Walter Neupert

A Mitochondrial Odyssey

Walter Neupert

Ludwig-Maximilians-Universität München and Max Planck Institute of Biochemistry, Martinsried D-82152, Germany; email: Neupert@biochem.mpg.de

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Abstract

Good fortune let me be an innocent child during World War II, a hopeful adolescent with encouraging parents during the years of German recovery, and a self-determined adult in a period of peace, freedom, and wealth. My luck continued as a scientist who could entirely follow his fancy. My mind was always set on understanding how things are made. At a certain point, I found myself confronted with the question of how mitochondria and organelles, which cannot be formed de novo, are put together. Intracellular transport of proteins, their translocation across the mitochondrial membranes, and their folding and assembly were the processes that fascinated me. Now, after some 30 years, we have wonderful insights, unimagined views of a complex and at the same time simple machinery and its workings. We have glimpses of how orderly processes are established in the cell to assemble from single molecules our beautiful mitochondria that every day make some 50 kg of ATP for each of us. At the same time, we have learned amazing lessons from the tinkering of evolution that developed mitochondria from bacteria.

Contents

ΔAIMΩN, Dämon
Wie an dem Tag, der dich der Welt verliehen,
Die Sonne stand zum Gruße der Planeten,
Bist alsobald und fort und fort gediehen
Nach dem Gesetz, wonach du angetreten.
So musst du sein, dir kannst du nicht entfliehen,
So sagten schon Sibyllen, so Propheten;
Und keine Zeit und keine Macht zerstückelt
Geprägte Form, die lebend sich entwickelt.

ΔAIMON, Daemon
As stood the sun to the salute of planets
Upon the day that gave you to the earth,
You grew forthwith, and prospered, in your growing
Heeded the law presiding at your birth.
Sibyls and prophets told it: You must be
None but yourself, from self you cannot flee.
No time there is, no power, can decompose
The minted form that lives and living grows.

(Goethe, Primal Words, Orphic, translated by Christopher Middleton)

PROLOGUE

This poem, written in 1817, has been interpreted many times, mostly by men of letters. For me, it has a firm scientific motivation. Goethe anticipated the ideas and discoveries of Darwin and Mendel made only some 30 to 50 years later. He had observed that nature works according to very strict rules once the individual constellation has been set. The metaphor of the position of the planets at the time when one enters the world means, in terms or our present knowledge: As your set of genes was arranged when egg and sperm fused, you have to be and stay that way for the rest of your life. You are subject to the dualism of chance and law.

I believe my entire life was governed by this principle. I feel in retrospect I was destined for a life of finding solid truth behind things. I realized there was another most enjoyable world full of excitement in humanities, philosophy, and literature, always a source of intellectual curiosity and delight, but I was apparently not made for this world. Now, after so many years thinking along the lines of Mendel and Darwin and the many giants of scientists who built up our present stronghold of wisdom in biology,

I am convinced that we are minted forms not only in our bodies but also in our minds.

GROWING UP IN POSTWAR GERMANY

I was born on October 24, 1939. My first largely unremembered years were the years of terror, ubiquitous misery, fear, and despair. My father was drafted in 1938. Aware of the disaster to come, he volunteered to become a paramedic. He survived the war, but it took him 10 years to return. My first memory of him must have been in 1943 or 1944, when he left after a vacation. I still see him jumping onto the open platform of the last car of the leaving train, which took him back to the Ukraine. He left my mother at home with three little boys. On one of his leaves from service at the eastern front, my father built a shelter for protection against the bombs, which had started to rain on Munich. I remember the family sitting at the radio, a Volksempfänger, listening for the announcement of the approaching bombers mixed with the "Preludes" of Liszt played to report about the great successes of the army when everybody knew that Germany was doomed. I still hear the sirens ringing. Sometimes this was during the day, more often during the night. And then we were running to the shelter, which was a hole in the ground covered with a concrete lid, was open at both sides to avoid the dangerous air pressure, had benches at each side, and had a few steps up to the garden on both sides. I remember in particular the cold during the winters. Later, when the bombing was more and more intense, our mother had just time to drag us out of our beds to the basement of the house.

As far as I can remember, I did not feel the danger and misery around me, like the children now who are playing in the ruins of the wars that still torment the planet. But I remember well the excitement when the war ended, the turmoil just before the Americans arrived, the weapons left behind, and the dark things happening during the last nights. I remember standing at the gate of our house when the American troops moved into Munich, the 1st of May 1945, with an endless chain of jeeps, tanks, and trucks. Later, our curiosity to see these strange people lured us into their camps. And indeed, we saw unbelievable wonders: chocolate, orange juice in gasoline canisters, and, for the first time, African American people. The GIs were very friendly to us kids, and they sometimes shared their Hershey bars.

But life grew darker and darker, and more and more misery was to come. Marauding criminals were a constant threat; there were burglary, looting, and killing. We did not know what had happened to our father. The adults had little hope that there would be better times ever. And starving started. Droves of people from Munich were cycling through the villages in upper Bavaria to exchange valuables for potatoes. Our mother was busy enough looking after her boys, which was necessary. With no fathers around, the boys looked for adventures and formed little gangs. The war had left plenty of exciting things in the woods and in deserted antiaircraft stands. There was a variety of discarded weaponry to play with. One day in the summer of 1945, three boys of families of our neighbors, 10 or so years old, were killed when playing with a hand grenade they picked up somewhere. We grew vegetables in our garden and had a goat and a few hens to provide us with milk and eggs.

In the autumn of 1945, I started elementary school in the village nearby. There was no paper, and there were no pencils. Some had slates and chalks. We had to bring logs to heat the classroom. In the second year, the class consisted of more than 60 kids, since the stream of refugees from the former East Germany had arrived in Bavaria. The school moved to the dancing floor of the village inn. A 20-year-old survivor of the war was our teacher. School was exciting but much more so was our daily march of some two or more miles, depending on our investigative mood. It took us along the road where the GIs were driving with their new miraculously flashy Mercurys and Studebakers between their barracks in Munich and a military airport in the vicinity. Occasionally, the dream happened, and they gave the little hitchhikers

a ride to school, unforgettable adventures. 1946 and 1947 were the worst years regarding food. In 1948, after three years of struggling to survive behind the Ural mountains, my father came home. He was lucky not to belong to the 1 million out of 3 million prisoners of war who died somewhere in boundless Siberia. And he was certainly very fortunate not to belong to those survivors who were released only in 1953. He was sent home because he suffered from severe dystrophy. We pushed our fingers into his swollen legs, and it took time until the dents disappeared. He spoke very little about the life in the prison camp, but many years later when he read *One Day in the Life of Ivan Denisovich* by Alexander Solzhenitsyn, he told me that the layout and the regime in the prison camp were exactly as in the gulag. My father immediately got a job, and everything became better. In June 1948, there was the Währungsreform (currency reform), and with this, we had food and a warm kitchen in winter.

My parents and grandparents were not academics. I guess they believed I should learn something useful in times in which survival was the essence. Fortunately, my teacher and the parish priest told my father he should send me to *Gymnasium*. I was the only one of the whole class who was thought to meet the requirements, yet at the same time, my parents and I were told: Many try, but few succeed. Certainly, this was not encouraging to me. I passed the admission examination to be exposed to teachers who were quite demanding, and some were grim or draconic. Most of them had participated in, or at least suffered from, the war and had been part of the prewar white-bread society. They felt school was a Darwinian institution that involved selection of the best. It was not for the fainthearted nor the sensitive. Only about one-third of those admitted to *Gymnasium*, the equivalent of high school and college in Germany, succeeded in getting through. Then, about 3% of an age group qualified for admission to university, compared to a proportion of 40% at present. After the first six years of *Gymnasium* and finding I had survived this insecurity, pressure, and threat, I enjoyed the remaining three years enormously. I began to take to physics, biology, math, and German classes. The American Forces Network in Germany had a daily outpost concert, with the overture to Donna Diana by Emil Nikolaus von Reznicek as the theme music. This started right when we came home from school, and for years, this was a regular pleasure after school stress. The years of the mid-1950s were the time when it became clear that there was a future for Germany. Munich, almost completely destroyed, was largely rebuilt. The impact of American culture, movies, music (including Elvis Presley), parties, and porch swings, which accompanied the reeducation of Germany, was enormous. America was the dream of a new, glorious life. But I also became fascinated by literature; I devoured Molière, Shakespeare, Shaw, Anouilh, even Corneille, and, of course, all the German classical writers, and later realized how little I had understood. When I finished *Gymnasium*, we were confident; life would be good for our generation, there would be jobs, and we would have to work hard, but there was a brave new world waiting for us. We enjoyed skiing in winter and mountain climbing in summer in the Bavarian and Austrian mountains, although everything, compared to present-day standards, happened under very frugal conditions.

When it came to decide what to study, I confess I was confused. I liked literature, but was this something really solid on which to build a profession? After half a year in law school, another one studying botany and zoology, and then one studying geology, I finally decided on chemistry. Rather irritated, not to say guilty, about my wanderings for two years, I rushed through my chemistry classes. I enjoyed the intellectual challenge of the analytical work, of synthesizing components, and the universe of possible novel compounds to be designed and made for the first time by clever experimentation. I considered then to do a PhD in organic chemistry, but when it came to decide on a supervisor, a deep fascination by the question of what really is at the bottom of living organisms made me choose biochemistry.

I should, however, admit what made my decision for biochemistry definite was an accident in my final year in an advanced inorganic chemistry course. This was in a lab that practiced the synthesis of boron tris azide, BN_9, a compound that has the tendency to explode by merely looking at it. Another student in the lab was to prepare lithium azide from sodium azide; both were not so dangerous substances as long as they were not mistreated. This step involved the use of ether, which then was to be removed by mild heating. Unfortunately, the student, after putting half a kilo of lithium azide in an oil bath on a tripod under a Bunsen flame, went for lunch together with his supervisor. The windows, with their frames, were blown into the street; I was grazed by a bullet, the tripod, and thrown out of the lab through the door and was deaf for a whole day. This finally cured my excitement for chemistry. The group leader devoted to BN_9 lost all but three of his fingers in the hood upon minor explosions at the time when he became professor.

GETTING HOOKED ON BIOGENESIS OF MITOCHONDRIA

I went through the biochemistry labs in Munich with complete ignorance of what biochemistry meant. There was one Nobel Prize winner, Adolf Butenandt, with his institute, but they were inaccessible to a boyish chemistry student. Feodor Lynen, not yet a Nobel Prize awardee, offered me a project to isolate the kinase that converted NAD to NADP, and then there was Theodor Bücher, the new holder of the chair of the Institute of Physiological Chemistry in the medical faculty. He was the last of Otto Warburg's PhD students. During the war, he continued to work with Warburg until the end when the Russians occupied Berlin and confiscated Warburg's lab and horses. Bücher's interest was in enzymes of glycolysis, metabolic regulation of the flow of hydrogen, and development of tissues and organs by the study of enzyme patterns. He had just completed a combined electron microscopic and enzymological analysis of the development of the flight muscle of African locusts [see Brosemer et al. (1)].

Bücher showed me the electron micrographs of the developing flight muscle (**Figure 1**). The development of a locust occurs through five instars, and the last molting changes the grasshoppers into little aeroplanes. This process includes the formation of mighty muscles in the thorax with powerful mitochondria densely filled with cristae. The muscle bundles are covered with oily fat, the fuel that enables the locusts to cover large distances. In the 1840s, a swarm of African locusts landed in Brandenburg near Berlin, some 2000 miles from the place where they had started. This high-performance motor, one may call it an insect Ferrari, develops over a period of a few days by an increase of mitochondrial volume by a factor of 20–50. It appeared to be an ideal model to study the biogenesis of mitochondria. I was deeply impressed by the question as to how these beautiful organelles are made and immediately was hooked.

