required for assembly of Rubisco, its functions should be studied, for they appear likely to tell us something about the regulation of the biogenesis of this key photosynthetic enzyme.

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Talking Point

How finicky is mitochondrial protein import?

Nikolaus Pfanner, Rupert Pfaller and Walter Neupert

Recently it was reported that artificial targeting signals or signals specific for organelles other than mitochondria could direct proteins into mitochondria. Here we discuss findings which suggest that specific steps of mitochondrial protein import can be bypassed. Non-specific targeting signals appear to use this bypass pathway. Such import occurs at very low rates under physiological conditions and therefore does not affect the uniqueness of mitochondrial protein composition.

Several features of the targeting of nuclear encoded proteins to mitochondria and the translocation of proteins across the mitochondrial membranes have been unravelled during recent years. Many precursor proteins carry amino-terminal peptide extensions (presequences) which contain mitochondrial targeting information. This was shown by fusing such presequences to non-mitochondrial 'passenger' proteins and transporting these chimaeric proteins into mitochondria *in vivo* and *in vitro*[1]. Recent studies have resolved the translocation across the membranes into several distinct steps[2–5], including: (1) binding to receptor proteins on the mitochondrial surface (which may require a cytosolic cofactor); (2) subsequent insertion into proteinaceous sites in the outer membrane; and (3) transport into or across the inner mitochondrial membrane via contact sites between both membranes (requiring the electrical potential, $\Delta\psi$, across the inner membrane). These findings suggested that the recognition of a targeting signal (usually contained in the presequence) by a receptor protein served as the control step for selective import of mitochondrial proteins.

Recently, however, it has been reported that sequences that are not specific for mitochondrial protein import could also target 'passenger' proteins to mitochondria. These included a chloroplast 'transit' sequence[6], sequences selected out of the *E. coli* genome[7], a region of the cytosolic protein dihydrofolate reductase[8], a mitochondrial gene product[9], and artificial presequences[10]. Furthermore, changes of individual amino acid residues at the amino terminus of the mature protein part allowed import of a precursor protein from which the presequence had been removed[11]. These results suggest that mitochondrial protein import is much less selective than previously thought. It is a generally accepted view, however, that all subcellular compartments contain a unique set of proteins.

The question thus arises as to how mitochondria are able to maintain the specificity of their protein composition. It might be proposed that misrouted proteins are degraded when they arrive in the wrong compartment. In such a case the degradation system would have to possess a high specificity to distinguish between misrouted proteins and correctly targeted and imported ones. Furthermore, it would be rather uneconomical for the cell to control intracellular sorting predominantly by selective degradation. How then can this conflict be resolved? The following observation may provide the answer. Import of proteins targeted by non-mitochondrial targeting signals seems to occur at low rates when compared to the import of physiological precursor proteins. In the studies mentioned above, it was already shown that the import rates of proteins carrying non-physiological targeting sequences were

