**Question 1**

Chloroplasts contain six compartments—outer membrane, intermembrane space, inner membrane, stroma, thylakoid membrane, and thylakoid lumen—each of which is populated by specific sets of proteins.

Many of these proteins are encoded by nuclear genes, translated in the cytosol, and then post-translationally directed to the appropriate chloroplast compartment. To investigate the import of proteins into chloroplasts, you have cloned the cDNAs for ferredoxin (FD), which is located in the stroma, and plastocyanin (PC), which is located in the thylakoid lumen. Furthermore, using recombinant DNA techniques, you have constructed two hybrid genes: ferredoxin with the plastocyanin signal peptide (PCFD) and plastocyanin with the ferredoxin signal peptide (FDPC). You translate mRNAs from these four genes in vitro, mix the translation products with isolated chloroplasts for a few minutes, reisolate the chloroplasts after protease treatment, and fractionate them to find which compartments the proteins have entered. The status of the normal and hybrid proteins at each stage of the experiment are shown in the Figure: each lane in the gels corresponds to a stage of the experiment as indicated alongside the experimental protocol.

**(I) Experimental Protocol:**

- Translate mRNA *in vitro* (lane 1)
- Add chloroplasts (lane 2)
- Treat with protease & reisolate chloroplasts (lane 3)
- Fractionate Chloroplasts  
  - inner and outer membranes (lane 4)  
  - stroma (lane 5)  
  - thylakoids (lane 6)  
  - thylakoids + protease (lane 7)

**(II) Gel Analysis:**

![Gel Analysis](image-url)
A. How efficient is chloroplast uptake of ferredoxin and plastocyanin in your \textit{in vitro} system? How can you tell?

Import of all 4 proteins is very efficient, as indicated by the small fraction of precursor that remains after incubation with chloroplasts. This precursor is still outside of the chloroplasts in all cases because it is protease-sensitive (see lanes 3 and 7).

B. Are ferredoxin and plastocyanin localized to their appropriate chloroplast compartments in these experiments? How can you tell?

Yes, both of these proteins are localized to their appropriate compartments. The vast majority of FD is in the stromal fraction and the majority of PC is in the thylakoid fraction.

C. Are the hybrid proteins imported as you would expect if the N-terminal signal peptides determined their final location? Comment on any significant differences.

Yes, the hybrid proteins are imported into chloroplast compartments that are consistent with their N-terminal sequences. This result is most obvious for FDPC, which is found exclusively in the stromal fraction just like FD. By contrast, only a small fraction of PCFD makes it into the lumen of the thylakoid. Some seems to be bound to the thylakoid membrane since it is digested when treated with a protease. However, the majority is found in the stromal fraction.

There are several potential reasons for the inefficiency of PCFD import into the thylakoid lumen. 1) It may be difficult for FD to cross the membrane. 2)FD normally picks up an iron-sulfur center at some point, and this may happen in the stroma preventing its entry into the thylakoid lumen. 3) FD normally functions as part of a complex in the stroma. Association of FD with other proteins may prevent its transport across the thylakoid membrane.

D. Why are there three bands in experiments with plastocyanin and PCFD but only two bands in experiments with ferredoxin and FDPC? To the extent you can, identify the bands and their relationship to each other.

The highest band for all proteins is the precursor (the primary translation product) since it is present \textit{in vitro} in the absence of chloroplasts. The lower bands have each had some portion of protein removed. The 2 band pattern is associated with the N-terminal segment from FD while the 3 band pattern is associated with the N-terminal segment from PC. The FD signal induces a single cleavage event upon import into the stroma. The PC signal induces two cleavage events, 1 upon import into the stroma and the second upon import into the thylakoid lumen. The lowest PC band corresponds to the functional protein in the thylakoid.

Question 2

To aid your studies of protein import into mitochondria, you treat yeast cells with cycloheximide, a drug that blocks ribosome movement along mRNA. When you examine these cells in the electron microscope, you are surprised to find cytosolic ribosomes
attached to the outside of the mitochondria. You have never seen attached ribosomes in the absence of cycloheximide. To investigate this phenomenon further, you prepare mitochondria from cycloheximide-treated cells and extract the mRNA that is bound to the mitochondria-associated ribosomes. You translate this mRNA \textit{in vitro} and compare the protein products with similarly translated mRNA from the cytosol. The results are clear-cut: the mitochondria-associated ribosomes are translating mRNA’s that encode mitochondrial proteins.

