Folding, Assembly, and Stability of Transmembrane Cytochromes

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Abstract: Heme cofactors of transmembrane cytochromes are crucial for mediating charge transfer across membranes during aerobic and anaerobic electron transport reactions. In addition, several *in vivo* observations indicate that heme cofactors directly or indirectly impact the folding, assembly, and stability of individual transmembrane cytochromes and also of oligomeric cytochrome complexes. In this article, we review the function of heme molecules for the formation of transmembrane cytochromes *in vivo* and *in vitro*, and discuss distinct steps during the assembly of cytochromes and cytochrome complexes. We furthermore highlight the need of *in vitro* studies using isolated apo-cytochromes for analyzing the role of the heme cofactor for folding and stability of single proteins or larger cytochrome complexes. In combination with *in vivo* studies, this approach holds the potential to obtain a comprehensive picture on how binding of the heme cofactor to its apoprotein determines not only the biological function of a heme protein but also its three-dimensional structure.

Keywords: Co-factor, cytochromes, cytochrome oxidase, electron transfer, membrane protein assembly, Two-Stage-Model.

INTRODUCTION

The determinants for folding and stability of membrane proteins are still largely enigmatic and many open questions remain to be answered. Although important details of the molecular machineries involved in integrating a membrane protein into the lipid bilayer have been worked out during the last few years, the mechanisms of how individual membrane proteins assemble into oligomeric complexes and how they acquire their final three-dimensional structure is still not entirely clear. This in particular holds true for cofactor containing membrane proteins, in which the cofactor is considered to be an important determinant for structure and stability. Although the biological functions of these cofactors are usually well defined and their biophysical features explored in many details, several important issues remain to be solved. These include the question of at which assembly stage cofactors bind to the apoprotein and whether binding of the cofactor is an essential step during folding and assembly of membrane proteins. Further important issues are whether these cofactors bind to an unstructured polypeptide chain and thus initiate the subsequent folding or whether cofactors bind to an at least partially pre-folded transmembrane protein. Equally important is the question of whether cofactor insertion in general is a catalyzed process involving specific assembly factors, like chaperones. In this review we will focus on the assembly of membrane integral cytochromes, which are crucial components of aerobic and anaerobic electron transfer chains in almost all living organism. We will discuss *in vitro* and *in vivo* data on the assembly of transmembrane cytochromes with a special emphasis on integral *b*-type cytochromes.

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TRANSMEMBRANE CYTOCHROMES

Heme molecules are frequently found in both soluble and transmembrane proteins, and these proteins are collectively called heme proteins. Depending on the nature of the proteinacous part they execute distinct, yet essential physiological functions like oxygen transport (hemoglobin & myoglobin), detoxification of reactive oxygen species (catalases & peroxidases), or electron transport as in the case of cytochromes. While most transmembrane cytochromes are involved in mediating charge transfer across membranes, in some cases the heme molecules are directly involved in catalyzing chemical reactions, like *e.g.* in the case of the terminal oxidases. In general, a heme group consists of a porphyrin ring system with a central iron atom, which is ligated to the pyrrol-nitrogen atoms. One or several heme molecules can be ligated covalently and/or non-covalently to a polypeptide chain and, depending on the exact structure of the heme group and its binding to the protein, several classes of cytochromes are distinguished (Fig. **1**). Differences in the side chains of the protoporphyrine ring system determine the specific spectral properties (α, β, and γ bands) of cytochromes which are diagnostic for the different classes (compare Fig. **2**). The prototype of a heme molecule is Fe-protoporphyrin IX, which is found in hemoglobin, *b*-type cytochromes, and *c*-type cytochromes. The major structural difference between the latter two is that in *b*-type cytochromes the heme group is non-covalently attached to the apoprotein, while in *c*-type cytochromes it is covalently attached. This covalent attachment is typically achieved *via* thioether bonds to a characteristic CxxCH amino acid motif of the polypeptide chain. The thioether is formed between the two cysteins of the motif and the original vinyl groups of the heme molecule. Chemical modifications of the basic Fe-protoporphyrin IX structure involves the farnesylation of a vinyl group by heme O synthase, resulting in heme *o*. In a second reaction, heme A synthase oxidizes a methyl side chain to an aldehyde, thus forming heme *a*. As a result, *a*-type cytochromes contain a long farnesyl side chain attached

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to the porphyrin ring and a formyl group instead of a methyl substituent as in protoporphyrin IX. So far, both heme *o* and heme *a* have been found only as cofactors of cytochrome oxidases. In *d*-type cytochromes one vinyl group of the porphyrin ring is oxidized to an alcohol and the C ring of the tetrapyrole is saturated (Fig. **1**).

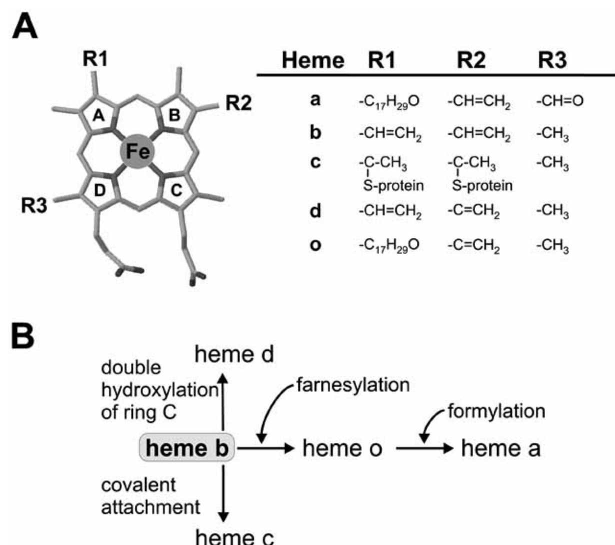

Fig. (1). Chemical structures of cytochrome heme groups and heme formation.

A. All heme molecules have the same basic structures and are derived from the *b*-type heme (protoporphyrin IX). The nomenclature of the porphyrine rings (A-D) is indicated in the heme structure. The individual hemes can have different substitutes at side chains as summarized in Table. **B**. Individual hemes are formed by chemical modification of the prototypic heme *b* structure. For details see the text.

With the exception of *c*-type cytochromes, the heme is non-covalently linked to the polypeptide chain. In most cytochromes the fifth and sixth coordination sites of the iron atom are occupied by strong field ligands, regardless of the oxidation state of the iron centre. These ligands are usually histidines, but methionine, cysteine, tryptophan, lysine, and tyrosine can also be found. In some cases the sixth ligand position remains unoccupied to allow an external ligand to bind, and in these cases the heme is directly involved in catalytic reactions (*e.g.* oxygen binding and reduction during the catalytic cycle of terminal oxidases). The central heme iron atom undergoes oxidation-reduction between the two oxidation states Fe(II) and Fe(III), and the spectral characteristics of the cytochromes change dramatically with the redox state of the central iron atom (Fig. **2**). Therefore, the characteristic absorbance spectra of cytochromes can be used to identify a certain member, and often the maxima of the α-band is used as suffix to discriminate between different cytochromes of the same type, *e.g.* cytochrome b_{556}.

TRANSMEMBRANE CYTOCHROMES IN RESPIRATORY AND PHOTOSYNTHETIC ELECTRON TRANSFER CHAINS OF BACTERIA

Transmembrane cytochromes are essential parts of electron transfer chains in bacteria as well as in eukaryotes [1-3]. The properties of these cytochromes have been mainly studied in bacterial model systems and a representative collection of cytochromes from phylogenetically distinct model organisms is listed in Table **1**.

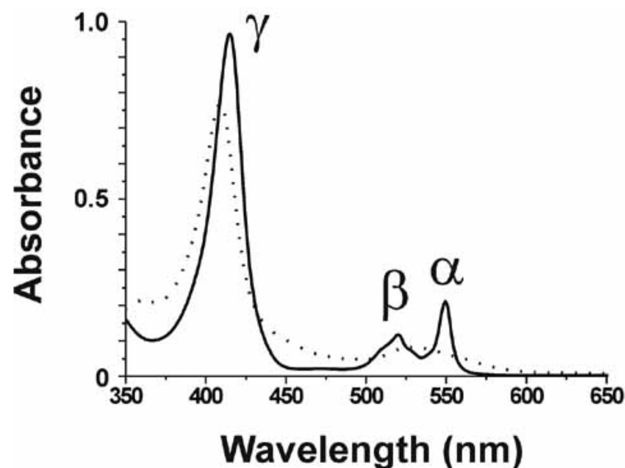

Fig. (2). Absorbance spectra of horse heart cytochrome c under reducing (solid line) and oxidizing (dashed line) conditions. The positions of the α, β, and γ absorbance maxima are indicated for the reduced cytochrome spectrum. The α and β maxima are often not as clearly defined under oxidizing conditions as under reducing.