The origin of mitochondria from bacteria had been a matter of speculation and debate for already many decades. Mitochondria were thought to be self-replicating entities in the cell. My supervisor told me to isolate mitochondria and to find conditions to make them grow in the test tube (they were bacteria!) with the unspoken proviso that I should come back when I had succeeded. This way of educating young scientists was the traditional German norm. Nowadays, this attitude has completely disappeared, and the principles of affirmation, close guidance, and supervision, traditionally practiced in the Anglo-American world, have become the gold standard. Looking back, I can see the problematic sides of the old education system, but also the benefits. It was not simply neglect of the young students, but rather respect for their own ways of growing up. By contrast, the old-world principle was clearly not the most efficient and productive one. Many young students got lost on their way sometimes because of minor deficiencies in their development, which could have been easily corrected by some advice and guidance at the right time.

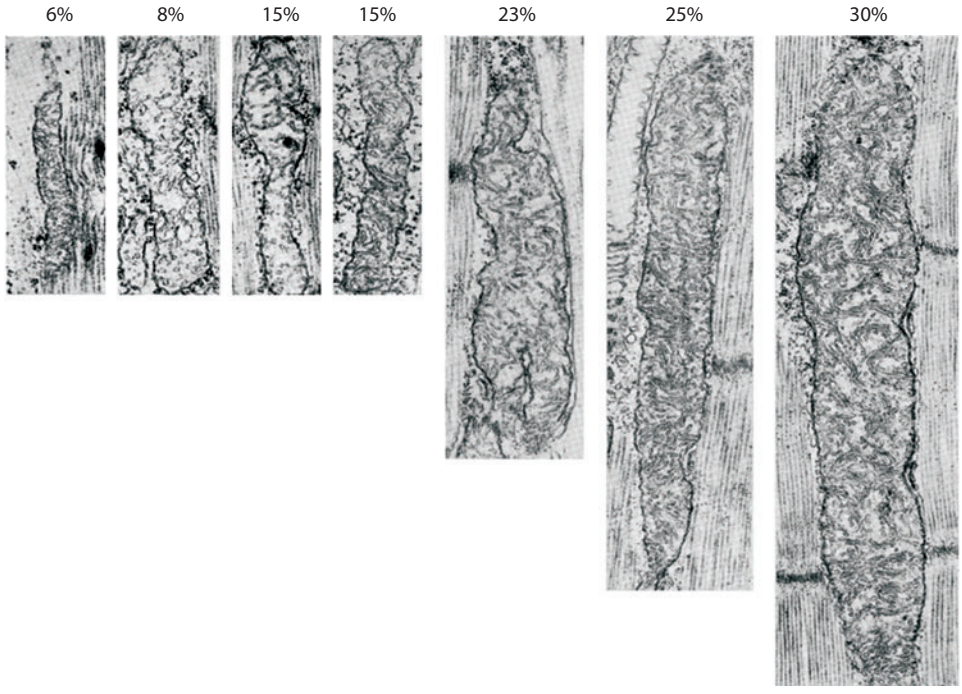

Figure 1

Mitochondria in the developing flight muscle of *Locusta migratoria* before and after the molt that leads it from the grasshopper stage to a flying insect. The sequence of electron micrographs shows mitochondria nine days, six days, three days, and one day before, as well as two hours, three days, and eight days after the imaginal molt. The percent values indicate the relative volume of the muscle occupied by mitochondria. Adapted from Reference 1.

I went to the Anti-Locust Research Center in London to obtain a stock of egg-laying locusts, which were the founders of hundreds of thousands of progeny. I scraped the flight muscles out of the thorax, isolated the mitochondria, and incubated them with radioactive leucine. One year after this start, I convinced myself that mitochondria can make proteins, and it was membrane proteins that were synthesized. This was supported by a few other reports studying the new field of mitochondrial protein synthesis in various organisms. My supervisor passed on to me his invitation to the first "Bari meeting." It was where, for the first time, I met the grand world of mitochondria research and its heroes. This was an exciting event for bioenergetics and biogenesis of mitochondria. The conference, in beautiful and then unspoiled Puglia, left a deep impression on me and so did the lectures. The fights and feuds among the various groups baffled me. There was a group challenging the existence of mitochondrial protein synthesis, arguing that it was an artifact owing to contaminating bacteria. The fiercest storm was on Peter Mitchell who presented his chemiosmotic hypothesis. Argument after argument destroying his hypothesis was thrown at him by the proponents of the "hypothetical intermediate." He listened quietly and after some time took out his hearing aids and put them on the table.

The work with locust mitochondria eventually led to the isolation and characterization of mitochondrial ribosomes. Surprisingly, they had a very small S-value, 55S to 60S, and they contained RNA of a very small size, almost half of that of the cytosolic ribosomes, which for quite some time made us believe they were degradation products (2). But then in several reports, mitochondrial ribosomal RNAs from

mammalian cells were reported to be of a similar small size. These ribosomes, during high-resolution electron microscopy, revealed somewhat smaller dimensions than cytosolic ribosomes and a very similar morphology. As it turned out, the extremely small size of the RNA and the lack of 5S RNA are compensated for by an increase of the number of ribosomal proteins.

Somehow, during all this work, I found the time to enlist in medical school and to go through the whole curriculum of preclinical and clinical studies. Studying medicine, in comparison to chemistry, was highly entertaining. The lectures and practical courses were eventful, patients were presented and examined, clinical pictures were complex and their analysis was intellectually demanding. And one got a glimpse of the satisfaction that one can derive from providing help to patients. This was a dangerous temptation, but after I had completed my state exam and received my MD degree and driven by the exhilaration of discovering new things, I dismissed medicine. Yet, I felt enriched by having experienced a completely different exciting world.

A burning question, arising from the finding that mitochondria have the capacity to make proteins, was to find out which proteins are made. What now seems a trivial task was extremely challenging in the late 1960s because there were practically no tools to separate and analyze membrane proteins. So we started to find out which of the mitochondrial membranes contained mitochondrial translation products. These experiments made it necessary to switch from the locust flight muscle to another source that would allow isolation of larger amounts of mitochondria. We chose regenerating rat liver, because mitochondria from this tissue were highly active in synthesizing proteins and new elegant procedures were available to separate outer from inner membranes. The result was that radioactive proteins were found only in the inner but not the outer membrane.

At this stage, it became obvious that further progress would require a different biological system that was more accessible not only to biochemical experimentation but also to genetic analysis. The experiments of Beadle and Tatum with the filamentous fungus *Neurospora* had brought a revolution to eukaryotic genetics. However, not only was this organism accessible to genetic manipulation, but also it was easy to grow on simple inorganic media, and kilogram amounts of cells could be obtained in a very short time. The fungal hyphae could easily be broken, and the content be released to yield morphologically and biochemically intact mitochondria. Moreover, in beautiful experiments, David Luck (3) had described the making of new mitochondria from preexisting ones. In addition, mitochondrial ribosomes were isolated and characterized. We found their proteins to be synthesized by cytosolic ribosomes, as it later turned out, with the exception of one protein of the small subunit (4).

The mid-1970s were a period of great excitement with mitochondrial biogenesis in the Bücher lab in Munich. Walter Sebald and Hanns Weiss had joined the mitochondria group. They were eager to find out which proteins were made by the mitochondrial ribosomes. They worked out methods to isolate cytochrome oxidase and then also cytochrome *b*. Walter Sebald developed the method of differential labeling of proteins in intact *Neurospora* cells by using cycloheximide and chloramphenicol, which specifically inhibited cytosolic and mitochondrial translation, respectively. The differential labeling involved the use of two different isotopes to discriminate between total protein and protein synthesized in the presence of one of the inhibitors. In this way, Sebald discovered the three largest subunits of cytochrome oxidase to be made in the mitochondria, in contrast to the smaller ones [see Sebald et al. (5)]. Hanns Weiss identified cytochrome *b* as a mitochondrial translation product [see Weiss & Ziganke (6)]. These results were confirmed for mitochondria of yeast by the groups of Alex Tzagoloff in New York, Gottfried Schatz in Basel, and Piotr Slonimski in Paris. They had performed pioneering work on mitochondrial genetics in yeast as well as biochemical work on the enzyme complexes

of mitochondrial oxidative phosphorylation. The results from all these labs came together in the early 1970s to provide an emerging picture of the genome of mitochondria of yeast and *Neurospora*. These studies were then completed by the discovery of three and two subunits of the ATP synthase in yeast and *Neurospora*, respectively, and one subunit of the mitochondrial ribosomal proteins.

EARLY WORK ON IMPORT OF PROTEINS INTO MITOCHONDRIA

In about 1975, I became interested in how the many mitochondrial proteins encoded in the nucleus and synthesized on cytosolic ribosomes would reach their sites of function in the mitochondria. Astonishingly, very few mitochondrial researchers found this question attractive. There seem to be several reasons for this. There was no immediate genetic approach, and this was the time when molecular biology made a steep rise. Maybe some people were simply put off by the obvious complexity of the process. Molecular geneticists and biologists may not have appreciated the importance of this problem, although it touched on many general aspects of cell biology, such as the mechanisms of movement of proteins across one or two membranes; the folding of proteins before, during, and after membrane passage; the addition of cofactors and assembly into multisubunit complexes; as well as the nature and specificity of signals for intracellular transport.

The processes involved in the intracellular and intraorganellar sorting of nuclear-encoded newly made mitochondrial proteins surfaced during a time when transport of proteins across and insertion into membranes came into the focus of cell biology. One of the major areas that developed at this stage was the secretion of proteins by eukaryotic and prokaryotic cells. Günter Blobel, David Sabatini, and others, inspired by the work of George Palade, studied the processes in the endoplasmic reticulum (ER) that, in 1976, led to the signal hypothesis by Blobel and Dobberstein. Bill Wickner, Jon Beckwith, and others were analyzing protein export and membrane insertion in bacteria with biochemical and genetic methods. Randy Schekman started to identify the proteins of the yeast secretory system by a combined genetic and biochemical approach. Cotranslational translocation and membrane insertion by membrane bound ribosomes were the predominant concepts.

The first proposal for mitochondrial protein import was made by Ron Butow [see Kellems et al. (7)]. He suggested that ribosomes would make mitochondrial proteins that sat at the mitochondrial surface, and therefore translocation would be cotranslational. However, under normal growth conditions, ribosomes were not seen in electron micrographs in association with the mitochondrial surface, in contrast to the ER. This suggested that import of proteins could occur after their synthesis. By contrast, in yeast cells treated with cycloheximide, which arrests translation, the mitochondrial surface was covered by ribosomes. This seemed to argue for a direct release of proteins by active ribosomes into the mitochondria.