You are astounded! Here, clearly visible in the electron micrographs, seems to be proof that protein import into mitochondria occurs during translation. \textit{How can you rationalize this result with the prevailing view that mitochondrial proteins are imported after they have been synthesized and released from ribosomes?}

\textbf{Normally, translation is much faster than mitochondrial import, so that proteins are completely free of the ribosome before interacting with the mitochondrial membrane. By blocking protein synthesis with cycloheximide, you have made the rate of translation much slower than the rate of import. Since the signal peptide for import into the mitochondria is at the N-terminus, some partially synthesized mitochondrial proteins that are still attached to ribosomes will be able to interact with the mitochondrial membrane. The attempted import of such a partially synthesized protein will tether the ribosome and mRNA to the mitochondrial membrane.}

\textbf{Question 3}

A number of serious human diseases are known in which the patients’ cells lack functional peroxisomes. Patients with Zellweger syndrome—the best known of these diseases—show multiple development and psychomotor abnormalities and usually survive no more than a few months after birth. To understand these peroxisome-deficiency diseases, more must be known about the biogenesis of peroxisomes.

You feel it is time to take a genetic approach to this problem and decide to see if Chinese hamster ovary (CHO) cells can be mutagenized to give cells that mimic those from Zellweger patients. (CHO cells are especially useful for isolating recessive mutations because they behave as if they were haploid for much of their genome.) You develop an assay for peroxisomal function in which mutagenized cell colonies are incubated with a soluble radioactive precursor that is converted into a readily detectable insoluble product by a peroxisomal enzyme. A laborious screen of 25,000 colonies is finally rewarded by the discovery of two colonies that do not have the insoluble radioactive product. Sure enough, these mutant cells lack typical peroxisomes, as judged by electron microscopy.

To confirm their peroxisomal deficiency, you test directly for two peroxisomal enzymes: catalase and acyl CoA oxidase. The levels of catalase in the mutants is virtually the same as in normal CHO cells, except that it is dispersed in the cytosol instead of localized in peroxisomes. By contrast, acyl CoA oxidase \textit{activity} is absent in both mutant cell lines.

To investigate the acyl CoA oxidase deficiency, you perform a pulse-chase experiment: you grow cells for 1 hour in medium containing $^{35}$S-Methionine, then transfer them to unlabeled medium and immunoprecipitate acyl CoA oxidase at various times after transfer (see Figure). To clarify the relationship between the 75kD and 53kD forms of the oxidase, you isolate mRNA from wild-type and mutant cells, translate it \textit{in vitro}, and immunoprecipitate acyl CoA oxidase: all three sources of mRNA rive similar levels of the 75kD form, but none of the 53kD form.
You are most curious as to whether the two mutant cell lines have defects in the same gene or different genes. To answer this, you fuse wild-type and mutant cell in various combinations and examine the fused cells (heterocaryons) for the location of catalase and the presence of acyl CoA activity. As shown in the table, when two mutant cells were fused, the heterocaryons had normal peroxisomes as judged by catalase localization and acyl CoA oxidase activity. [Note: Fusions were made using HAT selection. Any unfused cells or fusions of like cells will not survive selection.]

<table>
<thead>
<tr>
<th>Fusion</th>
<th>Cells Fused</th>
<th>Catalase Location</th>
<th>Acyl CoA Oxidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal x Normal</td>
<td>peroxisomal</td>
<td>present</td>
</tr>
<tr>
<td>2</td>
<td>Normal x Mutant 1</td>
<td>peroxisomal</td>
<td>present</td>
</tr>
<tr>
<td>3</td>
<td>Normal x Mutant 2</td>
<td>peroxisomal</td>
<td>present</td>
</tr>
<tr>
<td>4</td>
<td>Mutant 1 x Mutant 1</td>
<td>cytosolic</td>
<td>absent</td>
</tr>
<tr>
<td>5</td>
<td>Mutant 2 x Mutant 2</td>
<td>cytosolic</td>
<td>absent</td>
</tr>
<tr>
<td>6</td>
<td>Mutant 1 x Mutant 2</td>
<td>peroxisomal</td>
<td>present</td>
</tr>
<tr>
<td>7</td>
<td>Mutant 2 x Mutant 1</td>
<td>peroxisomal</td>
<td>present</td>
</tr>
</tbody>
</table>

A. As shown in the Figure, acyl CoA oxidase exists in two forms in normal CHO cells. How do you think the two forms are related? Which one, if either, do you suppose is the active enzyme?