In *E. coli*, transmembrane cytochromes can be found in several membrane protein complexes (Table **1**). The succinate dehydrogenase (complex II of the aerobic respiratory chain) contains a *b*-type cytochrome (cytochrome b_{556}), in which the heme group is sandwiched in between the two hydrophobic subunits SdhC and SdhD of the complex [4, 27]. Also in the transmembrane parts of formate dehydrogenase [5] and nitrate reductase [28] from *E. coli* non-covalently attached *b*-type hemes can be found (Table **1** and Fig. **3**). In the terminal quinole oxidases of *E. coli*, *b*-type, as well as *o*- and *d*- type hemes can be found in several distinct complexes (Table **1** and Fig. **3**). Importantly, due to the absence of heme A synthase, *E. coli* is unable to synthesize *a*-type hemes [29]. Since *E. coli* directly oxidizes quinol by quinol oxidases, a cytochrome bc_1 complex and the prototype of a terminal oxidase, the cytochrome aa_3 oxidase, are absent in *E. coli* membranes [1].

In the photosynthetic electron transfer chain of cyanobacteria *b*-type cytochromes can be found in both photosystem 2 and the cytochrome b_6f complex [3]. Cytochrome b_{559} is an essential subunit of photosystem 2 of both, chloroplasts and cyanobacteria. The protein consists of one α- and one β-subunit, which both span the membrane once. The two subunits sandwich one heme in between and the heme is non-covalently ligated by conserved histidine residues located in the transmembrane regions of the two subunits [30, 31]. Cytochrome b_6 is a core subunit of the cytochrome b_6f complex and the four-helix-bundle protein binds two hemes non-covalently (Fig. **3**). The two heme molecules are ligated by conserved histidine residues, which are located in helices B and D of the polypeptide [32]. Besides the classical components of the photosynthetic electron transfer chains, transmembrane cytochromes can also be found in the terminal oxidases of cyanobacteria (Table **1**).

Table 1. Representative Members of Bacterial Transmembrane Cytochromes

	Type of Cytochrome(s)	Function	Comments	Ref.
Escherichia coli				
Succinate dehydrogenase	SdhC + SdhD (one *b*-type)	Succinate-quinol oxidoreductase (complex II of the respiratory electron transfer chain)		[4]
Formate dehydrogenase	FdnI/Cytochrome b_{556} (two *b*-type)	Formate dehydrogenase Menaquinone reductase		[5]
Cytochrome *bd* oxidase	• Cyd A (one *b*-type) • Cyd A/B (one *b*-type, one *d*-type)	Quinol oxidase	Expressed at low levels of oxygen. A different cytochrome *bd* type oxidase is expressed under microaerobic conditions	[6]
Cytochrome bo_3 oxidase	Subunit I (one *b*-type, one *o*-type)	Quinol oxidase	Operates at high oxygen	[7]
Nitrate reductase	NarI (two *b*-type)	Nitrate-menaquinol oxidoreductase		[8]
Rhodobacter capsulatus				
Cyt c_y	*c*-type heme	Electron carrier in photosynthesis and respiration		[9]
DorC	Penta-heme *c*-type heme	Electron transfer from ubiquinol to DMSO-reductase		[10]
HupC (HupM)	*b*-type heme	Subunit of hydrogen-uptake hydrogenase		[11]
Sqr	Flavocytochrome *c*	Sulfide-quinone reductase	Probably only attached to the periplasmic side of the membrane	[12]
Succinate dehydrogenase	*b*-type heme	Complex II of the respiratory chain		[13]
Cytochrome bc_1 complex	PetC (one *c*-type heme) PetB (di-heme *b*-type)	Complex III of the respiratory chain		[14]
Cytochrome cbb_3 oxidase	• CcoN (two *b*-type hemes) • CcoO (*c*-type mono-heme) • CcoP (*c*-type di-heme)	Complex IV of the respiratory chain, Cytochrome *c* oxidase		[15]
Cytochrome *bd* oxidase	• CydA (two *b*-type hemes) • CydB (*d*-type heme)	Complex IV of the respiratory chain, Ubiquinol oxidase	Predicted features	[16]
***Synechocystis* sp PCC 6803**				
Photosystem 2	• Cytochrome b_{559} (one *b*-type) • Cytochrome c_{550} (one *c*-type)	Uses light energy to reduce plastoquinon	Cytochrome c_{550} is a peripheral subunit of photosystem 2 and not directly attached to the membrane	[17]
Cytochrome b_6f complex	• Cytochrome b_6 (two *b*-type, one *c*-type) • Cytochrome *f* (one *c*-type)	Quinol-plastocyanine (cytochrome *c*) oxidoreductase		[18]
Cytochrome aa_3 Oxidase		Cytochrome *c* oxidase	Plastocyanine can also serve as a electron donor	[19-21]
ARTO (Cytochrome bo_3 type oxidase ?)		Cytochrome *c* oxidase	Largely uncharacterized, function predicted	[19-21]
Cytochrome *bd* oxidase		Quinol oxidase	Only indirect evidence for the function as a plastiquinol oxidase	[20-22]
Solfolobus acidocaldarius				
Cytochrome $b_{558/566}$	One *b*-type	Function unknown		[13]
SoxABCD oxidase	• Cytochrome aa_3 (two *a*-type) • Cytochrome a_{587} (one *a*-type)	Quinol oxidase		[24, 25]
SoxM terminal oxidase	• SoxG/Cytochrome a_{587} (two *a*-type) • SoxM/Cytochrome b_{562} (two *b*-type)	Quinole oxidase; function as a cytochrome *c* oxidase proposed		[26]

As examples transmembrane cytochromes of the enterobacterium *Escherichia coli*, of the purple bacterium *Rhodobacter capsulatus*, of the cyanobacterium *Synechocystis* PCC 6803, and of the archaeon *Solfolobus acidocaldarius* are listed.

The cytochromes of the electron transfer chains of purple bacteria are well characterized in terms of structure and function. Three-dimensional structures of the aa_3-type cytochrome oxidase of *Rhodobacter sphaeroides* have been resolved and they disclose a topology very similar to those of other eukaryotic and bacterial aa_3-type oxidases. In *Rhodobacter sphaeroides* as well as in *Rhodobacter capsulatus* a cbb_3-type cytochrome oxidase is functional, which not only exhibits significant differences in subunit composition and cofactor content but is furthermore implicated in aerobic respiration under low oxygen concentrations. Besides the terminal oxidases, in the electron transfer chain of purple bacteria a *b*-type cytochrome can be found in the cytochrome bc_1 complex, which is structurally and functionally analogous to the cytochrome b_6f complex [33]. Here the two heme molecules are bound by the cytochrome *b* subunit, which, in contrast to cytochrome b_6, consists of eight transmembrane helices. Nevertheless, the first four helices of cytochrome *b* are homologous to cytochrome b_6 [34]. The heme molecules are bound by the transmembrane helices B and D, which each contain two highly conserved histidine residues [35, 36]. The cytochrome bc_1 complex is part of many respiratory electron transfer chains in bacteria as well as in mitochondria (complex III).

In the archaeon *Sulfolobus acidocaldarius* three different *a*-type and two different *b*-type cytochromes have been detected. Two of the *a*-type cytochromes can be attributed to cytochrome a_{587} (SoxC) and cytochrome aa_3 (SoxB), which are both part of the SoxABCD quinole oxidase of *Sulfolobus* [37]. Cytochrome c_{587} and cytochrome b_{562} are both part of the SoxM terminal oxidase complex [38]. A second *b*-type cytochrome, cytochrome $b_{558/566}$, could not be attributed to any known complex of the electron transfer chain so far [23].

The cofactor containing domains of *c*-type cytochromes always seem to be extra-cytoplasmatic, *i.e.* they are found in the periplasmic space of bacteria or the thylakoid lumen of cyanobacteria. Usually, the *c*-type cytochrome assembly machinery is localized in the periplasm or thylakoid lumen [39], because only there the cells maintain the reducing conditions required for thioether formation between the heme vinyl and a cysteine side chain. Although successful expression of *c*-type cytochromes in the *E. coli* cytoplasm has been reported [40, 41], the protein structures of these cytochromes appear to be distorted after expression. But in some cases the polypeptide structure is probably packed tightly enough so that a heme remains bound non-covalently even in the absence of the thioether linkage. This is in line with the recent observation that a *c*-type cytochrome can be converted to a *b*-type *in vitro* [42]. This observation generally raises the question why hemes have to be attached covalently to proteins, as in the case of *c*-type cytochromes. Although this question cannot be answered properly yet it is discussed in several recent reviews [1, 43, 44].

Recently the structures of the cytochrome b_6f complex from a cyanobacterium and from a green alga have been solved, and the structures revealed that a so far uncharacterized third heme is covalently attached to the cytochrome b_6 protein [18, 45]. Heme c_x is covalently linked to the cytochrome b_6 protein *via* a single thioether bond and the only identified axial ligand is a single water molecule. The sixth heme ligand still remains mysterious. Interestingly, heme c_x is localized in the membrane plane and faces the cytoplasmic side of the membrane (Fig. **3**). This localization is rather untypical and it is difficult to envisage how a heme can be covalently attached to its apoprotein on the cytoplasmic face of the membrane, since the *c*-type cytochrome assembly machinery is localized in the periplasm or the thylakoid lumen. Heme c_x seems to represent a special type of *c*-type cytochromes and future work has to elucidate the mechanism of cytochrome c_x formation.

The covalent as well as non-covalent binding of a heme cofactor to a protein will have an impact on the overall structure and stability of a protein/protein complex and also binding of the cofactor could influence membrane integration, folding and assembly of transmembrane proteins. In general, several pathways exist in parallel by which proteins are integrated into biological membranes *in vivo*.

