EVOLUTION AND GENETIC SIGNIFICANCE OF MITOCHONDRIA

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SUMMARY

Mitochondria are constituted of two different membrane systems which may well have quite different origins in replication and evolution. It is hypothesized that the outer membrane is entirely derived from cytosol constituents and activities. The inner membrane and contained matrix is derived from an aerobic promitochondrion which was originally a free living procaryote. It became an endosymbiont in an anaerobic host cell which supplied it with the end products of its glycolytic pathway. These the symbiont converted to CO_2, H_2O and energy through a system homologous to the present day Krebs TCA cycle and the electron transport system.

The original endosymbiont lost its cell wall and became a mitochondrion by a gradual process of integration into the host cell. In this process there was transferred to the nucleus of the host cell the genetic material in the form of DNA from the promitochondrion which was involved in the transcription of messenger RNA coding for the soluble components of the inner membrane-matrix system. This class of soluble inner membrane-proteins contains such enzymes as those involved in the synthesis of isoleucine and valine in Neurospora mitochondria, the Krebs TCA cycle enzymes and cytochrome c. These enzymes are apparently all synthesized in the cytosol and transferred to their appropriate positions within the mitochondria. Their functional integration within the inner membrane-matrix is dependent on the structure of the inner membrane. It is probable that most if not all are allotopic proteins.

The inner membrane contains insoluble components which must be synthesized within the mitochondria, since they cannot be transported from the cytosol into the mitochondria. The function of the mitochondrial DNA is to transcribe the RNA necessary for the constitution of a mitochondrial-protein biosynthetic system, and perhaps to furnish messenger RNA for the translation of certain proteins in the inner membrane as well as some that may function in the protein biosynthetic system. A minimal amount of DNA must remain within the mitochondrion both for this reason, and possibly also because some of the proteins must be synthesized by translation of RNA simultaneously with its transcription from DNA as occurs in bacteria.

INTRODUCTION

The discovery that both mitochondria and chloroplasts contain DNA, and separate and different systems from the cytosol for the synthesis of RNA and proteins, has made these organelles most important to the geneticist. A large part, if not all, of what has been

called cytoplasmic or extra-nuclear inheritance may be ascribable to these organelles, and the deep mystery formerly associated with this kind of inheritance is now at least partly dispelled.

However, mitochondria possess only a meager amount of DNA compared to the nucleus. In multicellular animals the length of the DNA averages only about 5µm and in green plants probably not more than 60 or 70 µm with fungi in between. These lengths appear insignificant when compared to the meters of DNA present in the nuclei of cells of plants and animals even after one considers the high degree of redundancy in eucaryote nuclear DNA as contrasted to the limited or absent redundancy reported for mitochondrial DNA (WELLS and BIRNSTIEL 1969, BORST 1970).

But the fact remains that this comparatively small amount of DNA persists, and it appears to be unique; that is, it is not represented in the nucleus (DAWID and WOLSTENHOLME 1968, FUKUHARA 1970). Making the assumption that DNA which persists has a significant function else it would have been lost in evolution is quite reasonable. The question then is what does this DNA do that is really significant and unique that the nuclear DNA cannot do? The answer to this is at best not known, but an attempt to answer this question with the observations and some of the data presently at hand leads one into some interesting areas of inquiry and speculation concerning the origin, evolution and functioning of the eucaryote cell.

CERTAIN ASPECTS OF THE STRUCTURE AND FUNCTION OF MITOCHONDRIA

Mitochondria, from whatever source, are always clearly constructed of two separate and distinct membranes, the inner and outer membranes. These two differ not only by virtue of their location but in their content of enzymes. The outer membrane (OM) contains enzymes with a quite different function than the inner membrane. Indeed it is currently believed by many that the outer membrane is derived completely from the endoplasmic reticulum in contrast to the inner as discussed below. In many instances one can see a direct physical connection between the outer membrane and the endoplasmic reticulum, and there does appear to be a chemical similarity between them (PARSONS 1966, SCHNAITMAN 1969).

The inner membrane (IM) is associated with enzymes of the Krebs tricarboxylic acid cycle (TCA cycle), the electron transport system and its oxidative phosphorylation system, and a host of other enzymes depending on the kind of plant or animal observed. Also associated with the IM and the matrix at the core of the mitochondria are the mitochondrial DNA, mitochondrial ribosomes, transfer RNA's, and the associated enzymes necessary to carry out the replication and transcription of DNA and translation for protein synthesis within the mitochondria. An important point to note is that the mitochondrial protein synthetic mechanism is very different from the synthetic system of the cytosol. It is sensitive to inhibition by antibiotics such as chloramphenicol which do not inhibit the cytosol system, and in general it resembles the translation process of bacteria which is also inhibited by chloramphenicol.