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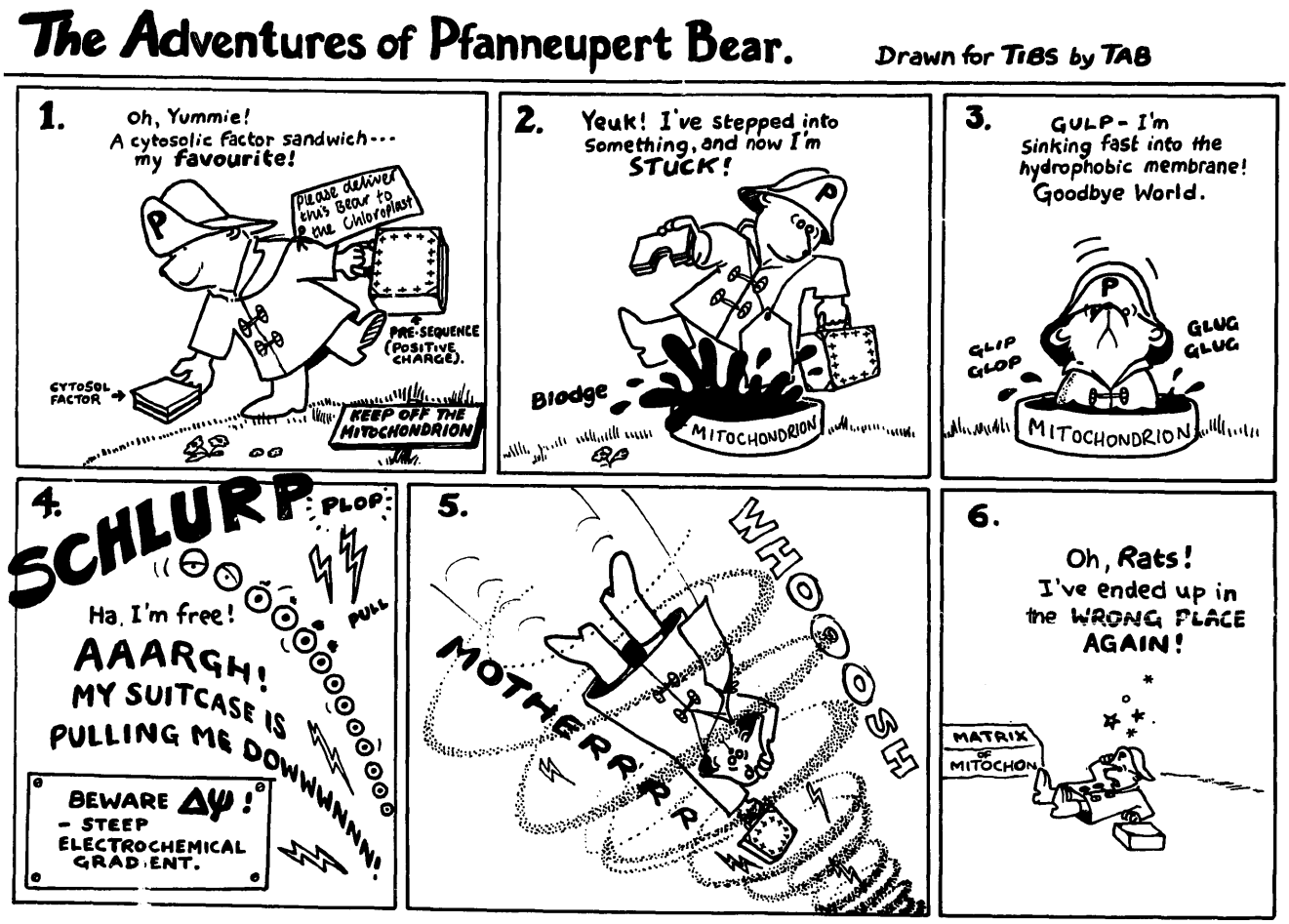
lower *in vivo* and *in vitro* than those of physiological precursors[6,8–10,12], whereas (at least in several cases) fusion proteins between authentic targeting signals (e.g. presequences) and 'passenger' proteins were imported with reasonable efficiency[13]. A study with plant cells showed no apparent mistargeting of proteins between chloroplasts and mitochondria[14].

We would like to point out here that mitochondrial protein import involves several subsequent steps, each of which can increase the efficiency and specificity of import. This includes the interaction of precursor proteins with putative cytosolic cofactors, the binding to receptor proteins on the mitochondrial surface, the insertion into proteinaceous sites in the outer membrane, and probably the interaction with other components in the mitochondrial membranes (e.g. in the contact sites). The following observations suggest that at least some of these steps can be bypassed. Some purified precursor proteins could be imported without addition of cytosolic factors[4,15]. Pretreatment of mitochondria with proteases was shown to strongly decrease the efficiency of overall import, however, a residual import (about 10% of control) was independent of protease-sensitive components ('receptors')[16,17]. The kinetics of this residual import were slowed down compared to the kinetics of the control import. Furthermore, the low efficiency import of a chloroplast protein (the small subunit of ribulose-bisphosphate carboxylase) into mitochondria was not inhibited by a protease-pretreatment of the mitochondria (Pfaller, Pfanner, and Neupert, unpublished). Hydrophobic sequences that are present in several precursors (class I precursors) supported a strong interaction of precursors with binding sites on the mitochondrial surface and allowed rapid import of the precursors. Precursors lacking these 'assistant' sequences (class II precursors) showed slower import and only weak interactions with binding sites[13]. Thus, a precursor protein does not have to interact properly with each component of the import machinery, or to participate in each possible mechanism of the import pathway. The resulting 'bypass' import is less efficient, but also less selective. It therefore allows a certain amount of import of precursors with artificial targeting signals to occur, especially if they are presented in excessively high amounts (expressed from high-copy-number plasmids).