MULTIPLE STRATEGIES FOR INTEGRATING MEMBRANE PROTEINS INTO THE LIPID PHASE

The integration of a membrane protein into the lipid bilayer is no simple task, as the cell has to prevent aggregation of these hydrophobic proteins in the hydrophilic milieu of the cytosol and it has to maintain the permeability barrier of the membrane during the actual insertion process. In order to cope with these restraints, cells have evolved sophisticated transport strategies, which can only briefly be highlighted here (Fig. **4**). A conceptionally simple strategy to prevent aggregation in the cytosol is to couple membrane protein integration with protein synthesis [46]. This co-translational insertion is mediated by the universally conserved signal recognition particle (SRP) pathway. The ribosome-bound SRP recognizes the hydrophobic signal-anchor sequence of an emerging membrane protein and targets these ribosome-associated nascent chains (*e.g.* the complex of ribosome, growing membrane protein and the mRNA) *via* its interaction with the membrane-bound SRP receptor (SR) to the SecY translocons at the bacterial cytoplasmic membrane or the membrane of the endoplasmic reticulum (ER) [47]. In eukaryotic cells, the initial contact between SRP and the growing polypeptide chain transiently slows down chain elongation (elongation arrest), opening a time window for efficient targeting to the ER membrane. Chain elongation resumes upon SRP-SR contact and the peptidyl-transferase activity of the ribosome threads the membrane protein into the protein conducting channel. The subunits required for elongation arrest are lacking in the prokaryotic SRP and it is assumed that elongation arrest is not a prerequisite for co-translational targeting in prokaryotes as there is usually only one membrane to target and distances are small [46]. Although, both SRP and SR are GTPases, the GTP-hydrolysis does not seem to provide energy for membrane protein insertion *per se* but is rather needed to dissociate the SRP-SR complex at the late stages of the targeting reaction. The major driving force for membrane protein integration is probably the peptidyl-transferase activity of the ribosome. In bacteria, however, this activity does not seem to be sufficient for the integration of membrane proteins harbouring large periplasmic loops. These proteins are also recognized and targeted *via* the SRP-pathway. They require, however, the ATPase SecA in addition for translocating the large periplasmic loops [48, 49].

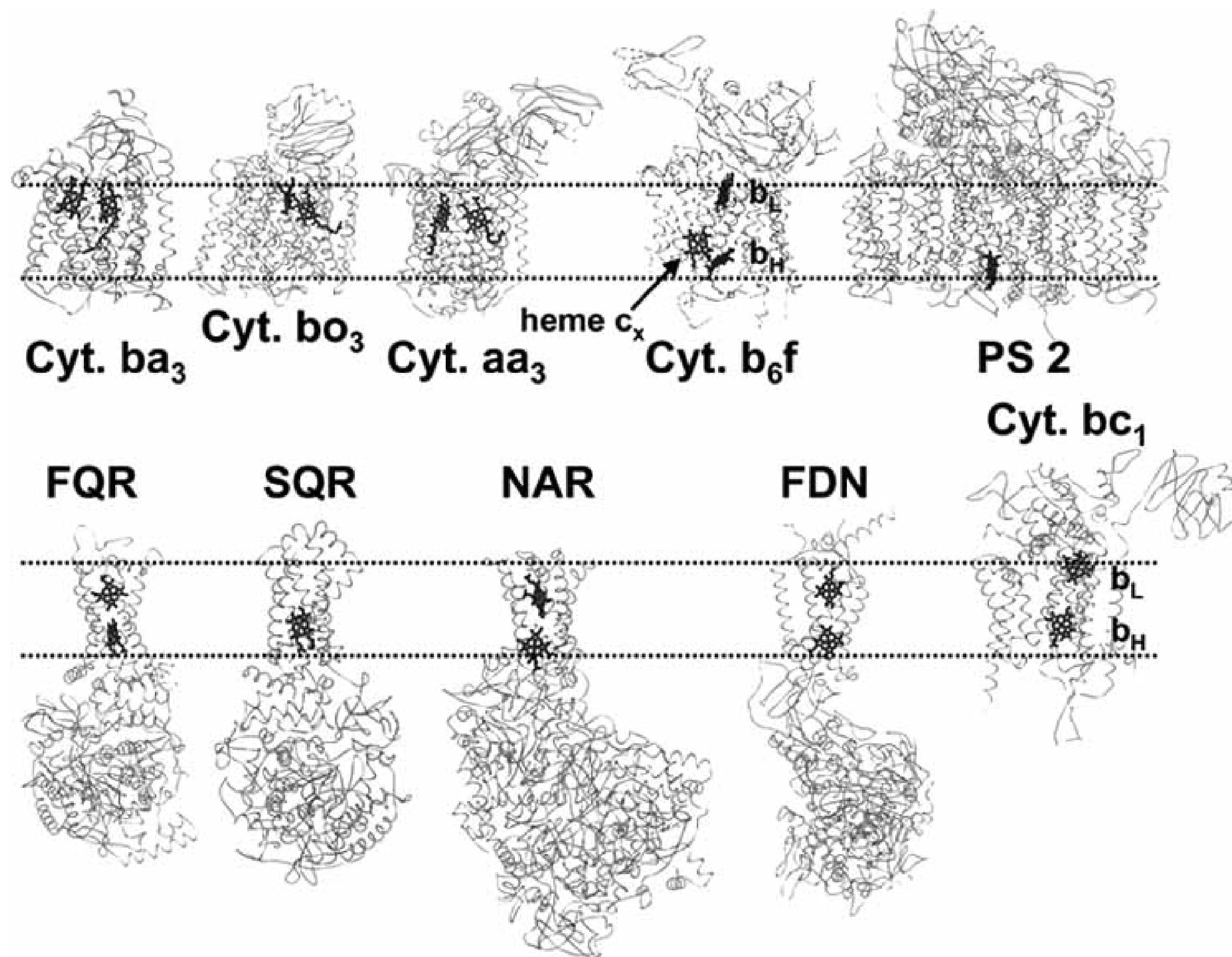

Fig. (3). Structures of transmembrane cytochromes. The pdb codes are given in the brackets.

The following structures are shown: Cyt b_6f: cytochrome b_6f complex (1UM3), Cyt ba_3: cytochrome ba_3 oxidase (1EHK), Cyt bo_3: cytochrome bo_3 oxidase (1FFT), Cyt. bc_1: cytochrome bc_1 complex core (1KYO), PS2:photosystem 2 (1S5L), SQR: succinate dehydrogenase (1NEK), FQR: fumarate reductase (1QLA), FDN: formate dehydrogenase (1KQF), NAR: nitrate reductase (1Q16).

Although the co-translational integration of membrane proteins *via* the SRP/Sec pathway is the dominating strategy in eukaryotes and prokaryotes, efficient SRP independent post-translational pathways are operating as well. In bacteria, the less hydrophobic β-barrel outer membrane proteins are post-translationally recognized and targeted by the bacteria-specific SecA/SecB pathway. SecA substrates harbour a cleavable signal sequence which is less hydrophobic than the signal-anchor sequence of SRP substrates. In the SecA pathway, SecA not only provides the energy for translocating the proteins across the cytoplasmic membrane, it is also required for the targeting reaction to the Sec translocon. This is achieved *via* its specific interaction with SecY, the central component of SecY translocon [50]. Once these outer membrane proteins have passed the SecY channel and reached the periplasmic side of the cytoplasmic membrane they get into contact with specific chaperones, like Skp before they finally reach the outer membrane [51]. The insertion into the outer membrane is most likely mediated *via* the universally conserved YaeT complex (Omp85 in *Neisseria meningitidis*; Sam35 in mitochondria and Toc75 in chloroplasts) [52].

The SecY translocon is not the only integration site at the cytoplasmic membrane of bacteria. A second integration site is provided by another universally conserved protein, YidC. In bacteria YidC appears to have a dual function, it functions in concert with the SecY translocon [53] and is probably involved in the lateral release of a transmembrane domain from the Sec channel into the lipid bilayer [54]. However, YidC seems to function also independently of the SecY translocon and it is assumed that it provides a hydrophobic scaffold that allows transmembrane domains to insert at the protein-lipid interface [55]. So far, only a few substrates have been identified and it appears that in particular small subunits of respiratory complexes like subunit c of the F_1F_0 ATPase use this YidC-only integration pathway. There is conflicting evidence as to the involvement of SRP/SR in targeting membrane proteins to YidC.