Together with Matt Harmey, a sabbatical guest from University College Dublin, and Gerhard Hallermayer, a graduate student, we reasoned that the question of whether import was posttranslational or cotranslational could be solved by performing pulse-chase experiments. The basic assumption was as follows: If proteins are delivered to the mitochondria by ribosomes attached to the mitochondria, the appearance of proteins in mitochondria should occur with the same kinetics as the release of cytosolic proteins from cytosolic ribosomes. In contrast, if proteins were initially released from cytosolic ribosomes into the cytosol and then imported, there should be (*a*) a lag and slower appearance of these proteins in the mitochondria and (*b*) observable extramitochondrial pools of mitochondrial proteins with precursor product kinetics. We designed the experiments in the following way: *Neurospora* cells were grown in the presence of [^{35}S] sulfate to obtain a homogeneous labeling of cellular proteins; were given a pulse of [^{3}H]-labeled leucine

for short periods, followed by addition of a large excess of unlabeled leucine to instantly terminate further labeling; were incubated for additional different periods of time (chase); were shock cooled to 0°C; were harvested with breaking of the hyphae; and were separated into cellular fractions; finally, we determined the $^{3}H/^{35}S$ ratios in proteins. It was necessary to develop methods to rapidly harvest and fractionate the cells, a relatively easy procedure with *Neurospora* hyphae, but this needed at least 10 hands. Total mitochondrial proteins and individual proteins were then analyzed by immune precipitation from cytosolic and mitochondrial fractions and sodium dodecyl sulfate gel electrophoresis. The $^{3}H/^{35}S$ ratios were determined in gel slices. This was before autoradiography of dried gels was introduced and had the advantage of allowing reliable quantitation. The kinetics varied from 90 to 1,500 s, and newly made proteins showed a distinct time lag for their appearance in mitochondria and continued to accumulate in the mitochondria after blockage by cycloheximide. Among different mitochondrial proteins, the lag phase of labeling varied. Together, this showed the existence of extramitochondrial pools of mitochondrial proteins of different sizes for the various proteins analyzed (**Figure 2**). In addition to these in vivo experiments, protein synthesis in a cell-free homogenate mimicked the delayed kinetics of the labeling of proteins in mitochondria as compared to proteins of the cytosol fraction (**Figure 3**). Altogether, these results demonstrated that transport of proteins into the mitochondria is essentially a posttranslational process. However, it did not exclude the possibility that some mitochondrial proteins could be translocated in a cotranslational manner. The accumulation of cytosolic ribosomes on mitochondria observed by Butow and colleagues (7) indicated the possible location of signals for transport at the N terminus of the nascent chains because translocation could apparently start if elongation was stalled by cycloheximide.

Two of the first proteins whose biogenesis we investigated in more detail were the ADP/ATP carrier and cytochrome *c*. We found that the latter protein was initially synthesized as apocytochrome *c* (8). Luckily, we obtained antibodies that were able to discriminate between both forms of this protein. The apo form was imported into the mitochondria where it was converted to holocytochrome *c* by addition of heme via thioether bridges.

These findings were presented at a conference on the biogenesis of mitochondria and chloroplasts, which we organized in Munich in 1976, and were published in 1977 (9, 10). They raised a large number of doubts and questions. What was the nature of the signals that directed the newly synthesized proteins to the mitochondria? How could completed proteins cross the mitochondrial membranes? What conformation would these proteins have? How could membrane proteins exist as precursors outside the mitochondria? What kind of driving forces would support translocation? How would the different proteins know to which subcompartment of the mitochondria they should go?

In 1979, the labs of Günter Blobel and Gottfried Schatz published a study demonstrating the synthesis of nuclear-encoded subunits of the ATP synthase with N-terminal extensions that were removed upon import (11). These experiments fully confirmed the results of our in vivo and in vitro studies, arriving at the same conclusion that import can be posttranslational. Later in the same year, we reported a study on the synthesis of the matrix protein citrate synthase for which we had observed a large extramitochondrial pool. Upon pulse labeling of intact cells, this protein was present initially as a larger precursor species, which was cleaved when it reached the mitochondria (12). In our initial experiments in 1976 and then in further studies, a difference in the molecular mass of the precursor and mature proteins with cytochrome *c* and the ADP/ATP carrier was not observed (9, 13). This led us to conclude that there is one class of precursor proteins with signals at the N terminus, which are generally cleaved after import, and another class with internal targeting signals. In retrospect, owing to bad luck in

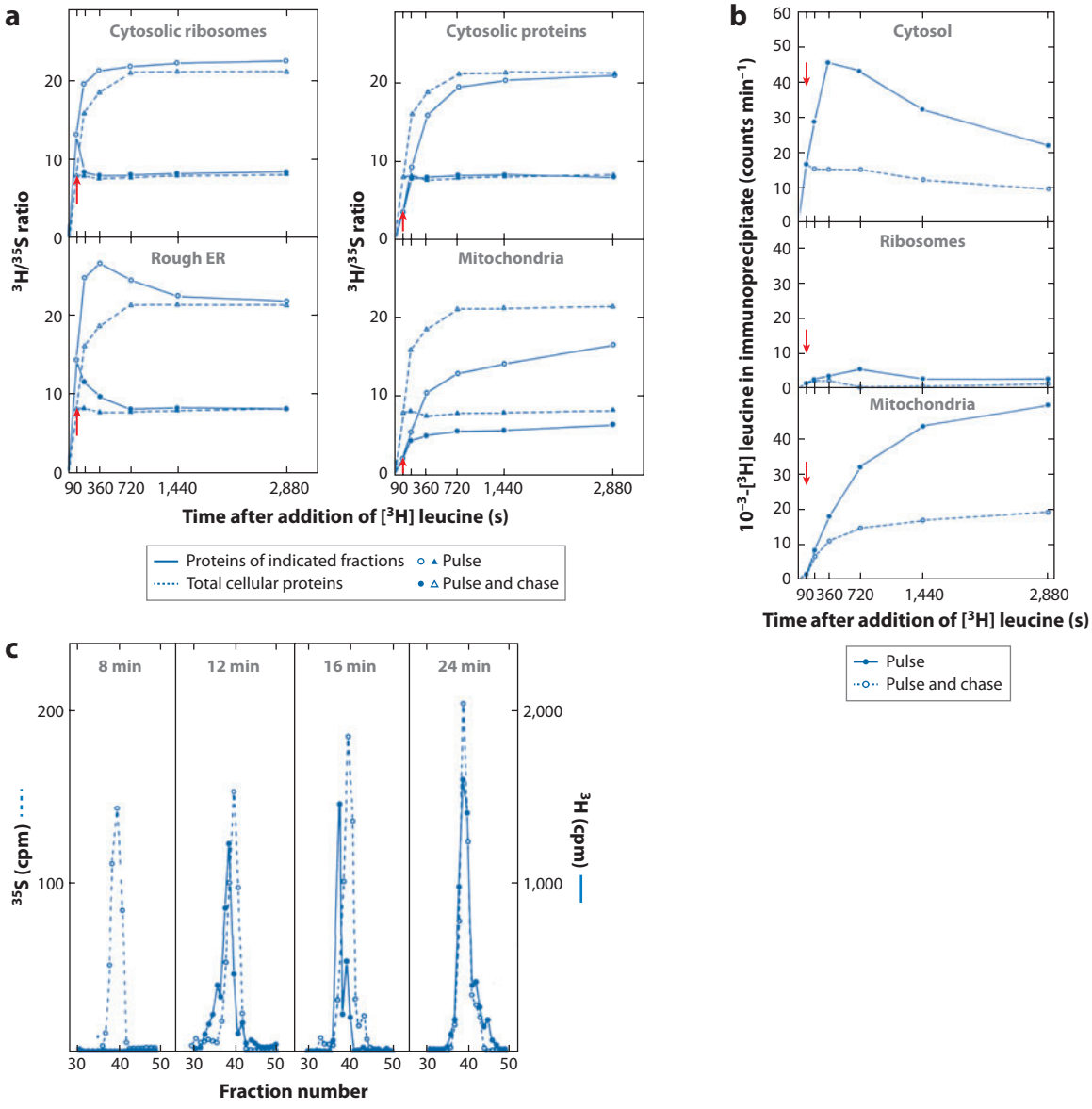

Figure 2

Kinetics of posttranslational protein translocation into mitochondria in intact *Neurospora* cells. (*a*) Kinetics of the appearance of newly synthesized proteins in mitochondria. Release of proteins from ribosomes is much faster than their transfer into the mitochondria, which shows a distinct lag phase. Cells were grown on [^{35}S] sulfate and then subjected to pulse labeling and pulse-chase labeling with [^{3}H] leucine at 8°C. Red arrows indicate the time of addition of a chase with unlabeled leucine; for comparison, the $^3H/^{35}S$ ratios of the total homogenate are included in all panels. (*b*) Translocation of matrix proteins into mitochondria is slow as compared to translocation of total mitochondrial proteins and indicates a large precursor pool in the cytosol. Cells were pulse and pulse-chase labeled with [^{3}H] leucine, and mitochondrial matrix proteins were immunoprecipitated from the indicated fractions. Arrows indicate addition of a chase of unlabeled leucine. (*c*) A larger precursor of citrate synthase appears upon pulse labeling, which is slowly converted to the mature citrate synthase. Cells were prelabeled with [^{3}H] leucine and pulse labeled with [^{35}S] sulfate. Citrate synthase was immunoprecipitated from cell homogenates after various times of labeling with [^{35}S] sulfate and analyzed by sodium dodecyl sulfate gel electrophoresis. Adapted from Reference 9. Abbreviations: cpm, counts per min; ER, endoplasmic reticulum; (s), seconds.

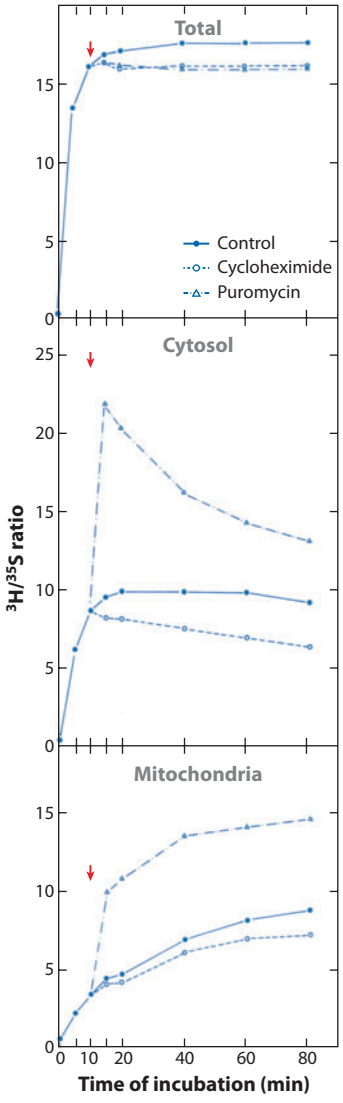

Figure 3

Rapid synthesis of proteins in an in vitro coupled translation-translocation system of *Neurospora* cells and slow uptake into mitochondria. Incorporation into various fractions of a cell-free homogenate were prepared from cells grown on [^{35}S] sulfate. Labeling was performed with [^{3}H] leucine. One aliquot was left untreated (control), or either cycloheximide or puromycin was added after 10 min (*red arrows*). After the indicated time periods, the homogenates were fractionated to separate the cytosol and mitochondria. Adapted from Reference 10.

our earlier experiments, we did not choose for analysis a precursor that belonged to the type with cleavable extensions. But importantly, our initial experiments revealed that it was possible to separate synthesis of mitochondrial proteins from import and to perform these steps in the test tube. Now, an experimental system was available to probe the many burning issues.

DIVERSITY OF IMPORT SIGNALS AND IMPORT PATHWAYS

In 1978, I accepted a professorship at the University of Göttingen. The town was small with some 100,000 inhabitants, including the 30,000 students, who were in the middle of a revolution dominated by Marxist students. The university was in a state of turmoil and paralysis, yet it still carried the historical reputation of a great place for science. At a time when the northern German Mescaleros, which included the progeny of the local professors, burned down buildings around my new lab, I was able to build up a larger group. This began with a few people, in particular Richard Zimmermann, who had started the work on the ADP/ATP carrier and came with me from Munich, and Matt Harmey, a regular guest from Ireland. Also, a number of new students and postdocs contributed to rapid growth of the lab. There was the most enjoyable atmosphere of a new beginning.