The importance of this bypass import may then be overestimated, when the screening system does not control the rates of import, but only distinguishes between growth and non-growth of cells. Rescue of mutants lacking a mitochondrial protein by cytoplasmic expression and import of the protein seems to be a very sensitive system. In several cases, very low import rates are sufficient for restoration to the wild-type phenotype[9]. So far, the only obligatory steps in mitochondrial protein import appear to be the insertion of precursors into the outer membrane and the $\Delta\psi$-dependent entrance of precursors into the inner membrane. The initial insertion into the outer membrane may require an amphiphilic structure of the targeting sequences[18]. $\Delta\psi$ is assumed to exert an electrophoretic effect on positively charged precursor domains[3,19,20]. This would explain why virtually all physiological and artificial targeting sequences that direct proteins to the inner membrane are positively charged. The studies with non-mitochondrial targeting signals thus provide important information on basic mechanisms of mitochondrial



protein import.

In summary, mitochondrial protein import can be dissected into at least three steps involving the targeting sequences. Accordingly, the targeting signals must contain the required functional elements for: (1) specific interaction with receptors; (2) insertion into the outer membrane; and (3) response to $\Delta\psi$. The interaction with the receptor is not an absolute requirement for import. Rather, as discussed above, this step can be bypassed, yielding 'residual' import. On the other hand, specific recognition can hardly be executed by unspecific or artificial signals. This would explain the low efficiency of import direct by artificial signals. Steps (2) and (3) above are essential for import, but artificial signals or sequences present in the mature protein part (or in a 'passenger' protein) may allow them to occur. This general model may be subject to variations since mitochondrial precursor proteins can contain several targeting signals which may act either in concert or sequentially at distinct stages of the import pathway[1,13,21].

Authentic mitochondrial precursor proteins probably make use of the several specific steps of the import pathway to increase the efficiency and specificity of import. Such a multiple-step system may thus represent a 'multiple check' system. Since bypass import of non-mitochondrial precursors occurs only at a very minor level, if at all, under physiological conditions (i.e. in non-transformed cells), it would not normally disturb the selectivity of mitochondrial protein uptake and the uniqueness of mitochondrial protein composition.

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Letters

Lipid terminology: 'free' fatty acid is ambiguous

In papers dealing with studies on long-chain fatty acids and derivatives a variety of terms is used to designate the various chemical and biological forms in which these substances occur. Some of these terms are redundant or even equivocal. A major confusion in terminology arises from the term 'free fatty acid', commonly abbreviated as FFA. In this term the adjective 'free' is mostly used to refer to 'non-esterified' or 'unesterified' fatty acids (abbreviated as NEFA or UFA, respectively)[1,2], but is also used to denote 'non-protein bound' (unbound) fatty acids (sometimes abbreviated also as UFA)[3].

In the former case the adjective is actually redundant, since a fatty acid by definition is non-esterified. Correspondingly, an esterified fatty acid should be referred to as fatty ester. This nomenclature was recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (e.g. Ref. 4).

The covalent chemical bond that forms between the naturally occurring fatty acids and other molecules is commonly an ester linkage. Thus, instead of using 'bound fatty acids' in this case it is more appropriate and specific to refer to fatty esters.

Fatty acids may also be bound non-covalently to proteins (e.g. albumin, fatty acid-binding protein), lipoproteins, membranes or other matrices. The IUPAC-IUB Commission does not recommend a term for these cases[4]. We suggest that the terms 'bound' and 'unbound fatty acids' should be reserved for use with these systems.

Some authors have employed the term 'free' fatty acid to distinguish it from its (protein-)'bound' form. This terminology has gained general acceptance particularly with the analysis of protein–ligand binding according to the method of Scatchard[2,5]. Although 'free' may seem a valid alternative for 'unbound' it should be avoided as much as possible because of the above-mentioned ambiguity of this adjective.

Another complication in fatty acid nomenclature appears in physiological systems. At neutral pH fatty acids are largely present in their ionized form, together with various cations. By standard biochemical convention, the suffix '-ate' (e.g. palmitate) denotes any mixture of the acid and its ionized form. Unfortunately, this suffix is also used to designate fatty esters, e.g. cholesteryl palmitate[4]. Distinction between the non-ionized and ionized forms can be important, e.g. in case of specific membrane-transport systems. Perhaps in this case

Table I. Fatty acid nomenclature

Commonly used term	Suggested appropriate term
Free fatty acid (FFA)	Fatty acid
Non-esterified fatty acid (NEFA)	
Unesterified fatty acid (UFA)	
Unbound fatty acid (UFA)	
Esterified fatty acid	Fatty ester
Bound fatty acid (covalent bond)	
(Protein-)bound fatty acid or ester (non-covalent bond)	Protein-bound fatty acid or ester