A third integration site is the Tat translocase, which is used by proteins carrying an N-terminal signal sequence with a typical SRRxFLK consensus motif [56]. A typical feature of Tat dependent proteins is that they contain redox-cofactors that need to be inserted prior to transport. As a con-

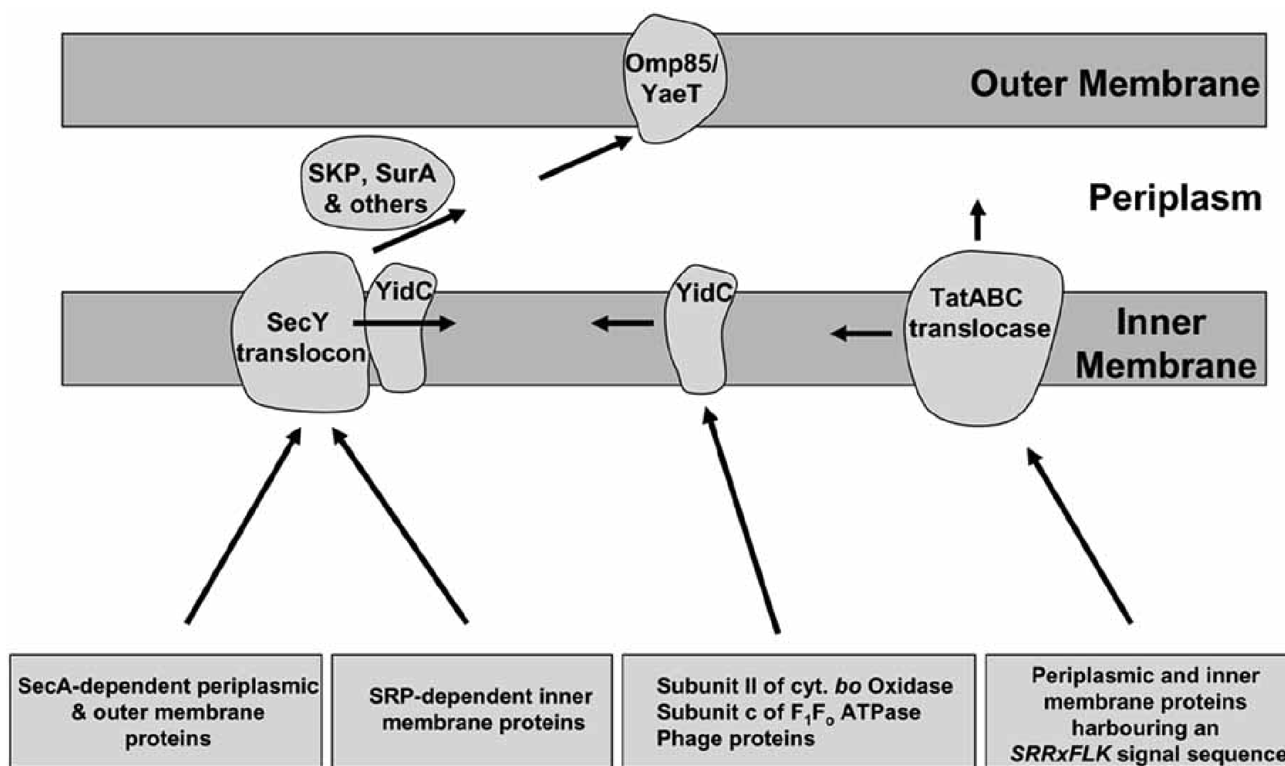

Fig. (4). Multiple strategies for integrating bacterial membrane proteins into the lipid phase.

The vast majority of bacterial inner membrane proteins are co-translationally targeted by the signal recognition particle (SRP) pathway to the SecY translocon. Lateral movement of the transmembrane domains presumably requires YidC. The SecY translocon is also required for the post-translational transport of secretory proteins, *e.g.* proteins destined for the periplasmic space or the outer membrane. Targeting and transport is mediated by the motor protein SecA. YidC does not seem to be involved in the transport of secretory proteins. Periplasmic chaperones like Skp or SurA are thought to accept secretory proteins on the trans side of the membrane. Integration of outer membrane proteins probably involves Omp85/YaeT. YidC can also function independently of SecY and some membrane proteins use this YidC-only pathway. However, their targeting, *e.g.* whether the SRP pathway is involved, is controversially discussed. Finally, a few membrane proteins have been shown to use the TatABC translocase. This pathway is predominantly used by periplasmic, co-factor containing proteins, which are translocated in their folded state.

sequence, these proteins fold already in the cytoplasm and can therefore not be transported *via* the Sec translocon, which is constrained to the transport of unfolded substrates. The size spectrum of folded Tat substrates encompasses roughly 10 to 100 kDa monomeric and oligomeric proteins and although mainly periplasmic proteins use this post-translational transport pathway, some integral membrane proteins have also been shown to use the Tat translocase. These proteins remain anchored to the cytoplasmic membrane either *via* a non-cleaved signal sequence as in the Rieske Fe-S protein of the cytochrome bc_1 complex or *via* a c-terminal stop-transfer-type transmembrane helix as in HybO of the [NiFe] hydrogenase [56].

MEMBRANE TRANSPORT OF CYTOCHROMES AND APO-CYTOCHROMES

Despite the vast number of different cytochromes, experimental evidence as to which of the above mentioned transport systems is employed for transporting them into or across the membrane is scarce. The available experimental data indicate that periplasmic *c*- and *b*-type cytochromes are synthesized as apoproteins with cleavable signal sequences. They are subsequently recognized by SecA which delivers them to the SecY translocon. As suggested originally by Page & Ferguson [57], heme attachment to the conserved CxxCH motif of *c*-type cytochromes and to the less conserved motifs of *b*-type cytochromes takes place after transport into the periplasm. This of course would make the Tat pathway for cytochrome transport dispensable since co-factor induced folding would be a post-secretion event. Nevertheless, at least one periplasmic heme protein (YcdB) is suggested to use the Tat pathway [58], but further experiments are required to determine unambiguously whether the heme group of YcdB is already inserted in the cytoplasm.

It can be predicted with reasonable confidence that most (if not all) soluble periplasmic cytochromes are recognized by SecA and are subsequently translocated *via* the SecY translocon. The transport route for membrane-bound cytochromes, on the other hand is less clear. The topology of the single-spanning *c*-type cytochromes CycM of *Paracoccus denitrificans* or CycY of *Rhodobacter* species is identical to the better characterized proteins FtsQ or Momp2. In all these proteins an N-terminal transmembrane domain is fused to a large periplasmic domain, which in the case of CycM and CycY carries the heme-binding motif [59]. Targeting of FtsQ and Momp2 to the Sec translocon is SRP-dependent, but for the translocation of the periplasmic domain the ATPase activity of SecA is required [48, 60]. A dual dependency on SRP and SecA is also expected for CycM, CycY and other

single-spanning *c*-type cytochromes like CcoO and CcoP, the subunits of the cbb_3-type cytochrome *c* oxidase [61]. The transport route of multi-spanning cytochromes, like subunit I of the heme-copper oxidases or the cytochrome *b* subunit of the bc_1 complex, is even less apparent. In mitochondria, which lack an SRP and Sec system, the mitochondrial-encoded cytochrome oxidase subunits Cox1, Cox2 and Cox3 are inserted into the inner membrane *via* the Oxa1 complex [62]. Oxa1 is homologous to the bacterial YidC, but carries an additional ribosome-binding domain at its C-terminus. Thus, it is predicted that the insertion is a co-translational process. Whether specific targeting factors are involved in directing mitochondrial ribosomes to Oxa1 during protein synthesis or whether proteins in mitochondria are generally synthesized by membrane-bound ribosomes, is currently not entirely clear. In contrast to the mitochondrial Oxa1 or the bacterial SecY translocon, the bacterial YidC appears to be unable to directly bind ribosomes. It lacks the C-terminal ribosome-binding domain of Oxa1 and is unable to functionally complement a yeast ΔOxa1 strain, unless it carries the fused ribosome binding domain. This rather points to a post-translational insertion of bacterial membrane proteins *via* the YidC-only pathway. If this assumption turns out to be correct, one would expect that the very hydrophobic cytochrome subunits of the bacterial respiratory complexes are preferentially integrated *via* the SRP/SecY pathway rather than *via* the YidC-only pathway. In agreement with this, the so far identified substrates of the YidC-only pathway are small phage proteins or simple, double spanning membrane proteins [55]. An up to now unique sequential insertion pathway has recently been discovered for subunit 2 of *E. coli* cytochrome *bo* oxidase. The N-terminal domain is inserted *via* YidC, while the large C-terminal periplasmic domain requires the Sec translocon and SecA, suggesting that for this particular protein the SecY translocon operates downstream of YidC [63-65].

HEME TRANSPORT

Like protein synthesis, heme synthesis in bacteria takes place in the cytoplasm, challenging the cell with the need to transport the heme molecule either into the membrane or across into the periplasmic space. In mammalian mitochondria, the last three enzymes of the heme biosynthetic pathway are suggested to form a loosely associated complex at the inner membrane, which might favour substrate channelling between the individual proteins and prevent the accumulation of potentially toxic intermediates. A similar mechanism has also been proposed for the *Bacillus subtilis* [66] heme biosynthetic pathway. Although orienting the last steps of heme biosynthesis at the membrane could in principle be advantageous for subsequent transport processes, it does not solve the problem of how heme molecules enter the periplasmic space or the membrane. The fact that the intrinsic hydrophobicity of heme molecules favours their spontaneous insertion into phospholipid bilayers also does not solve the problem, because the heme molecule would need to leave the phospholipid bilayer for entering the periplasmic space, which then can hardly be spontaneous.

Several studies on the maturation of *c*-type cytochromes indicate that specific transport proteins are responsible for transporting heme across the membrane. So far three different maturation systems have been identified (SI, SII, SIII; see [39, 67] for review), in which dedicated proteins are involved in the four common steps of *c*-type cytochrome biogenesis:

1. Transport of the apo-cytochrome across the membrane (see above).
2. Processing of the apo-cytochrome: thiooxidation/thioreduction.
3. Transport of heme across the membrane.
4. Heme ligation.

