite membrane topography. In the ER, cyt b_5 has a C-terminal hydrophobic membrane anchor (23 residues), and an active, cytosolic, N-terminal domain of $\approx$ 100 residues [12], while the reductase has an N-terminal myristylated anchor (24 residues) and a C-terminal active domain of $\approx$ 275 residues, which is oriented to the cytosol [13]. A good reason to use these proteins as models is that both are present as different isoforms with different localizations in mammalian cells. The availability of similar, but differently located forms of these proteins should be very helpful in the investigation of post-translational targeting pathways to intracellular membranes.

We will first give a little information on the structure and function of the ER forms of the two enzymes, and then review our work on the subcellular distribution of the isoforms and on the biogenetic relationships be-

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Abbreviations: cyt b_5, cytochrome b_5; ER, endoplasmic reticulum; OM cyt b_5, outer membrane cytochrome b_5; OMM, outer mitochondrial membrane(s); reductase, NADH-cytochrome b_5 reductase; SRP, Signal Recognition Particle.

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tween them. Finally, we will discuss the interactions of these enzymes with membranes and possible targeting mechanisms.

2. STRUCTURE AND FUNCTION OF THE ER FORMS OF CYT b_5 AND NADH-CYT b_5 REDUCTASE

Both cyt b_5 and reductase belong to protein families with members in evolutionarily distant organisms. Cyt b_5 belongs to a family of hemoproteins characterized by the so-called 'cytochrome b_5 fold' [14], while reductase is a member of a recently discovered flavoenzyme family of dehydrogenases-electron transferases [15].

Proteins of both families have diverse intracellular localizations. They may be cytosolic (as for plant NADH-nitrate reductase), peripherally associated with a membrane (as for chloroplast ferredoxin-$NADP^+$ reductase), or integrated in the membrane by a short hydrophobic stretch with the hydrophilic active domain exposed to the cytoplasm (as for mammalian reductase and cyt b_5). Members of both families are often found as domains of larger multicenter redox enzymes, suggesting that both cyt b_5- and reductase-like proteins arose early in evolution and that they were used as building blocks for the construction of more complicated proteins by gene fusion events (reviewed in [16]).

In mammalian cells (with the exception of erythrocytes - see below) cyt b_5 and reductase have been converted to membrane-bound enzymes, with their active domains exposed at the cytosol. The localization of this electron transport chain on membranes concentrates the components to a two-dimensional space, and is thought to promote a discreet orientation of the active domains, which would facilitate interaction of the enzymes with each other [17]. This interaction is thought not to involve the formation of stable complexes between the two enzymes [18]. Cyt b_5 is known to donate its electrons to a variety of acceptors involved in diverse aspects of lipid metabolism, such as fatty acid desaturation [19] and cholesterol biosynthesis [20]. Cyt b_5 can also accept electrons from an alternative reductase, NADPH-cyt P-450 reductase [19], and participate in drug metabolism, interacting with some (but not all) forms of cyt P-450 [21]. Thus, while reductase has only one acceptor on the ER membrane, cyt b_5 is promiscuous, using multiple acceptors and also more than one donor. In all these interactions, the same negatively charged surface area surrounding the exposed heme edge of cyt b_5 appears to be involved, with formation of complementary charge pairs between carboxylate groups of that region and appropriately spaced amino groups at the surface of the various acceptors or donors [22 and references therein].

3. ISOFORMS OF CYT b_5 AND OF NADH-CYT b_5 REDUCTASE AND THEIR SUBCELLULAR DISTRIBUTION

While cyt b_5 and reductase are present in most mammalian cells as membrane-bound proteins, both enzymes exist also in soluble form in erythrocytes. In the erythrocyte cytoplasm, soluble cyt b_5 reduces methemoglobin, and the reductase-cyt b_5 system constitutes the most important enzymatic system for the maintenance of hemoglobin in the reduced state. Erythrocyte reductase deficiency is the most common cause of hereditary methemoglobinemia in man [23].