We looked at the mechanisms by which various selected proteins of the different mitochondrial subcompartments are imported into the mitochondria. The import signals and pathways of the various precursor proteins turned out to be of unexpected diversity. We studied the import of the ADP/ATP carrier in more detail and found that it required a membrane potential for insertion into the inner membrane (14). Receptor-like components on the outer membrane were required for efficient import. As an example of an outer membrane protein, we chose Porin, the most abundant protein of this membrane. We isolated Porin for the first time, which, as we showed together with Roland Benz at the University of Würzburg, was responsible for the VDAC (voltage-dependent anion channel) activity (15). Porin belongs to the family

of β-barrel membrane proteins as was recently confirmed by X-ray crystallography. It required surface components, but not a membrane potential, for insertion into the outer membrane. Like the ADP/ATP carrier, it did not contain a cleavable signal. Therefore, we thought the difference between the precursor and mature forms might be just a matter of the folding state. Helmut Freitag, a postdoc with a very original way of thinking, converted the detergent-purified Porin into a water-soluble form. Magically, this then indeed behaved like a precursor and could compete with Porin synthesized in a cell-free system for import into the outer membrane (16). Thus, we could use it to quantify receptor sites and determine affinities. The nuclear-encoded subunit 9 of the ATP synthase from *Neurospora* served as a model protein for the inner membrane with a mitochondrial targeting sequence, which is exceptionally long and, as it turned out, is cleaved in two steps. This powerful matrix-targeting signal is still used when a passenger protein is to be sent to mitochondria for a variety of experimental purposes.

Along with the availability of efficient methods to study protein import in vivo and in vitro came the ability to analyze energy requirements. Roles for ATP and the electrical membrane potential were identified. However, these requirements were not uniform. Proteins of the mitochondrial matrix required both $\Delta\Psi$ (the electrical membrane potential) and matrix ATP. Some inner membrane proteins required $\Delta\Psi$ only, and outer membrane proteins neither. Some proteins of the intermembrane space needed neither matrix ATP nor a $\Delta\Psi$, others required both. Matrix proteins turned out to require $\Delta\Psi$ only for translocation of the N-terminal targeting signals across the inner membrane and matrix ATP for the translocation of the rest of the polypeptides. This apparently confusing picture was clarified only when we learned more about pathways and the import machineries. The precise role of $\Delta\Psi$ is still not clear; it might be involved in a kind of electrophoretic effect on the positively charged targeting signals or may have a role in gating the opening of the protein-conducting pore.

In 1983, I took over the chair for Physiologische Chemie of the medical faculty in Munich. I was excited by the offer of this prestigious chair. Although the academic atmosphere in Göttingen was very inspiring and the empty country around the town very charming, I knew I would be happier in Munich, where I was born and grew up. Indeed, both my hopes and fears came true. Not only did a number of students and postdocs come along with me to Munich, but I also immediately recruited excellent new people. Soon, my lab included Nikolaus Pfanner, who initially came to perform work for his MD degree and left seven years later to hold the chair of biochemistry at Freiburg University; Ulrich Hartl, who already had obtained his MD; Rupi Pfaller and Thomas Söllner, graduate students; Max Tropschug, a postdoc who introduced the art of cloning; Don Nicholson, a postdoc from London, Ontario; Matt Harmey, who was back from Ireland; and Rosemary Stuart, a graduate student from Dublin. Swiftly, the new lab was set up, the institute was reorganized, and the experimental work became productive. And I enjoyed immensely being back in Munich, where my family was and where my Bavarian heart felt at home.

As expected, my position in Munich brought many commitments on top of organizing the institute, including duties in the medical faculty, which I could avoid for a long time but not entirely. For a number of years, I was vice dean and dean of research. Serious work was also required from me because of my membership in the senate and the governing body of the German Research Council and several subcommittees of this main German funding agency. I served as president of the German Biochemical Society, as chair of the EMBO Council and on the boards of other international scientific organizations. I was sitting on advisory committees and organized conferences. I did my full share in working as a member of editorial boards and as a reviewer. When I cleared my office after retirement, there were some 100 thick folders with reviews, references, and statements to be destroyed. This did not happen without pondering about how much of my life had been

consumed. I never really liked all these responsibilities and did not get satisfaction from a single one of them. Still, I felt that as a member of a community that only works if everybody cooperates, I had to make a contribution.

The move to Munich also meant a large teaching load. In the beginning, there were some 500 medical students per year to be taught in lectures, seminars, and practical courses; when I left, there were some 1,000 students. It is a common experience that not all medical students are fond of biochemistry, but there were always enough extremely gifted and interested students who made teaching a pleasure.

FOLDING AND UNFOLDING OF PROTEINS BEFORE AND DURING IMPORT

How are proteins crossing the mitochondrial membranes? Proteins released from cytosolic ribosomes generally undergo rapid folding to their functional conformations. An obvious question was whether this was also true for mitochondrial precursor proteins, and another was whether these proteins would cross mitochondrial membranes in a folded state. As the experiments told us, this was not the case; rather most precursors are not tightly folded. ATP in the cytosol was found as a requirement for many precursors to remain import competent after release from the ribosomes. Further experiments identified a role of cytosolic Hsp70 proteins in interacting with precursors and in preventing them from misfolding and aggregation. Cytosolic Hsp70 and Hsp90 were later found to have an additional function in targeting precursor proteins to the receptors on the outer membrane. These observations hinted at the possibility of precursors crossing the mitochondrial membranes in an unfolded state. Could intermediates arrested in the translocation process be observed? I remember precisely the moment when Manfred Schleyer, a graduate student, showed me a sodium dodecyl sulfate gel in which bands could be seen representing precursor proteins, which were processed by the peptidase in the matrix but were still accessible to added proteases in intact mitochondria (17). Intermediates of this type, which are spanning the outer and inner membranes at the same time, then became invaluable tools for studying the import mechanisms of precursor proteins. Furthermore, binding of antibodies to precursors could lead to a permanent arrest of spanning intermediates, and such complexes were present at sites where outer and inner membranes come close together at contact sites (18). These observations suggested that precursors were crossing the membranes in an unfolded state. Conclusive experiments were then performed by Martin Eilers in the Schatz lab. Cytosolic mouse dihydrofolate reductase (DHFR) was fused to a mitochondrial targeting sequence. This chimeric protein contained a folded DHFR domain but still could be imported into mitochondria (19). This meant it became unfolded, a conclusion that was confirmed by adding the DHFR antagonist methotrexate, which stabilized the domain and completely prevented translocation. These results had important impacts on a number of issues. One immediate problem was that after reaching the matrix, proteins must be folded again. But this was a story that went far beyond the world of mitochondrial biogenesis, and that will be told below.

During the 1980s, the Schatz lab and my lab had very good exchanges of information, and we had regular meetings every year, alternating between Munich and Basel. These meetings were extremely productive and provided new views as well as inspiration for new avenues of research. Our groups were the largest working in this field. In later years, more and more overlap of interests developed, competition was difficult to avoid, and this began to limit free communication. Still, I am convinced these contacts promoted the field enormously, and cooperation is generally much more efficient than competition.

MITOCHONDRIAL PROCESSING PEPTIDASE

The year 1988 was when the mitochondrial processing peptidase was identified. Ulrich

Hartl and Gerd Hawlitschek took a biochemical approach and purified two proteins from *Neurospora*, which together efficiently cleaved mitochondrial targeting signals, initially named MPP and PEP, then α- and β-MPP [see Hawlitschek et al. (20)]. cDNA cloning was used to identify their sequences. In the same year, the Schatz group identified the *MAS* genes, out of a class of yeast mutants (*mas* mutants) that were deficient in mitochondrial assembly. The genes *MAS1* and *MAS2* encoded the yeast homologs of α-MPP and β-MPP, respectively (21, 22). In collaboration with Arthur Horwich at Yale University, who had isolated yeast mutants deficient in mitochondrial biogenesis, we also identified the *mif2* gene, which encodes β-MPP. That the competing groups in the field independently came up (more or less at the same time) with very similar results was a theme that was to prevail in the following years. Sometimes one is inclined to believe in the existence of something akin to parapsychology. But more likely, even if we do not speak openly about our new results, somehow these findings diffuse without our saying anything.

Yet there was one completely unexpected finding. We observed, together with Hanns Weiss in Düsseldorf, who was studying complex III of the *Neurospora* respiratory chain, that β-MPP was also present as core protein I of this complex (23). Core I and II and the MPP proteins belong to one family, and these proteins are apparently derived from bacterial proteases during evolution (24). So there was a protein involved in the processing of precursor proteins, and at the same time, this protein has an essential function in the respiratory chain. Absence of the core proteins leads to a nonfunctional complex III. In yeast, the core proteins and the MPP subunits are related but not identical. MPP proteases are metalloproteases that belong to the Pitrilysin family. X-ray structures showed the relationship to the core proteins in atomic dimensions and yielded profound insights into the enzymatic mechanism of this unusual protease.

MITOCHONRIAL RECEPTORS FOR PRECURSORS AND THE TOM COMPLEX

The approach used to study specific binding on the mitochondrial surface and to determine the numbers and affinity of sites enabled us to find proteins with a receptor-like function. The first one was MOM19, after unification of the nomenclature called Tom20. This protein is anchored to the outer membrane and has a hydrophilic domain facing the cytosol. Specific antibodies to Tom20, when added to isolated mitochondria, were able to inhibit import of a subset of precursors, including those of the outer membrane and the matrix, but very inefficiently inhibit import of the ADP/ATP carrier (25). Soon afterward, we identified another outer membrane protein, MOM72, then Tom70, which like Tom20 was anchored to the outer membrane by an N-terminal transmembrane segment, exposing its large hydrophilic domain into the cytosol. Antibodies against Tom70 strongly inhibited binding to the mitochondrial surface and import of the ADP/ATP carrier, but these antibodies had little effect on the import of precursors, which interacted with Tom20 (26). This work was performed with *Neurospora* mitochondria; Gottfried Schatz's group produced very similar results with yeast, a mutually reassuring progress.

Our previous work had led us to postulate the existence of a "general insertion pore" functioning as a protein-conducting channel in the outer membrane. Having specific antibodies available for the receptors, we isolated a complex, which in addition to Tom20 and Tom70, contained two more proteins, Tom40 and Tom22 (27). Tom40 was identified, and the cDNA sequenced by Michael Kiebler in my group. These results were published back to back with the identification of Tom40 in yeast (28). This exciting work on the translocase of the outer membrane was initiated by Nikolaus Pfanner, Thomas Söllner, and Rupert Pfaller and was one of several lines of research that afterward occupied our group and several others for many years. We found three

more subunits in the TOM complex that are important for the stability of this complex (29).

Our work on the TOM complex concentrated on its functional and structural characterization. We stayed mainly with the complex from *Neurospora* because of its accessibility in large amounts and efficient isolation. Frank Nargang of the University of Edmonton, an excellent *Neurospora* geneticist and collaborator, generated mutants and put tags on the individual subunits of the TOM complex and also on those of a number of other complexes of the mitochondrial import machinery. Tom22 was found to cooperate with Tom20 as well as with Tom70 in the passage of precursors to the general insertion pore. These three proteins are essential components required for the formation of intact mitochondria with cristae (29). Tom22 has abundant negative charges on its N-terminal domain, which faces the mitochondrial surface. A mutant analysis suggested the negative charges play an important role in the recognition of the positively charged side of the amphipathic targeting signals. Indeed, as shown recently by the fine structural and biochemical work of Toshi Endo and colleagues (30), this is what takes place when Tom20 and Tom22 together bind matrix-targeting signals. Roland Lill, a postdoc and then group leader together with Andreas Mayer, took over the part of characterizing the TOM complex functionally. They studied the TOM complex in isolated outer membranes and were able to discriminate two different binding sites: the *cis* site on the surface of the outer membrane containing the receptors, and the *trans* site on the inner face of the outer membrane (31, 32). Depending on the experimental conditions, the N-terminal targeting signals of precursors, but not the rest of the polypeptide chains, could transit from *cis* to *trans*. Precursors were observed to move to the *trans* site spontaneously, pointing to a higher affinity of the *trans* site compared with the *cis* site. Interestingly, when a DHFR domain was placed right behind the targeting signal, the DHFR became unfolded upon interaction with mitochondria. Binding of such constructs did not occur, if folding was forced by addition of methotrexate; when they were first bound to the TOM complex and then methotrexate was added, folding occurred as well as release from the mitochondria. Thus, binding and unfolding were equilibrium reactions, an important piece of knowledge for later studies on the mechanism of the import motor.