In α- and γ-proteobacteria, the putative ABC transporter CcmABCD has been suggested to translocate heme across the cytoplasmic membrane and consistently it has been shown that *E. coli* mutants lacking the Ccm components are unable to produce *c*-type cytochromes [68, 69]. Strikingly, however, these mutants are still able to produce periplasmic and membrane-integral *b*-type cytochromes. A recent report by Feissner *et al.* [70] has demonstrated that in *E. coli* heme ligation to apo-cytochrome *c* proceeds in a CcmABCD deficient mutant, if the *Helicobacter pylori* CcsAB protein is expressed. This could indicate that in organisms employing the SII cytochrome *c* biogenesis pathway like *Helicobacter* or *Bordetella pertussis*, CcsAB is not only involved in heme ligation but also in heme delivery. In agreement with this notion, an earlier report by Feissner *et al.* [71] had already indicated that CcsAB are the only dedicated components of the SII pathway in *Bordetella pertussis*. Alternatively, the observation that heme ligation proceeds in a *ccmABCD* mutant could suggest that either the primary function of CcmABCD is not heme transport or that alternative heme delivery pathways exist. The latter possibility would also be in agreement with the presence of periplasmic *b*-type cytochromes in this mutant background. Multiple heme-delivery systems have been suggested to exist in mitochondria [72], but like for bacteria, a detailed biochemical characterization has not been achieved so far. The identification of heme specific transport systems is complicated by the fact that many transport systems are indirectly involved in cytochrome biogenesis. This has been shown exemplary for the *E. coli* ABC transporter CydDC. Mutants in *cydCD* are impaired in the assembly of cytochrome *bd* oxidase [73] and of cytochrome b_{562} [74]. However, CydDC does not seem to be directly involved in cytochrome maturation but its contribution to this process is by maintaining the periplasmic redox homeostasis [73].

IN VIVO ASSEMBLY OF TRANSMEMBRANE CYTOCHROME COMPLEXES

The assembly of membrane-bound cytochromes and cytochrome complexes is an intrinsically complex process because the insertion of the apoprotein has to be tightly co-ordinated with the availability of the heme group. Due to the high toxicity of the heme group, it cannot be deposited in the membrane for later use and likewise, many membrane proteins do not fold properly in the membrane in the absence of their corresponding cofactor.

The best studied example for the biogenesis of a cytochrome complex is the aa_3-type cytochrome oxidase of eukaryotic mitochondria. In yeast, this complex consists of 12 subunits, of which the largest three, Cox1, Cox2 and Cox3, are mitochondrially encoded and form the catalytic core of

the enzyme. These three subunits are also present in all bacterial aa_3-type cytochrome oxidases [75]. Cox1 contains the heme *a* and the heme a_3-Cu_B binuclear centre, whereas the Cu_A centre is located in the periplasmic domain of Cox2. The additional subunits in eukaryotes are associated with the central COX1-2-3 core but their exact function is not completely understood. Some bacterial enzymes, like the one of *Paracoccus denitrificans* or *Rhodobacter sphaeroides* [75], also contain an additional subunit, but this subunit does not exhibit significant homology to the accessory subunits of eukaryotic aa_3-type cytochrome oxidases. Multiple assembly factors have been proposed to be involved in aa_3-type cytochrome oxidase maturation and in this review we will focus on factors predicted to be involved in cofactor insertion and those which are conserved between eu- and prokaryotes. Several recent reviews provide in-depth coverage of the complete assembly pathway [62, 76, 77].

The available structures of the bovine and bacterial aa_3-type oxidases demonstrate that the heme *a* and heme a_3-Cu_B cofactors of Cox1 are deeply buried within the membrane and connected to a network of α-helical transmembrane domains [78-80]. In contrast, the Cu_A centre in Cox2, which accepts the electrons from the donor cytochrome *c*, is exposed to the periplasmic side/intermembrane side of the membrane and is placed within a large β-barrel structure. The simplest scenario of co-factor insertion is that it proceeds simultaneously with the integration of the apoprotein into the lipid bilayer. Indeed, several observation point to a co-translational formation of at least the Cu_B-heme a_3-binuclear centre, as detailed below.

CU-DELIVERY TO CYTOCHROME OXIDASE

In eukaryotes, Cox17 is the key copper chaperone of the mitochondrial intermembrane space (compare Table **2**). Cox 17 orthologs are so far exclusively found in eukaryotes, although proteins with a characteristic cupredoxin-fold have been identified in bacterial species and are predicted to be at least functional homologues of the eukaryotic Cox17 [81]. Cox17 delivers Cu^{1+} to two additional Cu^{1+} binding proteins, Sco1 and Cox11 [82], which, in contrast to Cox17, are present in both eukaryotic and prokaryotic cells. Sco1 appears to be specifically involved in the formation of the Cu_A centre of Cox2, while Cox11 is required for the formation of the Cu_B centre [82]. In the fission yeast *Schizosaccharomyces pombe*, Cox11 is synthesized with an N-terminal extension, which exhibits a striking similarity to the ribosomal protein Rsm22p of *Saccharomyces cerevisiae* [83]. Although a Cox11-Rsm22p fusion is not observed in other organisms and even in *Schizosaccharomyces* the fusion seems to be cleaved into two separate domains after its translocation into mitochondria [84], an interaction between Cox11 and mitochondrial ribosomes has also been observed in *Saccharomyces cerevisiae* [83]. Although it is currently unclear whether this is a direct interaction or whether additional proteins are involved [85], the available data point to a strict coupling of Cu insertion and protein synthesis of Cox1.

In agreement with the general role of Cox11 in Cu_B centre formation, *Rhodobacter sphaeroides* Cox11 mutants lack CuB in the binuclear centre of aa_3-type cytochrome oxidase [86]. This, however, does, not impair the oxidase assembly or the insertion of the Cu_A centre or the heme a/a_3 cofactors [86]. This could suggest that different to eukaryotes, the bacterial Cox11 is involved in a late step of assembly. Alternatively, it could indicate that bacterial cytochrome oxidases follow a less ordered and less sequential assembly pathway than eukaryotic oxidases [75]. One important aspect of the study in *Rhodobacter sphaeroides* is that this organism contains a cbb_3-type cytochrome oxidase in addition to the aa_3-type cytochrome oxidase. The catalytic core of this additional oxidase also contains a Cu_B-centre, which is coupled to heme b_3. The deletion of *cox11* does not seem to impair the activity of cbb_3-type cytochrome oxidase, suggesting that copper insertion into this enzyme follows a different route. Indeed, the available data suggest that specific assembly factors are dedicated for the maturation of the cbb_3-type cytochrome oxidase [61, 87, 88].

For Cu-insertion into Cox2, it is currently not completely known whether this is also coupled with protein synthesis. In eukaryotes, Cox2 mRNA is tethered to the mitochondrial membrane for its translation which is mediated *via* integral translational activators like Pet309 [89]. Whether Sco1 is in close proximity to the site of translation and integration of Cox2 is not known, but available data indicate that the reduced level of Cox2 in Δ*sco1* strains is the result of increased degradation rather than reduced synthesis [90]. Importantly, the Δ*sco1* phenotype is partially suppressed by the addition of exogenous copper in human cell lines [91], pointing to a post-translational mode of action of Sco1. However, exogenous copper does not suppress the Δ*sco1* phenotype in yeast [92], which might be explained by the reduced stability of Cox2 in the absence of Sco1. In contrast to the Cu_B centre, which is sandwiched between transmembrane domains, the Cu_A centre is located in the soluble domain of Cox2. This domain might be flexible enough to accommodate the Cu_A centre after protein synthesis and integration is terminated.

The exact role of bacterial Sco1 homologs proteins is not entirely clear. In *Bacillus subtilis* the deletion of the gene encoding Sco1 leads to a loss of cytochrome oxidase activity but does not impair the activity of a menaquinol oxidase, which lacks the Cu_A-centre. PrrC, the ScoI homolog of *Rhodobacter sphaeroides*, has been implicated in both copper binding [93] and signal transduction [94]. Importantly, in Δ*prrC* strains, the activity of the aa_3-type cytochrome oxidase is not impaired [95]. There is also no effect on the cbb_3-type cytochrome oxidase in the absence of PrrC [94]. Although, this might have been expected, because the cbb_3-type cytochrome oxidases lack the Cu_A centre, one recent report suggests that in *Rhodobacter capsulatus* the ScoI homologs protein SenC is required for the assembly of cbb_3-type cytochrome oxidase [96, 97]. Importantly, the Δ*senC* phenotype is suppressed by adding exogenous copper. A role of SenC in protecting *Rhodobacter capsulatus* against periplasmic superoxide anions has also been shown [97], although it is not known whether this is a direct or indirect effect. These different effects might point to a dual function of Sco1 during the assembly of cytochrome oxidases. In addition to being involved in copper transfer, the protein is probably also involved in redox sensing *via* its characteristic thioredoxin fold [98].