In addition to these soluble isoforms and to the 'classical' microsomal enzymes, novel membrane-bound isoforms of cyt b_5 and of the reductase have been discov-

Table I

Mode of insertion* of mammalian ER proteins with cytoplasmically oriented active domains

Co-translational	Post-translational
Sarcoplasmic reticulum Ca^{2+} ATPase [1]**	Microsomal aldehyede dehydrogenase [5]
Cytochrome P-450 (phenobarbital-induced) [2]	Cytochrome b_5 [3,6]
Epoxide hydrolase [3]	Heme oxygenase 1 [7]
Hydroxymethylglutaryl-Coenzyme A reductase [4]	NADH-cytochrome b_5 reductase [8,3]
NADPH-cytochrome P-450 reductase [3]	α subunit SRP receptor [9]
	stearyl CoA desaturase [10]

* The evidence on the mode of insertion of the listed proteins is based on the identification of their site of synthesis (free or bound polysomes) and/or on the demonstration of SRP-dependence or independence in cell-free systems.

**Because of limitations in the length of the bibliography for FEBS Letters minireviews, the list of references quoted here is not complete.

ered, the biogenesis and significance of which will be discussed here.

3.1. *Isoforms of cyt* b_5*: soluble, microsomal and outer mitochondrial membrane forms*

Initial comparison of the primary structures of the soluble and microsomal forms of bovine cyt b_5 demonstrated that the soluble isoform was identical to the NH_2-terminal, cytoplasmic catalytic domain of the microsomal enzyme (residues 1–97) [24]. This suggested that the two isoforms were products of the same gene. The authors also proposed that the soluble form might derive from the microsomal enzyme by proteolytic release of the catalytic domain from the hydrophobic COOH-terminal membrane anchor during erythropoiesis. However, when sequence comparison was carried out in other mammalian species, it was found that the C-terminal residue of the soluble form had no counterpart in the microsomal enzyme (e.g. ref [25]). These data suggested that soluble and microsomal forms are generated from separate mRNAs. Indeed, Giordano and Steggles [26] recently isolated a cDNA from human reticulocytes which codes for soluble cyt b_5 and which has a 24 nt insertion between codons 96 and 97 of the corresponding liver transcript. The inserted sequence starts with two in-frame codons followed by a stop codon. Since the rest of the sequence of this cDNA is identical to the liver transcript, the data strongly suggest that the mRNAs coding for the microsomal and soluble forms of cyt b_5 are generated from the same gene by tissue-specific alternative splicing, as illustrated in Fig. 1.

It has been known for many years that outer mitochondrial membranes (OMM) contain spectrally detectable cyt b_5, and it was believed that microsomal cyt b_5 accounted also for the outer mitochondrial form. Indeed, anti-cyt b_5 antibodies recognized the cytochrome on OMM in immuno-electron microscopy [27] and en-