After a few years of work, Klaus-Peter Künkele and Marcus Dembowski, graduate students, and Roland Lill succeeded in isolating the TOM complex from *Neurospora* in a pure state (33). It came in two forms, (*a*) the core complex containing Tom40, Tom22, and Tom20 and the small subunits Tom7, Tom6, and Tom5, the latter three all C-terminally anchored proteins; and (*b*) the holocomplex containing in addition the receptors Tom20 and Tom70. Single-molecule electron tomography upon negative staining revealed a large structure with three holes in the case of the holocomplex and two holes of ~2 nm in diameter for the core complex (this work was performed by Stefan Nussberger with the Baumeister group in Martinsried). Still, an X-ray structure of the complex was missing; to our great disappointment, crystals, although easily obtained, did not sufficiently diffract. Together with the group of Michel Thieffry in Paris, we found the reconstituted complex had characteristic cation-selective and voltage-gated pores, which were regulated by mitochondrial targeting peptides (34). The TOM core complex displayed three different levels of conductance, suggesting the existence of two pores. The isolated TOM complex, as then shown by Doron Rapaport, a postdoc from Israel, retained a number of its in vivo properties, such as specific and high-affinity binding of precursor proteins (35). The TOM core complex could be dissociated to yield a subcomplex consisting of a single ring of Tom40 molecules with only two conductance states, demonstrating the central role of Tom40 as the pore-forming entity. Circular dichroism spectral analysis of

Tom40 suggested that β-strands are its main structural elements, which was in agreement with prediction programs (36). Still it is unclear as to whether the pore seen upon electron microscopy and made up by several Tom40 subunits is the protein-conducting channel or whether single Tom40 subunits constitute this channel. Recently, certain proteins that move through the TOM channel were observed to have the ability to exit laterally into the outer membrane. Thus, the TOM complex still poses many intriguing questions—even 20 years after its discovery. Roland Lill moved to the University of Marburg in 1995 to become professor of cell biology and discovered the complex machinery of mitochondria that provides the various cellular compartments with the iron sulfur clusters required for the formation of iron-sulfur proteins, an eminent contribution to understanding mitochondrial biogenesis.

HEAT SHOCK PROTEIN HSP60 AND THE DE NOVO FOLDING OF PROTEINS

Hsp60 and its cofactor Hsp10 appeared on the mitochondrial landscape when we entered into a collaboration with Arthur Horwich, at Yale University, to functionally characterize yeast mutants with defects in mitochondrial import. Ulrich Hartl and Joachim Ostermann analyzed the *mif4* mutant of the Horwich collection of temperature-sensitive mutants [see Cheng et al. (37)]. The phenotype of the *mif4* mitochondria was stunning. They could import precursors into the matrix, but their assembly in the matrix was defective. We proposed that protein-catalyzed assembly takes place in mitochondria and that the *MIF4* gene product had a role as a "workbench" in assisted acquisition of the native conformation of oligomeric proteins. We were particularly excited by this observation when Hsp60 turned out to be the protein encoded by the *MIF4* gene. Hsp60 is a homolog of the bacterial GroEL and the Rubisco subunit-binding protein, RBP60, of chloroplasts. GroEL was known for its role in mediating the assembly of λ-phage. RBP60 was found, by the group of John Ellis in Warwick, to be involved in the assembly of the nuclear-encoded small subunit and the chloroplast-encoded large subunit of Rubisco. They proposed the term molecular chaperones for components facilitating assembly of large protein complexes (38).

Ulrich Hartl then followed the scent of an exciting problem. We knew that proteins are unfolded when they enter the mitochondrial matrix, which is similar to the situation prevailing when polypeptides are emerging from the translocation channel of ribosomes during protein synthesis. Was it primarily the folding of the proteins in the mitochondria that required the action of Hsp60? This question was revolutionary because folding of proteins was generally considered a spontaneous process. As demonstrated by Anfinsen (39), purified ribonuclease A can refold spontaneously after denaturation. Convincing as the Anfinsen experiments were, it was, however, largely ignored that most proteins when unfolded would not refold in the test tube. This was attributed to the experimental conditions, which would be different in the cell. We showed that Hsp60 interacted with unfolded precursor proteins imported into the matrix and that they were released folded in an ATP-dependent manner (**Figure 4**) (40). The conclusion was that folding of proteins in mitochondria is catalyzed by a transient interaction with Hsp60, a process driven by ATP hydrolysis. As it turned out, not all proteins require Hsp60 for folding, but the general principle of chaperone-mediated folding in the cell was established. Within a few years, not without fights and feuds, the existence of catalyzed, chaperone-mediated protein folding was generally accepted. Ulrich Hartl became completely absorbed by protein folding in vivo and in vitro and quickly drifted away from mitochondrial biogenesis into the rapidly emerging new field of protein folding in the cell. He left the Munich lab to join the department of Jim Rothman as an associate member at Sloan-Kettering Institute in New York and returned five years later to become director at the Max Planck Institute of Biochemistry in Martinsried.

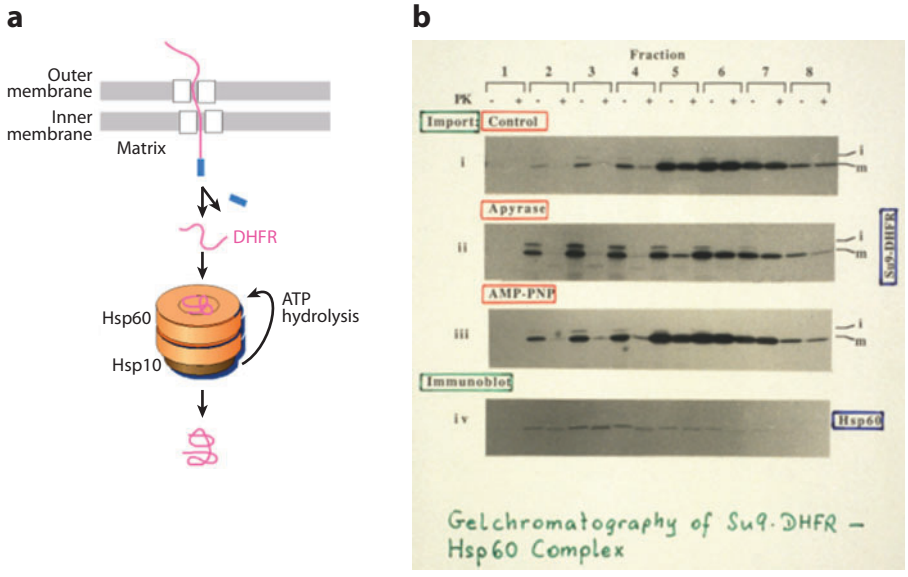

Figure 4

Hsp60-mediated folding in the mitochondria. The unfolded protein dihydrofolate reductase (DHFR) after import into mitochondria is bound to Hsp60 chaperonin in the matrix and released in the presence of ATP in a folded state. The white boxes in the outer and inner membranes symbolize the protein translocases TOM and TIM23. The blue bar represents the matrix-targeting signal. (*b*) The unfolded imported fusion protein Su9-DHFR (Su9, mitochondrial targeting-sequence of subunit 9 of the F_1F_O-ATP synthase of *Neurospora crassa*; DHFR, mouse dihydrofolate reductase) accumulates with Hsp60 in the absence of ATP, but not in its presence, where it is recovered as folded protein. Adapted from Reference 40. Abbreviations: PK, proteinase K; AMP-PNP, adenylyl-imidodiphosphate.

THE ROLE OF MITOCHONDRIAL HSP70 IN PROTEIN IMPORT

Support of protein folding in the mitochondrial matrix is not the only role of ATP in the import and assembly process. As was found early on, precursor proteins would not enter mitochondria at low levels of matrix ATP. So the question was: Which components would transduce the free energy of ATP hydrolysis into the energy for movement of polypeptide chains across both mitochondrial membranes? Experimental access to this question became possible once Betty Craig had created temperature-sensitive mutants of Ssc1, the mitochondrial member of the Hsp70 family in yeast. The mtHsp70 (Ssc1) is an essential protein and the closest relative of bacterial DnaK among all the various Hsp70s (41). Nikolaus Pfanner and Joachim Ostermann studied the effects of functional depletion of mtHsp70 on mitochondrial protein import [see Kang et al. (42)]. Import was interrupted at a stage in which the N-terminal targeting signals had crossed the inner membrane and were processed by MPP, but the bulk of the precursors were still outside the outer membrane. MtHsp70, therefore, is required for translocation of the mature parts of the precursor proteins, but not for translocation of the targeting signals. Yet, unfolded DHFR as passenger domain was still imported to a low degree in the mutant, apparently owing to residual activity of mtHsp70, whereas folded DHFR was not. We concluded that mtHsp70 is not only required for translocation, but also for the unfolding of folded domains in order to pass through the

mitochondrial membrane barrier. Moreover, imported DHFR did not become folded after being completely transferred into the matrix, in contrast to wild-type mitochondria. This indicated that mtHsp70 is also required for folding in the matrix. In further experiments, a direct interaction of mtHsp70 with precursor proteins was demonstrated. In summary, mtHsp70 turned out to be part of a matrix-based motor for the import of polypeptide chains.

Over the years following this initial discovery, the central role of mtHsp70 in protein import was substantiated by a series of functional studies. Precursors arrested by a folded DHFR domain on the cytosolic side of the mitochondria, as found by Doug Cyr, a postdoc from the University of North Carolina at Chapel Hill, and Christian Ungermann, a graduate student, were held in place by mtHsp70 (43, 44). At low levels of ATP in the matrix, they underwent reverse movement as long as the segments having reached the matrix were short, but not when they were long enough to form folded structures. We determined the length of segments of precursors spanning both TOM and TIM complexes, about 45–50 residues, translating into about 15 nm of length of an extended polypeptide chain. The TOM complex measures ~7 nm across the membrane, and the TIM23 complex should not be smaller, meaning the precursor chain must be essentially unfolded. This substantiated our previous conclusion that precursors crossed the both membranes in an extended fashion. Furthermore, we could dissect the roles of the driving forces, ATP hydrolysis and the membrane potential. In the early stages, the electrical membrane potential drives translocation of the matrix-targeting signal, and its binding to mtHsp70 is necessary to make this step irreversible. The precursor chain can oscillate in the import channel, and the cycling of mtHsp70 on and off the incoming polypeptide chain, powered by ATP hydrolysis, then leads to vectorial movement.

The process of reverse movement (or backward slippage) is a salient feature of a specific dual targeting pathway. A number of matrix enzymes are present both in the mitochondria and in the cytosol. In case of fumarase, a single gene encodes a precursor with a matrix-targeting signal. As demonstrated by Ohad Yogev & Ophry Pines (45), a fraction of the precursors end up in the matrix as processed mature enzyme. Another fraction is localized to the cytosol. It is also processed by MPP but then undergoes reverse movement. Folding of part of the precursor in the cytosol and interaction with the Hsp70s in matrix and cytosol appear to determine the degree of the eclipsed distribution.