Table 2. Important Assembly Factors for Cytochrome Oxidase Maturation

Factor	Predicted function	Localization	Distribution	Comments
Cox17	Cu-binding protein	Mitochondrial intermembrane space	Eukaryotes only	Is suggested to deliver Cu to ScoI and Cox11
Sco1	Cu-binding protein; thioredoxin-like protein; predicted to be involved in Cu_A-centre formation	Mitochondrial and bacterial inner membrane	Eu- and prokaryotes	Some species contain a highly homologous protein designated Sco2; The nomenclature of the bacterial Sco1 homologues is not consistant: R. capsulatus Sco1 is termed SenC and its homologue in R. sphaeroides PrrC.
Cox11	Cu-binding protein; predicted to be involved in Cu_B-centre formation	Mitochondrial and bacterial inner membrane	Eu- and prokaryotes	Cox11 is predicted to interact directly with ribosomal proteins in eukaryotes, pointing to a co-translational Cu-insertion into Cox1
Surf1	Predicted to be involved in heme a_3 insertion	Mitochondrial and bacterial inner membrane	Eu- and prokaryotes	Some active aa_3-type cytochrome oxidase is assembled even in the absence of Surf1
Cox10	Heme O synthase	Mitochondrial and bacterial inner membrane	Eu- and prokaryotes	Catalyzes the farnesylation of the C2 vinyl side chain at ring A (Fig. **1**)
Cox15	Heme A synthase	Mitochondrial and bacterial inner membrane	Eu- and prokaryotes	Heme-dependent monooxygenase; hydroxylates the methyl side chain at ring D (Fig. **1**), electrons are provided by NADPH+H^+ *via* an electron transfer chain involving a ferredoxin (Yah1) and ferredoxin reductase (Arh1)

For details and references see text.

HEME FORMATION AND DELIVERY

Heme *a* is formed by the conversion of heme *b* (Fe-Protoporphyrin IX), the final product of the heme biosynthesis pathway (Fig. **1**). Two universally conserved enzymes catalyze this two-step conversion. The first step leads to the formation of heme *o* and is catalyzed by Cox10 (heme O synthase). This farnesyl transferase converts the C2 vinyl side chain at pyrrole ring A into a hydroxyethylfarnesyl side chain. Cox10 is an integral membrane protein located in the inner membrane of mitochondria or in the cytoplasmic membrane of bacteria. The second step is catalyzed by the monooxygenase Cox15 (heme A synthase), which hydroxylates the methyl side chain at pyrrole ring D into an aldehyde. This reaction involves a ferredoxin (Yah1 in yeast) and a ferredoxin reductase (Arh1 in yeast). In bacteria, Cox10 and Cox15 have been shown to form a complex [99] and it is assumed that a similar complex exists in eukaryotic mitochondria.

As for the insertion of the Cu_B co-factor, it is assumed that the heme *a* cofactors are cotranslationally inserted into Cox1. In yeast Cox10 mutants, Cox1 is translated but almost undetectable in mitochondrial membranes [100]. Similar observations have been made also in *Paracoccus denitrificans* [101], suggesting that heme insertion is required for correct folding and stability of Cox1. On the other hand, in *Rhodobacter sphaeroides*, the assembly of cytochrome oxidase proceeds even in the absence of heme *a* [102], suggesting that the assembly of aa_3-type cytochrome oxidases does not necessarily follow a fixed pathway but that instead multiple pathways might exist to form an active enzyme. It is still largely unknown whether specific assembly proteins aid the insertion of the heme groups into Cox1. A recent report has implicated a role of the assembly protein Surf1 in heme a_3 insertion in *Rhodobacter sphaeroides* aa_3-type cytochrome oxidase [95]. Surf1 is a conserved integral membrane protein which is located in the inner mitochondrial membrane of eukaryotes or the cytoplasmic membrane of bacteria (Table **2**). Mutations in the human *surf*1 gene are responsible for most cases of the Leigh syndrome, which is characterized by cytochrome oxidase deficiency. However, in both eukaryotes and bacteria, some active aa_3-type cytochrome oxidase is formed even in the absence of Surf1, suggesting that Surf1 is not essential. Assuming that Surf1 is an assembly factor dedicated for heme a_3 insertion into aa_3-type cytochrome oxidase, this would indicate that either additional assembly factors can promote heme a_3 insertion or that spontaneous heme *a* insertion occurs at a rate that at least allows the formation of some active aa_3-type cytochrome oxidase. It should be noted that Surf1 is predicted to be required for heme a_3 insertion only. No specific factors have been implicated in the insertion of the low-spin heme *a*. Whether the different molecular environment of this cofactor might allow for sufficient spontaneous insertion or whether so far unknown factors are involved, remains to be analyzed.

In general, the process of heme insertion into the corresponding apoprotein is still largely enigmatic even for the well studied aa_3-type cytochrome oxidases and basically nothing is known about other cytochromes which contain non-covalently ligated heme groups.

LOSS OF THE HEME GROUP AND ITS EFFECT ON COMPLEX STABILITY *IN VIVO*

Respiratory complexes often exhibit a modular composition in which the transmembrane *b*-type cytochrome serves as a membrane anchor which tethers large periplasmic or cytoplasmic modules (subunits) to the membrane. The transmembrane subunits are usually required for connecting the quinol pool to soluble redox partner, thus maintaining electron flow. For some of these enzymes it has been shown that the cytoplasmic subunits assemble correctly and even retain their oxidoreductase activity when the membrane anchoring subunits are absent [103, 104]. So even when the enzymatic redox reaction is uncoupled from electron trans-

fer, the catalytic activity of the soluble domain is preserved. These observations suggest that the soluble subunits interact and form the catalytically active domain prior to binding to the transmembrane domain, and this binding step therefore appears to be a rather late step during complex assembly. An important issue is whether soluble modules bind to their transmembrane module if it lacks its heme co-factor bound. Likewise, for multi-subunit transmembrane modules, it is an important question of whether binding of the heme co-factor is a prerequisite for the assembly of the individual transmembrane subunits. These issues are still mainly unsolved although some experiments have been performed which begin to address these questions.

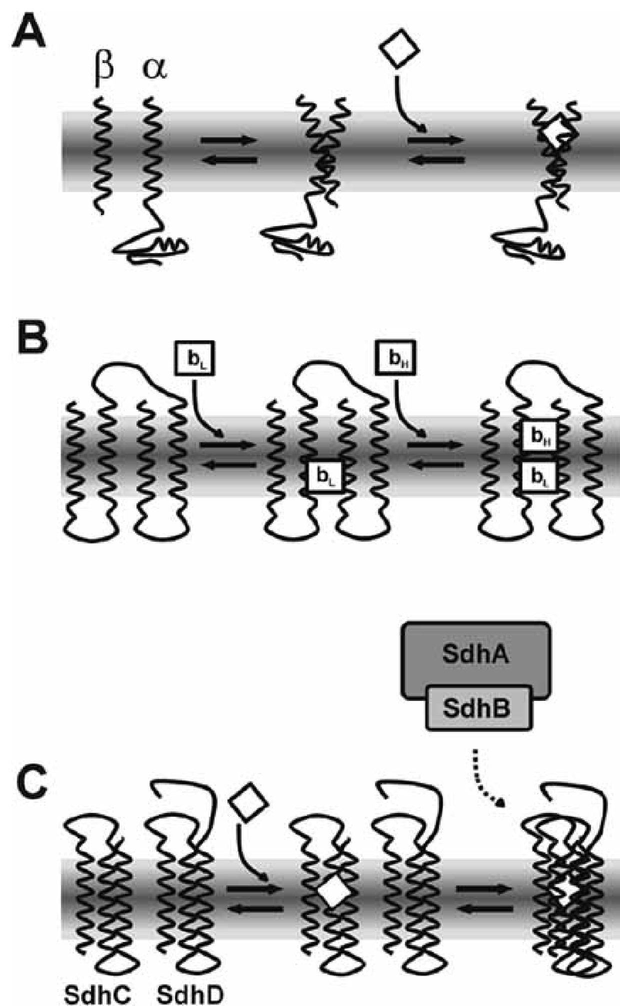

Fig. (5). Assembly of transmembrane cytochromes is predicted to involve multiple steps.

A. Cytochrome b_{559} probably folds in a three stage process: (1) after integration of individual transmembrane helices into a membrane, (2) the helices interact and form an apoprotein. In the third stage the heme is bound to the apo protein resulting in the formation of the functional cytochrome. **B**. During assembly of cytochrome b/b_6 the heme b_L binds first to the apoprotein, which is a prerequisite for subsequent binding of heme b_H. **C**. Assembly of the succinate dehydrogenase is initiated by binding of heme to one transmembrane subunit. Afterwards SdhD binds and the heme molecule is sandwiched in between the two hydrophobic subunits. In a last step the pre-assembled soluble domain binds to the membrane anchor domain.

Cytochrome b_{559}

Cytochrome b_{559} is a core subunit of photosystem 2 from chloroplasts and cyanobacteria [30]. The cytochrome is composed of two small hydrophobic polypeptides, termed alpha (PsbE) and beta (PsbF), each of which spans the membrane once with an α-helical conformation. Recent structural data obtained from cyanobacterial photosystem 2 has confirmed an alpha/beta heterodimeric structure in which each subunit provides one His residues for ligating the heme molecule [17, 105].