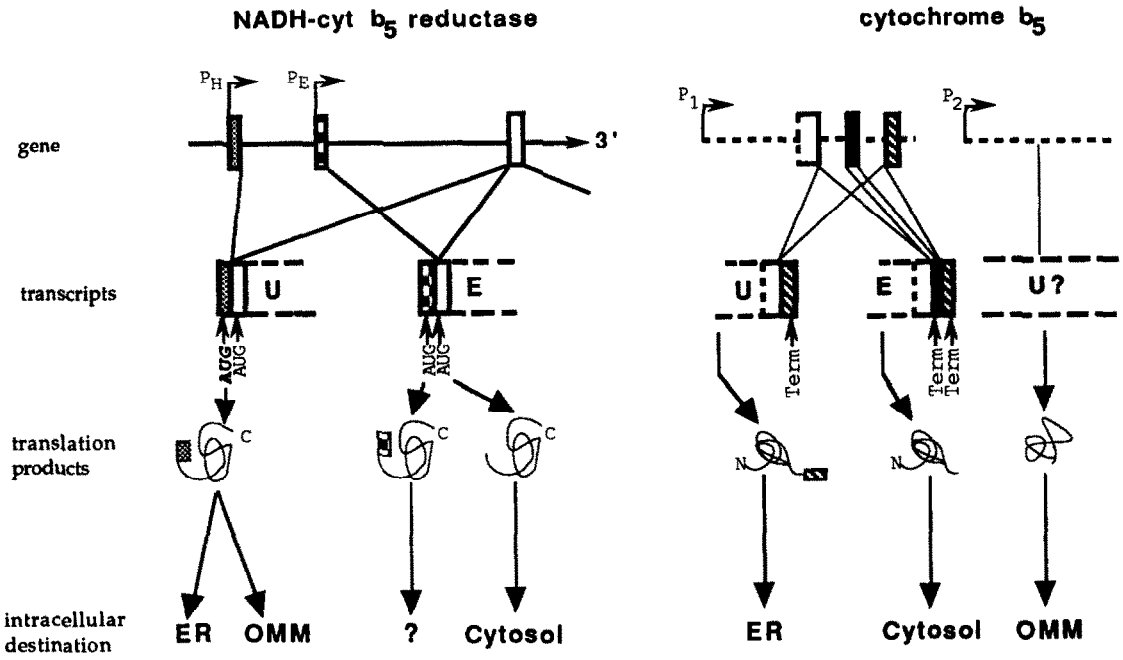

Fig. 1. Schematic representation of the biogenesis and intracellular targeting of rat NADH-cyt b_5 reductase and cyt b_5 isoforms. The figure illustrates the following points: (i) one gene generates the known reductase isoforms by a combination of alternative promoters and alternative initiation of translation, while two genes are involved in the generation of the ER and outer mitochondrial membrane (OMM) forms of cyt b_5; (ii) while the same (myristylated) form of reductase appears to be targeted both to ER and to OMM, the cyt b_5 forms on these two membranes are products of different genes; (iii) the soluble anchorless forms of the reductase and cyt b_5 differ from the ER forms at the N-terminus and C-terminus respectively, and are generated by an alternative promoter mechanism combined with initiation of translation from a downstream AUG in the case of the reductase, and by alternative splicing at the 3′ end of the gene in the case of cyt b_5. The symbols in the left part of the figure (reductase gene) represent: P_H, housekeeping promoter; P_E, erythroid promoter; stippled rectangle, exon which contains the coding sequence for the myristylation consensus; checkered rectangle, reticulocyte-specific exon, encoding 13 non-charged aminoacids; open rectangle, first exon common to the 2 transcripts (only the 5′ portion of the gene is shown); U, ubiquitous transcript; E, erythroid transcript; AUG in boldface, initiation codon in optimal context for translation initiation; AUGs in normal printing, initiation codons in suboptimal contexts for initiation. The symbols at the right of the figure (cyt b_5 genes) represent: P_1 and P_2, unidentified promoters of genes coding for cyt b_5 isoforms (both genes are shown as dashed lines to indicate that they have not yet been cloned and characterized); open rectangle, exon common to ubiquitous and erythroid transcript, coding for the C-terminal portion of the cytosolic domain (the 5′ border is not known, and is therefore represented as a dashed line); filled rectangle, alternatively spliced exon, which is included in the erythroid transcript, and whose third codon is a termination codon; striped rectangle, exon coding for the C-terminal membrane-anchor of the ER form of cyt b_5; U, E, and U?, ubiquitous, erythroid, and probably ubiquitous transcripts, respectively. See text for further explanations.

zyme inhibition experiments [28]. Subsequently, however, a tryptic hemopeptide with b_5 spectral characteristics was purified from mitochondria, sequenced [29], and found to be different from the microsomal tryptic hemopeptide, with which it shared ≈ 60% of its sequence. Antibody inhibition studies suggested that the newly characterized hemopeptide was derived from an OMM isoform of cyt b_5 (OM-cyt b_5) [30]. Since the sequence difference between microsomal and OM- cyt b_5 are distributed throughout the hemopeptides, the results of Lederer et al. [29] also indicated that these two isoforms are products of different genes.

An interesting question opened by the discovery of OM-cyt b_5 was whether any microsomal cyt b_5 was on the OMM at all. Indeed, the two isoforms would be expected to cross-react with polyclonal antibodies, thus casting doubt on all previous immunological studies [27,28,30]. An exclusion of microsomal cyt b_5 from OMM would be in contrast with the widely held belief that cyt b_5 can insert into any phospholipid bilayer, thanks to its C-terminal insertion sequence (e.g. [1]).

D'Arrigo et al. [31] have recently investigated this problem, using antipeptide antibodies monospecific for each of the two cyt b_5 isoforms. They first used the anti-OM-cyt b_5 antibody to identify the holocytochrome as a 23 kDa polypeptide, bound to the OMM in an alkali and urea-resistant fashion. They then used the two antibodies to probe Western blots of well-characterized subcellular fractions. Quantitative analysis revealed that OM-cyt b_5 was not present on ER membranes, while microsomal cyt b_5 was present on OMM at extremely low concentrations, less than 5% its concentration on ER membranes. The analysis also showed that most (if not all) of the spectrally detectable cyt b_5 on the surface of mitochondria is the OM-specific form, which, however, is expressed at lower concentration on its target membrane than its microsomal counterpart is on the ER membrane. These data suggest that novel post-translational targeting mechanisms result in the exclusion of microsomal cyt b_5 from OMM and direct it to the ER. In agreement with our conclusion, it has recently been shown that microsomal cyt b_5, overexpressed in COS cells, co-distributes with microsomal markers in cell fractionation experiments [32].