Since mRNA from normal cells yields both the 75kD and 53kD forms, while the mutants only yield the 75kD form, the 53kD form is likely the active enzyme. This active enzyme probably arises from the 75kD precursor when it is imported into the lysosomes.

B. Why do the mutant cells have only the 75kD form of acyl CoA oxidase, and why do you think it disappears during the chase in the pulse-chase experiment? How does this differ from the case of catalase?

The mutant cells have only the 75kD form of the enzyme because thier defective peroxisomes cannot import it and process it to the active 53kD form. Because the 75kD form disappears so quickly in the pulse-chase expts in the mutant cells (without giving rise to the 53kD form), the 75kD form must be unstable in the cytosol and rapidly degraded. Catalase, by contrast, although prevented from entering the defective peroxisomes in the mutant cells, is stable in the cytosol. This accounts for the equal
amounts of catalase activity in normal cells & mutant cells (as mentioned in the text of the question).

C. Do the mutations in the two mutant cell lines affect the same gene or different genes? How can you tell?

The mutations in the mutant cell lines must affect different genes because when the cells are fused (fusions 6 & 7) the heterocaryon has normal peroxisomes. This is a classic example of complementation. If the two mutant cell lines were defective in the same gene, the heterocaryon would be no better off that the original cells and would still be peroxisome deficient.

D. Are the mutations in the mutant cells recessive or dominant? How can you tell?

The mutations in the two mutant cell lines must be recessive. When either of the mutant cell lines are fused to normal cells (fusions 2 & 3), the heterocaryon has the phenotype of the normal cells: functional peroxisomes. If either of the mutations was dominant, the resulting heterocaryon would still be peroxisome deficient.

Question 4

Please do 2001 Problem Set 5, Question 1

Question 5. (12-23)

Four membrane proteins are represented schematically below. The boxes represent membrane-spanning segments and the arrows represent sites for cleavage of the signal peptides. Using the rules for co-translational insertion, predict how each of the mature proteins will be arranged across the membrane of the ER. Indicate clearly the N and C-termini relative to the cytosol and the lumen of the ER, and label each box as a start-transfer or stop-transfer peptide. (Boxes represent membrane-spanning segments and arrows indicate sites at which signal peptides are cleaves. The pluses and minuses indicate the charges at the ends of the transmembrane segments.) [Hint: + charges on the ends of transmembrane segments will preferentially associate with the cytosol rather than the lumen of the ER.]
Question 6:

Cystic Fibrosis (CF) is the most common genetic disease in the US. It is caused by mutations in the gene encoding the cystic fibrosis transmembrane receptor (CFTR) which is normally localized to the plasma membrane where it functions as a Cl- channel. Patients suffering from CF have mutations in both of their genes encoding CFTR. There are hundreds of different mutations that can result in CF with varying degrees of severity. The most common mutation, ΔF508, results in a single amino acid deletion. Interestingly, although the gene is expressed normally and the ΔF508 mutant protein is partially functional, none of it gets localized to the plasma membrane and patients develop severe CF. CFTR-ΔF508 is thought to fold much more slowly than the wildtype CFTR protein.

A) Where might the CFTR-ΔF508 protein be accumulating in the cell since it is not reaching the plasma membrane?

Misfolded or unfolded proteins remain bound to their ER chaperone and accumulate in the ER.

B) Design an experiment to test where CFTR-ΔF508 is located in the cell.

One could incubate ΔF508 cells with a gold conjugated antibody against CFTR and then perform electron microscopy on thin sections and look at where the protein localizes. Alternatively one could perform double immunofluorescence using an antibody against CFTR conjugated to one fluorophore and an antibody against a known ER localized protein conjugated to another fluorophore, and look for co-localization of the proteins.
C) Researchers are testing whether the use of proteasome inhibitors can help increase Cl-conductance in cells carrying the ∆F508 mutation by allowing more CFTR to become localized to the plasma membrane. What is the rationale behind this potential therapy? When unfolded or misfolded proteins are held in the ER they become ubiquitinated and transported back into the cytosol for degradation. Since it is thought that CFTR-∆F508 folds more slowly than WT protein, if one could delay its degradation (by inhibiting the proteasome) some of the protein may have time to complete its folding and then be transported to the plasma membrane.