Deletion of the gene encoding the cytochrome b_{559} alpha subunit in *Chlamydomonas reinhardtii* resulted in a complete loss of photosystem 2 assembly and it has been suggested that cytochrome b_{559} is involved in early steps of photosystem 2 assembly [106]. If the single histidine residue of the alpha subunit, which serves as one axial heme ligand, was exchanged by genetic methods, the entire photosystem 2 was highly destabilized, although photosystem 2 still assembled to some extent and the cytochrome b_{559} subunits were present [107]. The isolated photosystem 2 complexes contained undetectable amounts of heme, although the complexes were still active. These data indicate that *in vivo* apo-cytochrome b_{559} forms from the α and β transmembrane subunits and is stable even in the absence of the co-factor (compare Fig. **5A**). Furthermore, the lack of the heme group does not affect the interaction with the other subunits of the photosystem 2. The assumption that heme binding is not a prerequisite for formation of the α/β heterodimer is highly supported by recent *in vitro* experiments as further discussed below.

Cytochrome b/b_6

Cytochrome *b* is a core subunit of the cytochrome bc_1 complex which can be found in bacterial electron transfer chains as well as in mitochondria (complex III) [108]. The protein spans the membrane with eight transmembrane α-helices and two heme molecules are bound non-covalently by the protein [109]. The two hemes are assigned as the low-potential heme b_L and the high-potential heme b_H (Fig. **3**). Substitution of one of the two histidine residues, which ligate the heme b_H in *Rhodobacter sphaeroides*, resulted in formation of a cytochrome *b* subunit with only the b_L heme bound. The lack of the b_H heme did also not prevent subsequent assembly of cytochrome *b* with the other two core subunits of the cytochrome bc_1 complex. In contrast, replacement of any of the two histidine residues, which ligate b_L, resulted in a complete loss of both heme molecules and blocked subsequent complex assembly. It also appeared that in the absence of heme b_L the overall stability of cytochrome *b* was drastically reduced, because it was almost undetectable in membranes of this mutant. As a consequence also the steady-state stability of cytochrome c_1 subunit was significantly impaired [110]. These observations indicate that the two hemes bind to apo-cytochrome *b* sequentially, and that binding of heme b_L is a prerequisite for binding of heme b_H (Fig. **5B**). Furthermore, binding of at least heme b_L is essential for assembly of the entire cytochrome bc_1 complex in *Rhodobacter*.

Essentially identical observations have been made for the cytochrome b_6 subunit of the cytochrome b_6f complex [111]. The cytochrome b_6f complex is a structural and functional homologous protein complex from cyanobacteria and chloroplasts. In contrast to the cytochrome bc_1 complex, the cytochrome b_6f complex has four large core subunits (Cytochrome *f*, cytochrome b_6, the Rieske protein, and subunit IV) [32, 112]. Cytochrome b_6 is homologous to the N-terminal part of cytochrome *b* and subunit IV shows a high degree of sequence homology to the cytochrome *b* C-terminus [34]. Thus the cytochrome *b* subunit is split in the cytochrome b_6f complex (when compared to the cytochrome bc_1 complex).

After substitution of any of the four heme ligating histidine residues in the cytochrome b_6 subunit from *Chlamydomonas reinhardtii*, the amount of cytochrome b_6 as well as of the other three core subunits in the thylakoid membrane was significantly reduced. Whether the lack of a functional cytochrome b_6 subunit also impairs the interaction between the other subunits has not been shown so far [111]. Nevertheless, similar to cytochrome *b* from *Rhodobacter*, in the case of the cytochrome b_6 subunit from *Chlamydomonas reinhardtii* substitution of any of the two heme b_L ligands had also a different effect than substitution of any of the heme b_H ligands and also for cytochrome b_6 it has been proposed that heme b_H binds prior to heme b_L.

Initially, four nuclear factors have been suggested to be involved in binding of heme b_H to the apoprotein [111]. However, at least *in vitro* both *b*-type hemes bind to the apoprotein without the need for additional proteins, like chaperones. It has been believed for some time that one of the two cytochrome b_6 hemes is covalently attached to the protein moiety and therefore the protein should be described as a *c*-type cytochrome [111]. This assumption has been based on the observation that after denaturation of the protein and SDS polyacrylamide gel electrophoresis at least one heme was still found to be attached to the polypeptide chain. However, the structure of the cytochrome b_6f complex revealed that a third heme, heme c_x, is attached covalently to cytochrome b_6, and the original observations can now be attributed to the presence of the additional heme c_x. Thus it is possible that the four predicted assembly proteins are not involved in heme b_H binding but rather required for the biogenesis of heme c_x. Interestingly, *Bacillus subtilis* also contains a cytochrome b_6f-type complex, and at least one heme molecule seems to be covalently bound to the cytochrome *b* protein (QcrB) [113]. Therefore, a third, covalently attached heme is most likely also part of the cytochrom *b* subunit in this organism.

Succinate Dehydrogenase

In the succinate dehydrogenase (SQR) from *E. coli*, the heme molecule is sandwiched between the two hydrophobic subunits SdhC and SdhD, and each subunit provides one axial histidine ligand for heme binding [4, 114]. Interestingly, while in the *E. coli* SQR only one heme cofactor is bound by the transmembrane domain, in *Bacillus subtilis* two hemes are embedded in the SQR membrane domain [115, 116]. The structure of the SQR from *E. coli* has been resolved recently as well as the structure of the homologous fumarate reductase (FQR) from *E. coli* and *Wolinella succinogenes* [117, 118]. In the structure of the FQR from *Wolinella* two hemes are bound within the transmembrane domain as in the case of the SQR from *Bacillus subtilis*. Surprisingly, the FQR from *E. coli* contains no bound heme cofactor, although the catalytic activities and the overall 3D structures of the two enzymes are identical. The observation that the number of heme molecules in the transmembrane domains of SQR/FQR enzymes can vary from 0-2 may indicates that the heme is structurally and functionally of no importance, and it is still puzzling why similar enzymes utilize different numbers of hemes for their catalytic activities. In the structure of the *E. coli* FQR the packing of the transmembrane helices might preclude heme binding, and also for the function, assembly, and stability of this complex heme binding is obviously not essential.

It has initially been observed that after replacement of any of the two histidine residues, which serve as heme ligands of the *E. coli* SQR, the entire complex assembled and retained functional [27]. In a later study the generated mutants have been characterized in more detail and it has been found that substitution of single histidine residues still allowed the heme molecules to be incorporated into the heme pocket of cytochrome b_{556} [119]. Therefore, it has been suggested that binding of the heme cofactor could be essential for assembly of the enzyme complex. A study of SQR assembly in *E. coli* mutants, which are ferrochelatase deficient, has shown that the heme iron centre is indispensable for the functional assembly of SQR in the *E. coli* cytoplasmic membrane. Furthermore, the hydrophobic SQR subunits SdhC and SdhD seem not to accumulate in the membrane when heme synthesis is abolished. Based on all these observations, a possible mechanism has been proposed for the assembly of the *E. coli* SQR which involves various steps [120] (Fig. **5C**). In the presence of heme the two transmembrane subunits SdhC and SdhD integrate individually into the *E. coli* membrane and the heme molecule binds first to the SdhC subunit. This suggestion is based on the observation that heme still binds to this subunit in the absence of SdhD but no heme binding has been observed when just the SdhD subunit is present. After heme binding to SdhC, the SdhD subunit interacts with SdhC resulting in formation of the heterodimeric transmembrane cytochrome b_{556}. Following the assembly of the soluble SQR domain in the cytoplasm, this domain binds to the preformed transmembrane cytochrome resulting in formation of the final SQR structure.

All the above mentioned examples highlight the importance of heme binding for both the biological activity as well as for the folding and stability of heme proteins. *In vivo* it is, however, difficult to discriminate between the effects on the catalytic function of a heme protein and those that primarily affect protein stability and/or assembly. Therefore *in vitro* analyses on isolated proteins can be beneficial to determine the kinetic and thermodynamic impact of co-factor binding on folding and stability of transmembrane cytochromes.

INVOLVEMENT OF COFACTORS IN FOLDING AND STABILITY OF TRANSMEMBRANE PROTEINS

About fifteen years ago a simple model for α-helical membrane protein folding has been proposed, in which individual α-helices integrate independently into a membrane (stage I) and afterwards these independent helices interact to form the final oligomeric structures (stage II) [121, 122].

Although this model does not take into account that some transmembrane domains cooperate during their transfer from the SecY translocon into the lipid phase, it reduces the folding of entire membrane proteins to the interaction of individually stable α-helices, and therefore provides a very useful framework for analyzing folding of α-helical membrane proteins. Based on some theoretical assumptions the Two-stage-Model has recently been extended and to account for the role of soluble domains and cofactor binding on membrane protein folding [123].

The role of cofactors for folding and stability of transmembrane proteins has been studied in a few cases only and the results do not resolve the folding mechanism of cofactor containing transmembrane proteins in general, as suggested [123]. For the light harvesting complex II of higher plants the experimental data indicate that folding of the protein and binding of the cofactors are coupled [124-126], although it has been speculated that these could be separate assembly steps *in vivo* [126]. On the other hand, bacteriorhodopsin, a light driven proton pump from *Halobacterium salinarium*, folds into a folding intermediate to which the retinal cofactor binds, followed by formation of the final bacteriorhodopsin structure (summarized in [127]). For transmembrane *b*-type cytochromes essentially nothing is known about the function of the co-factors for folding and stability, whereas folding of several soluble *b*-type cytochromes is investigated more comprehensively.