3.2. *Myristylated and non-myristylated isoforms of NADH-cyt* b_5 *reductase*

The membrane anchor of microsomal reductase is N-myristylated [13]. The first 7 residues of the primary translation product (which contains the initiator Met) constitute the signal for myristylation, a co-translational modification catalysed by a cytoplasmic enzyme (for review see [33]. The myristylation consensus sequence is followed by a group of 3 residues, of which the third one is basic, and a subsequent stretch of 14 uncharged amino acids.

As in the case of cyt b_5, also for the reductase, amino acid sequence analysis revealed that the soluble form was identical to the cytoplasmic catalytic domain of the membrane-bound myristylated enzyme, and it was suggested that the soluble enzyme was generated by post-translational proteolysis during erythrocyte maturation [34]. Recently, however, we have shown [35] that soluble reductase is encoded by a separate mRNA, which is generated from the reductase gene by an alternative promoter mechanism (see Fig. 1). The first exon of the reductase gene contains the 5′ non-coding sequence, the initiator AUG, and only 6 codons which specify the myristylation consensus (stippled rectangle in reductase gene in Fig. 1). This exon is preceded by a housekeeping promoter. It is expressed ubiquitously and spliced to the third exon of the gene.

A specific erythroid mRNA is generated by initiation of transcription from exon 2 (checkered rectangle in Fig. 1), which is preceded by an erythroid-specific promoter. Thus, the codons specifying the myristylation consensus can be excluded or included in the transcript in a tissue-specific manner. The erythroid mRNA was found to be bifunctional (Fig. 1). It generates two polypeptides: a minor product, which is an N-terminally extended form of the reductase and which starts from the first initiation codon (product with checkered rectangle at its N-terminus in Fig. 1), and a major product which begins from a downstream AUG. The generation of these two products can be explained by 'leaky' scanning [36]. The first initation codon is weak, so that a large proportion of small ribosomal subunits bypass it and initiate translation at the downstream AUG. This downstream AUG is in the common portion of the two reductase transcripts and its use leaves the entire membrane anchor out of the polypeptide product, with generation of the soluble form of the reductase. It is not used in the ubiquitous transcript, because the first AUG, which precedes the myristylation consensus, is in a strong context for initiation (bold-faced AUG in ubiquitous reductase transcript of Fig. 1) and does not permit 'leaky' scanning.

One unexpected finding of this study [35] was the existence of the hitherto undescribed third reductase isoform (the N-terminally extended erythroid polypeptide). This isoform has at its N-terminus 12 uncharged reticulocyte-specific residues in addition to 17 residues of the membrane anchor of myristylated reductase (residues 7–23 of the myristylated form). Thus, it has a hydrophobic, non-myristylated N-terminal region, and, indeed, it was found that it interacts with microsomes in vitro. Since reductase is present on the plasma membrane of rat erythrocytes [37], it is tempting to speculate that the N-terminal anchor of this third isoform has specific targeting information which differs from that of the anchor of the ubiquitously expressed myristylated enzyme.

In liver cells, reductase is present on OMM as well as on ER membranes ([38] and references therein). West-

ern blot analysis of well characterized liver subcellular fractions showed that the reductase is most concentrated in OMM, followed by ER membranes, and that it is present in low concentration or absent in other membranes [38]. In contrast to the situation for cyt b_5, the OMM and microsomal forms of the reductase appear to be the same protein. They have the same apparent molecular weight, generate identical peptide maps, and are immunologically indistinguishable [39]; they also have the same amino acid composition and are both myristylated [40]. Moreover, only one reductase transcript was detected in rat liver, and results of Southern blotting of rat genomic DNA were consistent with the presence of a single reductase gene [41]. Thus, it appears that the same protein inserts into two biogenetically unrelated membranes. Nontheless, it cannot at present be excluded that minor differences exist between the mitochondrial and ER forms, which have escaped the analyses carried out so far.