Like all members of the Hsp70 family, mtHsp70 interacts with cochaperones. Together with Neil Rowley, a graduate student from Cambridge, Benedikt Westermann and Elisabeth Schwarz in my lab identified Mdj1 as the equivalent of bacterial DnaJ [see Rowley et al. (46)]. A surprise was the discovery of a protein we named Hep1, for Hsp70 escort protein. This protein exists in all eukaryotes, and its deletion leads to aggregation of mtHsp70. It appears that Hep1 has a role in stabilizing mtHsp70 in certain stages of its ATP-dependent conformational cycle.

THE TIM23 COMPLEX OF THE INNER MEMBRANE

With the growing insights into the complexity of the import pathways, it was realized that the molecular machinery mediating translocation into and across the inner membrane would have a multiplicity of tasks: Recognition of targeting signals in the intermembrane space after their initial recognition by the TOM complex, opening and closing of a precursor-conducting pore in the inner membrane while protecting the membrane potential, differentiation between precursors destined to the matrix and precursors to be laterally released into the inner membrane, and coordination of the precursor-conducting channel with the TOM complex and with the import motor to prevent idling.

The first three constituents were identified by genetic screening. Michel Meijer and coworkers (47) in Amsterdam discovered a gene encoding Tim44, a hydrophilic protein; this was also found in a biochemical assay by the

Schatz group. Rob Jensen's group (48, 49) identified Tim23 and Tim17 as constituents that were in contact with the translocation intermediates.

At this juncture, Michael Brunner came to our lab after a postdoc period with Jim Rothman in New York. He set out to study how the translocase of the inner membrane works. In a first set of experiments, together with several graduate students, he looked at the interaction of mtHsp70 with the membrane components identified. They found that mtHsp70 interacts with Tim44 in an ATP-dependent manner (50, 51). In the presence of ATP, the Tim44-mtHsp70 complex locked precursor polypeptides in a position in which they cannot undergo reverse movement. When a folded DHFR domain was placed at the C terminus of a precursor, this was held in tight apposition to the TOM complex. These results and others described above led us to propose that the import motor works as a molecular ratchet (52). This view was not shared by others who instead suggested a power stroke model in which mtHsp70 actively pulls on the incoming polypeptide chain. The molecular ratchet relies on the ability of the polypeptide chain to slide in the protein-conducting channel after translocation has been initiated by the effect of the membrane potential on the targeting signal. It further relies on the equilibrium of the (partial) folding and unfolding of precursors at the entry of the TOM-TIM complex. Since the time when these opposing models were put forward, a large amount of experimental data have been accumulated in favor of the molecular ratchet model (52a, 53).

In a next step, we found out that Tim44 forms a complex with the two other components, Tim23 and Tim17. We first termed this complex MIM; later, it became the TIM23 complex. In the isolated complex, we found additional components, in particular a 14-kDa protein, but this had to wait eight more years to be identified. The complex was unfortunately rather unstable in the presence of detergents; it was only with digitonin that it could be isolated together with accumulated precursor polypeptides and the TOM complex, indicating a close cooperation of TOM and TIM23 in the import process.

Tim23 and Tim17 are proteins that are predicted to span the inner membrane four times. These segments show sequence similarity, which points to a common origin of both proteins. In addition, yeast Tim23 has a hydrophilic part at the N-terminal side, comprising ~100 amino acid residues. We performed an analysis of the latter part of Tim23 and found the segment comprising residues 50–100 to be essential for the function of the TIM translocase. We suggested that this part of Tim23 acts as a receptor for mitochondrial targeting signals in the intermembrane space and responds to the membrane potential by changing the conformation and oligomeric state of Tim23 (54). A specific transmembrane helix of Tim23 appears to play an important role in the opening of the translocation channel (55). Altogether, Tim23 seems to be a voltage sensor in mediation of the membrane potential–dependent step of translocation of matrix-targeting signals across the inner membrane. The mechanistic details of the opening and closing of the protein translocation channel, however, are still unclear. Tim17 and Tim23 interact with Tim44 by forming dimers or higher oligomers. On the basis of these findings, we suggested a basic mechanistic property of the import machinery, namely that it acts in a hand-over-hand mechanism in which two neighboring Tim44 and mtHsp70s work together at the outlet of the translocation channel to hold the translocating chain, a plausible element of a ratchet-like import machinery.

A further component of the TIM23 translocase, Tim50, was discovered independently by the groups of Pfanner (56) and Endo (57) in yeast, and in my lab by Kai Hell and Dejana Mokranjac in *Neurospora* [see Mokranjac et al. (58)]. Tim50 is anchored to the inner membrane; its intermembrane space domain interacts with that of Tim23, a process responsible for the recognition of matrix-targeting signals and their passage to the translocation channel. Tim50 is apparently the first component of the TIM machinery to

recognize the targeting signals of precursors coming in through the TOM complex. Tim50 can be cross-linked to precursors even in the absence of a membrane potential and stays in their vicinity until the complete polypeptide chain has reached the matrix (58). It took some three years more until the most recently found components of the membrane sector of the TIM23 translocase were discovered, Pam17 and Tim21 (59–61). These are components that are not essential for the viability of yeast. Their association with the other constituents is not a firm one, and they change their interaction with the other subunits depending on the type of precursor translocated. Their precise functions are not known, yet Tim21 has the ability to interact with the TOM complex and thus may have a role in tethering both translocases.

The number of constituents of the import motor of the TIM23 translocase has also expanded since the discovery of Tim44 and mtHsp70. The nucleotide exchange factor Mge1 was found to be absolutely necessary for driving import because it catalyzes an essential step in the binding and release cycle of mtHsp70 to Tim44 and the precursor polypeptide to be imported (62). Then, we identified Tim14, the 14-kDa component observed previously in a complex with Tim44, Tim23, and Tim17 (63). This was again a case where three labs at the same time, in this case at the same meeting, presented a new component of mitochondrial import machinery (63–65). Tim14 (synonym Pam18) is a J domain protein and is membrane anchored. It has the ability to stimulate the ATPase activity of mtHsp70 and can be considered as the "accelerator" of the import motor. Immediately thereafter, we discovered Tim16, which forms a complex with Tim14 (66). The complex, in contrast to the isolated Tim14, did not stimulate mtHsp70 ATPase activity. Thus, it is acting as a "brake" of the motor. In further studies, using *in organello* cross-linking experiments, Tim14 and Tim16 were found in close contact with Tim44 and mtHsp70. These interactions were dependent on the nucleotide present, suggesting that extensive conformational changes take place during the cycles of the motor, involving binding and release of mtHsp70 to and from the precursor polypeptides (67). A more-detailed model of the motor was possible when Dejana Mokranjac and Michael Groll obtained a crystal structure of the Tim14-Tim16 pair. This pair forms a heterotetramer of two subunits each. The structure explains how Tim16 inhibits Tim14 and suggests the way they control the activity of the import motor (**Figure 5**) (68).

With 10 components in total found so far in the TIM23 translocase, models proposed for the mechanisms of action are not are not likely to be convergent. Thus, different views exist about how the divergent processes of complete translocation into the matrix versus lateral release into the inner membrane are triggered and regulated. Likewise, it is a matter of debate whether the membrane part and the import motor act as a single entity or by assembly of these modules upon demand (60, 69–71). The latter model would imply an additional signal in precursor proteins to trigger the assembly reaction for which there is no evidence so far.

And there are more unsolved questions. It is not understood how the electrical membrane potential is involved in the opening of the protein-conducting channel. The roles of Tim17 and Tim23 in forming the protein-conducting channel are still a mystery. The coordination of the various components of the membrane module and the motor as well as the interaction with the TOM complex are also poorly understood. X-ray structural analysis, it is hoped, will shed light on these questions.

SURPRISING ASPECTS OF THE TIM23 TRANSLOCASE

The TIM23 complex surprised us with unexpected variability in its functions. I want to illustrate this by three examples. A first one is the import of Bcs1 type substrates. The AAA-ATPase Bcs1 is involved in the biogenesis of the Rieske FeS protein (72). It is anchored to the inner membrane by a transmembrane domain close to the N terminus, and its

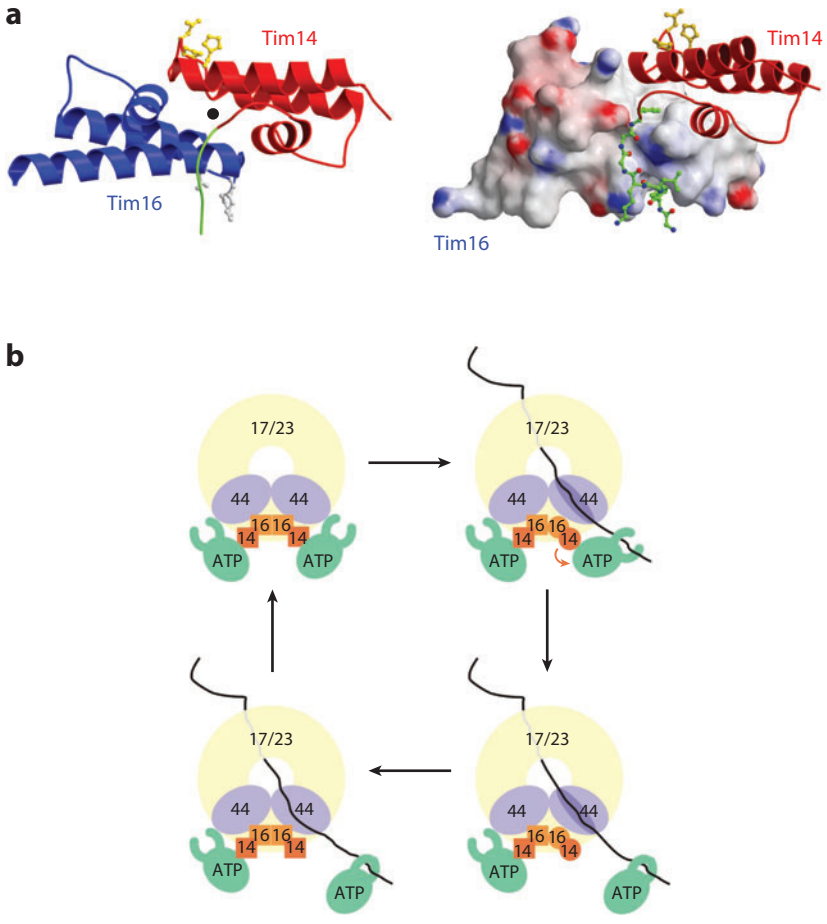

Figure 5

The import motor of the TIM23 translocase. (*a*) X-ray structure of the Tim14-Tim16 subcomplex and (*b*) molecular ratchet model of the motor. Adapted from References 68 and 116. The numbers of the protein components represent affixes to their names as Tim components and reflect their respective molecular mass in kDa.

ATPase domain is exposed to the matrix (73). This protein is synthesized without an N-terminal targeting signal, but such a sequence is present internally right after the transmembrane domain. Taken out of context and put in front of a passenger protein, this sequence works perfectly well as a targeting signal. In Bcs1, however, it works only in conjunction with the transmembrane domain. Rosemary Stuart and Heike Fölsch showed that the Bcs1 precursor threaded into the TIM23 channel as a loop structure (73). This leads to insertion of the membrane segment with an N-out orientation and to the sliding of the rest of the polypeptide through the channel into the matrix.