FORMATION OF SOLUBLE *B*-TYPE CYTOCHROMES

The role of the heme cofactor for folding and stability of a soluble *b*-type cytochrome has been studied extensively in a few cases. The periplasmic cytochrome b_{562} from *E. coli* retains much of the secondary and tertiary structure after removal of the heme and the cytochrome forms spontaneously after simply mixing the apoprotein with free heme [128]. These observations indicate that the primary sequence of the cytochrome contains all information for correct folding and that a preformed three dimensional structure is able to acquire the heme group. However, binding of the heme cofactor significantly increases the stability of the protein [128, 129] and in addition the holo-cytochrome is more stable under reducing than under oxidizing conditions [130]. An influence of heme binding on the thermodynamic stability has also been observed for the soluble cytochrome b_5 protein [131]. These observations point to a role of the heme group in stabilizing the preformed molecular contacts.

Heme protein assembly has not only been studied with naturally occurring cytochromes but was also investigated using designed heme proteins, which in most cases are derivatives of natural heme proteins [132]. Some of these designed proteins form stable helix bundles in both, the apo- and holo-states, indicating that the heme co-factors bind after formation of helix bundles or are at least are not required for stable helix formations and interactions. Although the synthetic peptides are water soluble, they bind the cofactors in the hydrophobic interior and mimic some features of transmembrane *b*-type cytochromes. In several cases the proteins are derived from transmembrane cytochromes and the helices were rendered water soluble. Based on the cytochrome *b* subunit of the cytochrome bc_1 complex, a four helix bundle *marquette* has been designed, which showed about the same secondary structure in its apo- and holo-form, although binding of the two hemes apparently stabilized the protein scaffold [133].

Designed soluble heme proteins haven been proven to be very useful to study *b*-type cytochrome formation; however, assembly of these proteins relies on an *in vitro* aqueous system and addition of free heme. In contrast, the assembly of transmembrane cytochromes occurs in a hydrophobic environment with a low dielectric constant, and a direct comparison of interactions critical for the formation of water soluble and membrane-embedded cytochromes is limited.

IN VITRO STUDIES ON TRANSMEMBRANE *B*-TYPE CYTOCHROME ASSEMBLY

In recent years soluble proteins have been designed, which mimic cytochrome b_6 and the N-terminal half of cytochrome *b* (see above), but in these cases the naturally hydrophobic proteins were rendered hydrophilic, which makes it almost impossible to draw any conclusion for the role of the heme co-factors in folding and stability of real transmembrane *b*-type cytochromes.

Francke *et al.* described the chemical synthesis of the 44 amino acid long cytochrome b_{559} β-subunit (PsbF) and the authors were able to reconstitute a homo-dimeric cytochrome with spectral properties very similar to those of the natural cytochrome b_{559} protein [134]. For successful reconstitution, the heme needed to be in its reduced state to get incorporated into the cytochrome. Based on a more detailed analysis we were very recently able to show that this cytochrome folds in a three step process [135] (Fig. **5A**): After integration of individual helices into a membrane (step 1) the helices interact and form an apoprotein (step 2) to which the heme cofactor can bind (step 3). Various point mutations have been used to characterize dimerization of the protein as well as incorporation of the heme cofactor *in vivo* as well as *in vitro*. In several cases substitution of single amino acids affected binding of the co-factor and assembly of the holo-cytochrome, although dimerization of the PsbF transmembrane helix and formation of the apo-cytochrome was not affected. The *in vitro* approach has clearly demonstrated that assembly of the apo-cytochrome is not affected by mutations affecting cofactor binding. Further studies have shown that also the redox state of the heme cofactor does neither stabilize nor weaken the transmembrane helix-dimer [136]. On the other hand, substitution of a highly conserved glycine residue abolished dimerization of the protein as well as binding of the cofactor. These observations suggested that transmembrane helix-helix interactions and formation of the apo-cytochrome is independent of cofactor binding whereas the cofactor can only bind to a preformed helix-dimer. The PsbF transmembrane part is consequently divided into two domains: a dimerization domain and a cofactor binding domain. It is remarkable that substitution of a single glycine residue in the dimerization domain completely disrupted formation of the transmembrane cytochrome. Glycine residues are often involved in mediating and stabilizing transmembrane helix interactions [137-140]. This residue allows two helices to closely interact, resulting in stabilizing Van-der-Waals interactions between surrounding residues. Furthermore, the close packing of two helices does in general

allow the formation of intra-helical hydrogen bonds, and such bonds have indeed been predicted for several membrane proteins [141, 142].

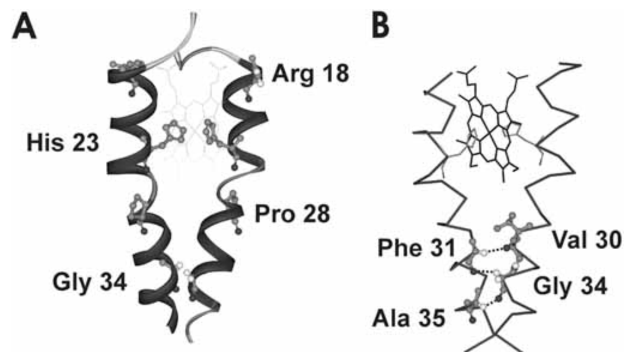

Fig. (6). Structure of cytochrome b_{559}´.

A. The amino acids Arg18 and His23 are essential for heme binding. Pro28 induces a helix kink which is essential for holo-cytochrome formation. Gly34 is vital for helix dimerization and subsequent cytochrome formation. **B**. The transmembrane helix dimer is stabilized *via* hydrogen bonds. At least three hydrogen bonds between a Cα hydrogen and a backbone carbonyl oxygen are predicted based on the available structural information (pdb code 1S5L).

Based on the structure of cytochrome b_{559} from the cyanobacterium *Thermosynechococcus elongates* we predict the presence of at least three hydrogen bonds, which could be important for the stability of apo-cytochrome b_{559} (Fig. **6**). In the three cases the predicted distances between the hydrogen and the oxygen atoms are less than 3 Å, which strongly argues for an electrostatic interaction. It is remarkable that close packing between the two transmembrane helices and the (predicted) hydrogen bonds can highly stabilize the transmembrane helix dimer, since only very few amino acids are involved in dimerization of the transmembrane helices.

In addition to cytochrome b_{559}, the successful heterologous expression of cytochrome b_6 from spinach has been reported [143, 144]. The protein was expressed as a fusion protein in *E. coli*, and the protein accumulated in the *E. coli* cytoplasmic membrane after expression [144]. Initially, the protein has accumulated in its apo-form and no typical cytochrome b_6 spectrum has been observed in the *E. coli* membrane after expression. If, however, heme was present during heterologous expression of cytochrome b_6, a typical spectrum of the holoprotein has been observed by UV/Vis spectroscopy. These observations suggest that the apoprotein can incorporate and accumulate in the membrane regardless of the presence of heme. Nevertheless, since heme was present during expression of cytochrome b_6 it is possible that the heme binds to a cytochrome b_6 folding intermediate, as in the case of bacteriorhodopsin (see above), and it remains an open question if heme can be incorporated into a preformed apo-cytochrome b_6, or if heme has to be present during membrane incorporation of apo-cytochrome b_6. In this case membrane incorporation, heme binding, and further structural organization would be coupled. Besides the assembly of cytochrome b_6 in the *E. coli* cytoplasmic membrane, cytochrome b_6 has also been reconstituted successfully *in vitro*, and the assembled protein displayed the typical characteristics of natural cytochrome b_6 [143], although it has not been shown conclusively yet how many hemes are bound to the *in vitro* reconstituted cytochrome. Interestingly, neither after expression of cytochrome b_6 into the *E. coli* membrane nor after *in vitro* reconstitution of the protein, a covalently attached heme molecule (heme c_x, see above) has been detected. This indicates that this heme *c* does not spontaneously bind to the apo-cytochrome and supports the suggestion that special factors are involved in covalent attachment of this heme. Furthermore, binding of heme c_x is most likely not critical for binding of the *b*-type hemes. In general, since cytochrome b_6 can be heterologously expressed and *in vitro* reconstituted [143, 144], as well as purified from natural hosts [145-147], this protein could serve as a simple model system for future *in vitro* and *in vivo* studies of transmembrane *b*-type cytochrome assembly.

CONCLUDING REMARKS

Some progress has been made in the recent years in terms of understanding membrane protein folding and assembly. Based on studies with several model systems the roles of individual helices and even single amino acids have been described. While for soluble proteins the role of cofactors for folding and stability is investigated in more detail, for membrane proteins the structural role of co-factor binding is not well defined. Transmembrane cytochromes could be powerful model systems to study the role of the heme cofactor for folding and stability of membrane proteins *in vivo* as well as *in vitro*. Furthermore, due to the importance of transmembrane cytochrome complexes in electron transfer chains in all kingdoms of life it will be important to further reveal the individual steps during assembly of transmembrane cytochrome complexes *in vivo*, and to identify the assembly proteins and machineries involved.

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