What is the function of the OMM reductase–cyt b_5 system? The microsomal acceptors of cyt b_5 have not been found on OMM, but Ito et al. [42] showed that OM cyt b_5 is involved in ascorbate regeneration from semidehydroascorbate. Why this system has been placed on the surface of mitochondria rather than on the ER is an open question.

Fig. 1 summarizes the available information on the biogenenesis and subcellular localization of cyt b_5 and reductase isoforms reviewed above.

4. INTERACTIONS WITH MEMBRANES AND POSSIBLE TARGETING MECHANISMS

What are the molecular mechanisms which result in the subcellular distribution of cyt b_5 and reductase isoforms described above? While it is likely that OM cyt b_5 shares its targeting mechanism with other OMM proteins (for review see [43]), the presumptive post-translational targeting pathways to the cytosolic face of the ER remain to be discovered.

A first point of ignorance concerns the exact topography of the membrane anchors. Although it is known that they penetrate deeply into the interior of the bilayer, there is no general agreement as to whether they span the entire bilayer, or whether they are confined to the cytoplasmic leaflet in a hairpin conformation (e.g. [12,44–46] and references therein). In the latter case, the insertion of the enzymes in the target membrane would not involve the translocation of any amino acid residues.

Another point of confusion has arisen from in vitro binding studies to liposomes. Early experiments showed that both microsomal cyt b_5 and reductase could associate in an active form with preformed liposomes. These results lent support to the idea that proteins like cyt b_5 and reductase can opportunistically insert into any bilayer also in vivo. Later, however, it was observed that both proteins, bound to preformed liposomes, although interacting directly with the phospholipid fatty acyl chains, were in a 'loose', exchangeable conformation different from that of the endogenous tightly bound proteins [17,47]. Thus, the in vitro binding experiments carried out in the past (e.g. [1]) which made no distinction between the two types of interaction, are of doubtful significance.

Future research will be directed to uncovering on the one hand the signals and, on the other hand, the features of the membranes involved in these as yet uninvestigated targeting pathways. The availability of cDNA clones for cyt b_5 and reductase, and the possibility of expressing wild-type, mutant or chimaeric forms of the enzymes in vivo and in vitro, should be very helpful in the search for putative targeting signals. Using this approach, Mitoma and Ito [32] have recently reported that, in COS cells, the carboxy-terminal 10 amino acid residues of microsomal cyt b_5 are necessary for its targeting to the ER. Vergeres and Waskell [46] found that a mutant rat microsomal cyt b_5, in which Ala-131 and Glu-132 (at positions −2 and −3 from the COOH terminus) were changed to lysines, remained normally membrane associated. Further work with mutants is required to nail down the targeting sequence of microsomal cyt b_5. No experiments with mutant forms of the reductase have been carried out yet. An interesting point to investigate is the role of the N-terminally bound myristic acid. Covalently bound myristic acid is thought to be involved in protein-protein interactions [33], as, for instance, in the case of the interaction of the myristylated tyrosine kinase $p60^{v\text{-}src}$ with a 32 kDa receptor [48]. Another point of interest in the targeting of the reductase is whether the same molecular feature targets it to OMM and ER membranes or whether two distinct signals coexist in the same protein.

Concerning the target membranes involved in recognition, it seems reasonable to search for specific protein receptors involved in targeting, since lipids alone are not sufficient for physiologically relevant binding of the enzymes (see above). To do this, it will be necessary to establish cell-free systems which distinguish between physiologically relevant and irrelevant binding. There are two ways in which these putative protein receptors could work. A 'stoichiometric' receptor would work by binding with high affinity to the post-translationally targeted protein, forming a stable complex with it. A 'catalytic' receptor would act by somehow facilitating insertion of the post-translationally targeted protein, which would then dissociate from the receptor and remain stably integrated in the bilayer.

In conclusion, mammalian cells use a combination of genetic tricks (alternative genes, alternative promoters, alternative splicing, alternative initiation codons) to produce differently localized isoforms of cyt b_5 and cyt b_5 reductase. The mechanisms by which these, and other membrane proteins with similar topography, reach their

target membranes from their site of synthesis on free polysomes, remain to be elucidated.

Acknowledgements: We thank Dr. K. Howell for critically reading the manuscript. Work of the authors reported in this paper was partially supported by C.N.R. grants: Progetto finalizzato Ingegneria Genetica, Progetto finalizzato Biotecnologie e Biostrumentazione, and Progetto Speciale PS0408.

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