A second example is the biogenesis of the two forms of the mitochondrial dynamin-like GTPase, Mgm1 (74, 75). This protein plays an important role in the fusion of mitochondria. Mgm1 occurs as a long form, l-Mgm1, anchored by a transmembrane segment some 100 residues from the N terminus, exposing its large GTPase domain to the intermembrane space. About half of the protein exists in this form. The short form, s-Mgm1, lacks the

N-terminal anchor of l-Mgm1 to the inner membrane. It is generated by cleavage at a site that is located at the C-terminal side of the transmembrane anchor present in the l-Mgm1, a reaction catalyzed by the rhomboid protease Pcp1. Pcp1 is an integral membrane protease that cleaves a peptide bond present in or near the lipid phase of the inner membrane. Thus, about half of the Mgm1 precursors are not arrested in the inner membrane when the membrane anchor sequence crosses TIM23 but slips through to allow the more C-terminally located cleavage site to be seen by Pcp1. Because the presence of both forms of Mgm1 is required for mitochondrial fusion, the ratio must be carefully regulated. The factors responsible for this are not completely clear. One factor is the activity of the import motor, which again depends on the ATP levels in the matrix. The homolog of Mgm1 in higher eukaryotes is Opa1, a protein that is involved in crista structure formation and that plays a role in apoptosis. Interestingly, Opa1 is not cleaved by the mitochondrial rhomboid protease, but rather by the m-AAA protease paraplegin, implying a surprising change in the regulation of its synthesis during evolution (76, 77)

A third example is the ability of yeast Tim23 to interact with the outer membrane so that its N terminus becomes exposed on the surface of the outer membrane (78). This reaction was most surprising because in this way a protein is generated that is anchored to the inner membrane by four transmembrane segments, has a domain of ~50 residues in the intermembrane space, and crosses the outer membrane with a sequence that is not of the usual transmembrane type. Insertion of the N terminus is triggered by the presence of a translocating chain in the TIM23 complex and is reversible (69). The function of this reaction is not clear, but it may help TOM and TIM23 to find each other for import of precursors. Fusion of stably folded protein domains to the N terminus of Tim23 leads to proteins that are permanently spanning outer and inner boundary membranes without impairing the functionality of Tim23. These fusion proteins proved to be extremely useful as they can serve as markers for the inner boundary membranes and contact sites between outer and inner membranes, as described below.

A protein whose sorting pathway was controversial is yeast cytochrome b_2. This protein is present only in yeasts. It is located in the intermembrane space as a soluble protein. Its precursor contains a matrix-targeting signal and a sorting signal, which are successively cleaved off when the precursor, mediated by TIM23, reaches the matrix. Its sorting has been intensely investigated and different pathways were suggested. We had initially proposed a conservative sorting pathway. This turned out not to be the case; rather the protein appears to belong to the class that is laterally released. It is, however, still possible that the precursor makes a partial entry into the matrix, in a way similar to that of Mgm1. In the case of cytochrome b_2, this might be necessary for translocation of the heme-binding domain across the outer membrane, which has a tendency to fold tightly in the presence of heme. Rather frequent observations have convinced me that Goethe was right when he said: *"Die Gelehrten sind meist gehässig, wenn sie widerlegen; einen Irrenden sehen sie gleich als ihren Todfeind an."* (Scholars are usually venomous when they refute somebody; they view an erring peer immediately as a deadly enemy.)

THE TIM22 TRANSLOCASE

Shortly after the initial experiments to identify the TIM23 translocase, Michael Brunner and Christian Sirrenberg detected an open reading frame in yeast showing sequence similarity to Tim23 and Tim17 and the same overall predicted structure of four membrane-spanning segments. Likewise, the encoding gene was essential for viability of cells. Downregulation of the protein led to reduction in the expression of solute carrier proteins, in particular the ADP/ADP carrier, whereas the levels of proteins transported by TIM23 were unaffected. This was the result of a defect in import as observed by in vivo experiments. We named this protein Tim22 (79). Tim22 was present in

a large complex not associated with the import motor, which was different from the TIM23 complex. We concluded that mitochondria had two import machineries in the inner membrane with different client precursors. This fitted perfectly with our previous results on the import of carrier proteins. In further studies on the complex, the groups of Jensen (80, 81) and Schatz (82) found additional components of the complex, Tim54 and Tim18, that are not essential but important for the stability of the TIM22 complex.

Following the identification of Tim22, we discovered this membrane protein needs cooperation of other components, Tim10 and Tim12 (83). These proteins were identified through their association with Tim22. In independent studies, they were found also by Carla Koehler and coworkers in the Schatz group (84). Without our groups apparently knowing of each other, the manuscripts were submitted on October 9, 1997, and accepted on November 25, 1997, ours in *Nature*, theirs in *Science*. Both manuscripts reported the interaction of these proteins with the ATP/ADP precursor, a major transport substrate of the pathway, suggesting that these proteins assist translocation of the hydrophobic carriers across the intermembrane space. Then Tim9, present in complex with Tim10, was discovered as another protein required for translocation (85, 86). In this case, we submitted six weeks later to the same journal. Another complex of "small Tim proteins," Tim8 and Tim13, was then described by Koehler and Schatz, similar in its structure to the Tim9-Tim10 complex. It helps, but is not essential for, import of the Tim23 precursor by the TIM22 translocase into the inner membrane. These results revealed an interesting and unexpected interdependence of the TIM23 and TIM22. Both are indispensible for mitochondrial biogenesis and have apparently a related central component responsible for transport across and insertion of precursors into the inner membrane in a membrane potential–dependent manner. Yet, they have a different molecular clientel, and TIM22 is necessary for the biogenesis of TIM23.

THE DISULFIDE RELAY SYSTEM

The discovery of the small Tim proteins in the late 1990s triggered an unforeseen burgeoning area of mitochondrial research. Initially, we were interested in their import pathways and observed that they could enter the intermembrane space via the TOM channel and somehow were retained then in the intermembrane space. We thought some factor would be bound to make translocation vectorial (87). The presence of two CysXXXCys motifs in the small Tim proteins was apparent from the point of their identification. We did not consider that these cysteines could form disulfide bonds. Proteins with S-S bridges so far were not found outside the endomembrane system. However, when other groups provided support for this assumption, we followed this line carefully. An important step was the discovery of Mia40 by the groups of Pfanner (88), Endo (89), and ourselves (90). Clients of this pathway enter the intermembrane space in the reduced state and interact with oxidized Mia40 to form mixed disulfide bonds. Mia40 acts as the initial retention component in the intermembrane space. Then the disulfide bonds are formed, and the proteins are released from Mia40 to undergo oxidative folding, leaving Mia40 in the reduced state. A next important step in our lab was the discovery of the role of Erv1 as disulfide oxidase in the intermembrane space by Nikola Mesecke, Johannes Herrmann, and Kai Hell [see Mesecke et al. (91)]. Erv1 catalyzes the removal of electrons from the reduced Mia40, which thereby returns to the oxidized state for further import cycles. Erv1 delivers the electrons to the respiratory chain to complete the reduction cycle. We termed this pathway the disulfide relay system. This pathway is probably more complex than known so far, as several other components assist in these reactions. Furthermore, this import system works also for other mitochondrial disulfide-containing proteins, such as the CuZn superoxide dismutase together with components mediating the addition of copper ions. This enzyme is of considerable interest to those studying the biology

of oxygen radicals and various diseases, such as amyotrophic lateral sclerosis (92–94). In this way, mitochondria became a new important player on the stage of disulfide biochemistry, with many groups exploring these new avenues further.

THE OXA1 COMPLEX

Import of precursor proteins, directed by an N-terminal signal in the majority of cases, implies that the N terminus resides in the matrix. However, early on, we realized that this was not always the case and that the assembled proteins with such a signal expose their N terminus into the intermembrane space. A prominent example is subunit 9 of the F_1F_O-ATP synthase (Su9), which forms the core of this enzyme complex's rotor. Su9 consists of two membrane-spanning helices with a loop on the matrix side. It is encoded in most eukaryotes in the nuclear genome, synthesized with an N-terminal targeting signal, and imported into the mitochondria by TIM23. Su9 of yeast is an exception; it is encoded by mitochondrial DNA, made on mitochondrial ribosomes, and inserted into the inner membrane from the matrix side.

How are these proteins put in place? In 1994, the identification of a nuclear gene termed *OXA1* was reported to be involved in the assembly of cytochrome oxidase (95, 96). Rosemary Stuart and Kai Hell found out that the Oxa1 protein is required for the insertion of cytochrome oxidase subunit II and of nuclear-encoded Su9 into the inner membrane. These observations are a very nice illustration of the events underlying the evolutionary processes involved in the transition from endosymbiosis to present mitochondria. Oxa1 is used both by mitochondrially encoded proteins and by nuclear-encoded proteins. The latter ones, which in the endosymbiont have entered the inner membrane from the inner face, are returned from the cytosol first into the mitochondrial interior, where they then use the "ancient" pathway they used in the bacteria. We have named the Oxa1-mediated pathway "conservative sorting" (97). Tom Fox and his coworkers arrived independently at the same conclusions (98). Ideas and concepts obviously mature at the same time at different places. This finding led to an intense investigation not only of the functional mechanism of Oxa1 but also of the bacterial homolog, YidC. Usually, the mitochondrial researchers have kept and continue to keep a close eye on their bacterial colleagues; this was a case where the latter could learn from the former ones.

We also found that Oxa1 has five transmembrane segments and faces the intermembrane space with its N terminus (99). It is synthesized in the cytosol with a matrix-targeting signal and, intriguingly, undergoes conservative sorting. The Oxa1 precursor can use the assembled preexisting form of Oxa1 for its assembly. Because it can be restored by artificial expression after deletion of its gene, there must be an additional topogenic component involved in its assembly. Perhaps Cox18, a homolog of Oxa1, plays a role, yet its function is not entirely clear. Further studies then showed a number of exciting properties of Oxa1. It exposes a C-terminal helical domain into the matrix, which serves as an anchor for mitochondrial ribosomes to the matrix face of the inner membrane, suggesting a cotranslational insertion pathway of mitochondrially encoded proteins (100). Oxa1 can replace yidC in bacteria, exhibiting an amazing degree of functional conservation during evolution (101).

These findings raised a large number of questions. One of them was: How are the precursors of the hydrophobic membrane proteins that are subject to this conservative sorting imported by the TIM23 translocase without being arrested and laterally released? Some answers are available regarding the distinguishing characteristics of the precursors. As Johannes Herrmann and Stefan Meier observed, the hydrophobicity of the transmembrane segments does not strongly differ between arrested and passing proteins; in both cases, they are considerably lower than those of bacterial or eukaryotic membrane proteins sorted via the endomembrane system. The flanking charges do matter, and charges on the C-terminal side favor arrest. Furthermore,

some of the conservatively sorted proteins have an increased number of proline residues in their transmembrane segments, and these residues might prevent formation of a helical arrangement required for lateral release (102).

THE TOB COMPLEX

Mitochondria together with chloroplasts are unique among cellular organelles in that they possess β-barrel proteins in their outer membranes. Mitochondrial Porins, Tom40, and Mdm10 are examples. In 2003, we identified another putative β-barrel protein in *Neurospora*, which we termed Tob55 (103); the yeast counterpart was termed Sam50 (104). They all are apparently inherited from their gram-negative prokaryotic ancestors. How are these proteins put in place? A simple answer would have been by direct insertion from the cytosol. However, Porin and Tom40 use the pore of the TOM complex for entry into the outer membrane, and small Tim proteins assist in their assembly. Tob55 showed sequence similarity to bacterial Omp85 and its homologs. These latter proteins have an essential function in the insertion of β-barrel proteins in prokaryotes. There, these proteins are made in the cytosol, exported by the SecYEG machinery, and inserted after passage through the periplasm into the outer membrane. Important elements of Omp85 are the β-barrel forming 16 β-strands and the Potra domains facing the periplasm. These elements are also present in the mitochondrial counterpart. Tob55 is essential for cell viability in yeast, which is obviously because of its role in the import of the essential protein Tom40. Tob55 forms the central part of a complex that contains two additional proteins on the cytosolic side of the complex, Tob38/Sam35 and Tob37/Sam37 (105). We termed this pathway also as a conservative pathway because the precursor has to return to the side of the membrane from which it was inserted in the prokaryotic ancestor to use the conserved insertion machinery. Many questions regarding this pathway are still open.

The TOB/SAM complex and the disulfide relay system were the last of the mitochondrial import and sorting machineries discovered. The number of protein components known to be involved has increased between 1984 and the present from zero to some 50 (**Figure 6**). Yet, more are to be discovered (105a).

MITOCHONDRIAL ATP-DEPENDENT PROTEASES

For a long time, regulated degradation of proteins has been suspected as playing an important role in mitochondrial biogenesis. When Thomas Langer joined the lab, we became interested in ATP-dependent proteases, which cooperate with molecular chaperones. Mitochondria contain a homolog of the bacterial Lon protease, Pim1. As we observed, the mtHsp70 system, including Mdj1 and Mge1, cooperates with Pim1 in the degradation of misfolded proteins (106). Apparently, also in terms of the quality control of proteins, the mitochondria have retained prokaryotic pathways.

A further example of the same principle came up with the discovery of AAA-ATPase proteins, Yta10/Yta12, proteins of unknown function, and Yme1, described to have a role in DNA escape from mitochondria. We observed that Yta10 is involved in the degradation of incomplete polypeptide chains made by mitochondrial ribosomes. Yta10 and Yta12 were then recognized as subunits of a heterohexameric complex that is able to degrade mitochondrial proteins, in particular mitochondrial membrane proteins (107). The subunits of the complex are anchored to the inner membrane and are composed of two domains, an AAA-ATPase domain proximal to the membrane and a distal metalloprotease domain. The AAA-ATPase has chaperone function, apparently pulling proteins out from the membrane to degrade them with their protease domain. We termed this complex m-AAA protease because it faces the matrix space (108). Yme1 then proved to form a similar, however, homohexameric complex termed i-AAA protease because it faces the intermembrane space. Both complexes are related to the FtsH protein of

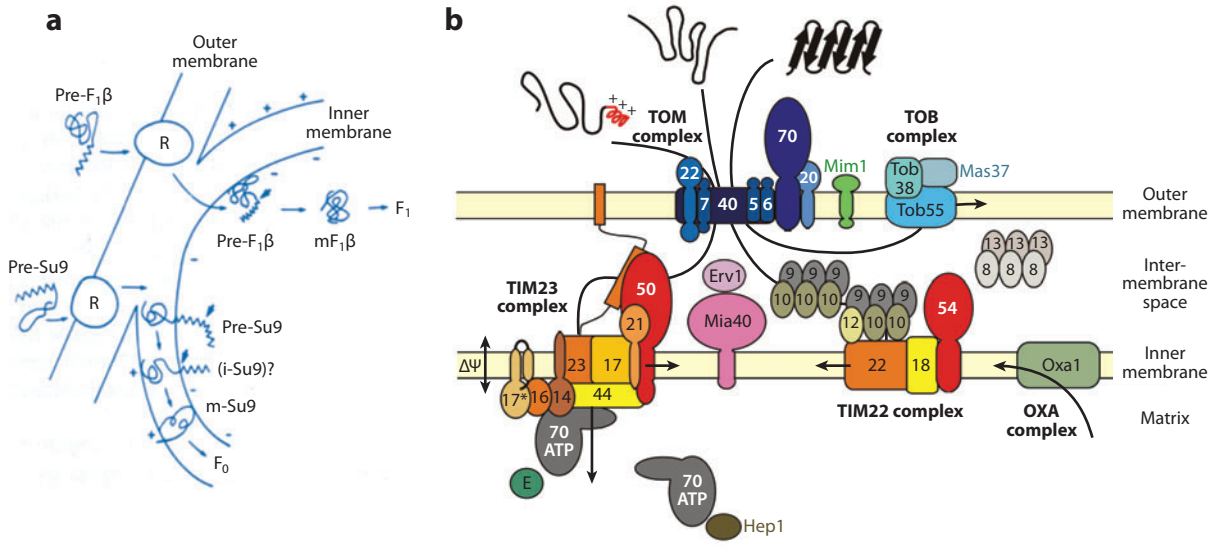

Figure 6

The machinery of mitochondrial protein import. (*a*) Models from 1984 and (*b*) from 2011. Panel (*a*) is adapted from Harmey & Neupert (119). The subunits of the TOM complex, the TOB complex, the TIM23 complex, and the Tim22 complex are indicated by numbers, which represent their apparent molecular mass. Abbreviations: R, receptors for mitochondrial precursor proteins; F_1, F_O, soluble and membrane sectors of the F_1F_O-ATP synthase, respectively; pre-Su9, i-Su9, m-Su9, precursor, intermediate, and mature forms of subunit 9 of the F_1F_O-ATP synthase, respectively; pre-F1 β, mF1 β, precursor and mature forms of subunit β of the F_1F_O-ATP synthase; ΔΨ, electrical membrane potential; Mia40, a protein that interacts with cysteine-containing client proteins upon import into the intermembrane space; Erv1, mitochondrial disulfide oxidase; Oxa1, a protein that mediates insertion of a group of mitochondrial membrane proteins into the inner membrane from the matrix side; 70 ATP, mitochondrial ATP hydrolyzing heat shock protein Hsp70; Hep1, Hsp70 escort protein; E, nucleotide exchange factor Mge1; Mim1, protein of the outer membrane involved in the assembly of the TOM complex; 17*, Pam17.

bacteria, which was studied at the same time by Koreaki Ito in Kyoto [see Ito & Akiyama (109)].

These findings were the beginning of a long story about the functions of AAA-proteases in mitochondria. Soon after their identification in yeast, mutations in the genes for m-AAA protease in human were described by Andrea Ballabio's group in Milan (110); this work suggested a mechanism involved in the neurodegenerative disorder hereditary spastic tetraplegia. The situation in human is much more complex than in yeast, and deep insights into the genetics of a whole group of related hereditary diseases were obtained in the following years. Thomas Langer, now professor at the University of Cologne, followed up the functions of the AAA-proteases in yeast and mice, which turned out to be quite diverse and surprising [see Tatsuta & Langer (111)]. The m-AAA ATPase was found to play a role in the topogenesis of yeast cytochrome *c* peroxidase (112). It pulls on an intermediate to adjust the sorting signal into a position in which it can be cleaved by the mitochondrial rhomboid protease. For this pulling reaction, the enzyme uses only its chaperone function.

These insights raise many exciting questions as to how evolution works as it, on the one hand, conserves established components and pathways and, on the other hand, finds surprising new solutions. They also taught me how prudent one has to be when drawing general conclusions on the basis of solid and virtually sound concepts arising from evolutionary considerations.

As Goethe said: "*Das Gewisse erfährt man aus der Regel, aber die Regel führt zum Übersehen der Ausnahme und die Natur ist voller Regeln*

und Ausnahmen, die nach ihrer Aufklärung eine begründbare Variation der Regel sind." (Certitude is experienced through the rule, but the rule leads to us ignore the exception, and Nature is full of rules and exceptions, which, after their elucidation, are a reasonable variation of the rule.)

THE FUTURE: THE MOLECULAR ARCHITECTURE OF MITOCHONDRIA

I believe my long, enduring fascination by mitochondria has several origins. For me, they presented a continuous journey of discovery, which is as yet not complete. My obsession has also an emotional root, and this is the beauty of mitochondrial architecture, which was triggered when I first saw electron micrographs of mitochondria. I immediately wanted to know how this complex architecture is built and maintained; how cristae are formed; how cristae junctions, the narrow short tube-like structures connecting the cristae and the inner boundary membrane, are shaped; and how the various morphologies of mitochondria in different cells and tissues are generated.

During the time when I was a student, Charles Hackenbrock demonstrated by electron microscopy that outer and inner membranes are held together by contact sites, which became visible when mitochondria underwent condensation of the matrix (113). What are the components responsible for these structures? And what is their biological meaning? Now, 35 years later, these questions are still not answered. But now we have the tools to address these questions, and I am returning to the times of my early scientific steps. As Goethe wrote: "*Der ist der glücklichste Mensch, der das Ende seines Lebens mit dem Anfang in Verbindung setzen kann.*" (Happiest is the man who can link the end of his life with its beginning.)

Many years ago, I made attempts to fractionate isolated mitochondria to separate different parts of the mitochondria and to study their composition and biogenesis, but this failed mainly because there were no markers for mitochondrial substructures. In the past years, initially together Andreas Reichert in the group and with Frank Vogel of the Max Delbrück Center Berlin-Buch, we studied the submitochondrial distribution of a series of proteins by immune electron microscopy. A number of components of the respiratory chain were found preferentially, but not exclusively, in the crista membrane as compared to the inner boundary membrane. Conversely, proteins of the mitochondrial import machinery, such as the subunits Tim17 and Tim23 of TIM23, were present preferentially in the inner boundary membrane. The latter distribution depended on active protein import; upon clearance of import sites, the distribution between the cristae and inner boundary membrane became equal. This suggested that the TIM23 complex can be recruited to the inner boundary membrane upon demand. Thus, a specific and dynamic distribution of membrane proteins among different parts of the inner membrane prevails.

Continuing these experiments, we discovered Fcj1, a protein concentrated at crista junctions of yeast. Its deletion resulted in an altered mitochondrial morphology with accumulation of large membrane stacks in the matrix and a virtually complete absence of crista junctions. Fcj1 has low sequence similarity to mitofilin, a mammalian protein; a similar absence of crista junctions was observed upon its downregulation. We then observed that Fcj1 antagonizes the formation of oligomeric F_1F_O-ATP synthase, whereas the subunits e and g of this complex promoted oligomerization. Fcj1 apparently has a key role in the formation of cristae and crista junctions, and oligomers of F_1F_O-ATP synthase play an important role in cristae morphology (113a). Quite recently, we identified a complex of at least six proteins, which is required for the formation of contact sites. One key to these experiments was the establishment of a marker, the above-mentioned GFP-Tim23 (113b). I am confident that these findings are the beginning of what will lead us to a deeper understanding of mitochondrial architecture, particularly to the relationship between the architecture and function of mitochondria.

EPILOGUE

The story told here represents a very personal view and does not describe the work of the many researchers who have contributed to the growth of this field. I apologize to them for missing or only referencing fragments of their contributions. A complete account of all the work would require a whole book and many thousands of quotations. Therefore, I refer the reader to three of my own reviews, two of them in this series (114–116), and a number of others (30, 111, 117, 118). In particular, I have not included references to the work of all of my colleagues, whose names appear in the text, because PubMed will easily lead to their work.

Looking back at almost 50 years of research, I have a sincere feeling of gratitude to the many colleagues who went through my lab as students, postdocs, and group leaders. Without them, nothing would have been achieved. Innumerable concepts, ideas, and practical solutions arose from the continuous open discussion in the lab. The Thursday evening lab seminars were almost always intellectually stimulating, a bouncing back and forth of thoughts and inspirations. My final Goethe quotation: "*Viele Gedanken heben sich erst aus der allgemeinen Kultur hervor, wie die Blüten aus den grünen Zweigen. Zur Rosenzeit sieht man Rosen überall blühen.*" (Many ideas emerge just from a common culture, like the blossoms from the green branches. At the time of the roses, one can see roses in flower everywhere.)

It also makes me happy that almost all of the alumni found their place in life and had either a successful or outstandingly successful career. Sad to say, a few got lost. Sometimes, there is no way one can help. I greatly appreciate the collaboration with quite a number of colleagues all over the world. Joining forces has not only been a great support for scientific advancement, but also led to many lasting friendships. And, mercifully, I was saved from deceiving experiments, fabricated data produced in the lab, and their devastating effects. I am also extremely grateful to the Max Planck Society, which after my retirement from university offered me the opportunity to continue research with a smaller group, in a way a "life after death." Finally, I am deeply grateful to my wife Monika, who tolerated my addiction to research and my lack of devotion to life's sensual outlets.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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