When isolated "intact" mitochondria are incubated in a proper supporting medium they can be shown to synthesize protein which makes up about 10% to 15% of the mitochondrial protein (HAWLEY and GREENAWALT 1970, SWANK et al. 1971). All of this synthesis apparently occurs at the inner membrane and/or the associated matrix. (It does not seem from the many experiments that have been done with mitochondria from a variety of organisms that any of the outer membrane proteins are synthesized by the mitochondrial system.) The IM-synthesized proteing have rather special properties. They are insoluble at physiological pH's and in the standard buffer media ordinarily used to solubilize protein. They seem to constitute a good part of the structure of the IM. They may in fact also have soluble components

such as cytochrome oxidase, a complex consisting of several components (SCHATZ et al. 1972).

At least six of these proteins have been tentatively identified by molecular weight in Neurospora mitochondria after having been separated on sodium dodecyl sulfate-acrylimide gels (SWANK et al. 1971). A similar number of this insoluble type protein have been isolated from mouse mitochondria (BUBSEE et al. 1972). Nine inner membrane proteins have been identified in yeast (SCHATZ et al. 1972). Since it is possible to determine the molecular weight of the protein on these gels one can calculate how much DNA would be required to code for the polypeptides. In the case of Neurospora, it is found that about 3,400 nucleotide bases are needed, in yeast 9,942, while in the mouse, according to the available data, the number of bases required is 16,578.

The length of the Neurospora and yeast mitochondrial DNA is about 25µm, and in the mouse 5µm. These lengths correspond to 75,000 and 15,000 nucleotide pairs respectively. It is highly probable that in both the mouse and Neurospora (although in this latter case the evidence is less direct) the mitochondrial robosomal RNA and a number of tRNA's (twelve to fifteen) are transcribed directly from the mitochondrial DNA (ALONI and ATTARDI 1971). Transcription of these would involve the use of about 3,700 nucleotides. Obviously, then, there is adequate DNA in Neurospora and yeast mitochondria to code for these inner membrane proteins provided there is not a great deal of redundancy, and this, as mentioned above, does not seem to be the case (see also, BROOKS and HUANG 1972). Thus it is possible that in yeast and in Neurospora all this protein can be coded by mRNA made in the mitochondria and translated on mitochondrial ribosomes. On the other hand, the mitochondrial DNA of the mouse may be too short and some or all of the IM protein synthesized on the mitochondrial ribosomes may be translated from messenger originating in the nucleus. Of course it is also possible that in the mouse the polypeptides may not be independent, i.e. there may be first made polypeptides of longer length which are then broken up into shorter chains, but at different places in the chains to give a multiplicity of different molecular weight chains derived from just a few original chains.

For the present it must be recognized that the mitochondrial DNA of yeast, Neurospora and higher animals is too short to participate in the synthesis of a great deal of protein, and in fact there may be good reason to believe that in animals part at least is coded for in the nucleus.

In addition to these so-called "insoluble" proteins of the IM there are a large number of "soluble" proteins, some associated, for example, with the TCA cycle. These enzymes are readily solubilized when the mitochondria are broken open in buffers without the use of the drastic methods necessary for the solubilization of the "insoluble proteins." For example, treatment of mitochondria with digitonin disrupts the outer membrane of the mitochondria and allows the soluble proteins of the IM to go into solution readily. It is probable that all of these proteins are coded for in the nucleus and synthesized in the cytosol, although this has been proved for only a very few of them such as cytochrome c (KADENBACH 1970, SHERMAN et al. 1966) (one of the soluble proteins of the electron transport system) and mitochondrial malate dehydrogenase (MUNKRES and RICHARDS 1965, KITTO et al. 1967, MUNKRES et al. 1970).

The mitochondria of Neurospora crassa also possess a number of enzymes for the synthesis of amino acids which have been studied rather extensively in our laboratory. These enzymes are in the soluble class, and, as will be shown below, probably are associated with the IM-matrix complex. They are considered here because the discovery that Neurospora mitochondria are centers of synthesis of certain amino acids lends further evidence to the possibility that mitochondria may be related to promitochondria which were originally free living organisms.

AMINO ACID SYNTHESIS BY NEUROSPORA MITOCHONDRIA

If mitochondria from Neurospora mycelium are isolated and purified in a sucrose gradient they remain in a reasonably intact condition as evidenced by the fact that they are able to carry out electron transport and oxidative phosphorylation with the production of ATP and the uptake of O_2. In addition we have found that when incubated in the presence of labeled pyruvate (^{14}C-2-pyruvate) they will produce labeled amino acids as indicated in Figure 1 (BERGQUIST et al. 1969). A total of six amino acids carry the pyruvate label after incubation periods ranging from 10 minutes to 2 hours. Proline and lysine may also be labeled, but our data are not decisive on this point for various reasons. Labeled glutamate also contributes carbons to isoleucine, threonine, and aspartate. Labeling of methionine in the presence of glutamate is questionable, but may occur.

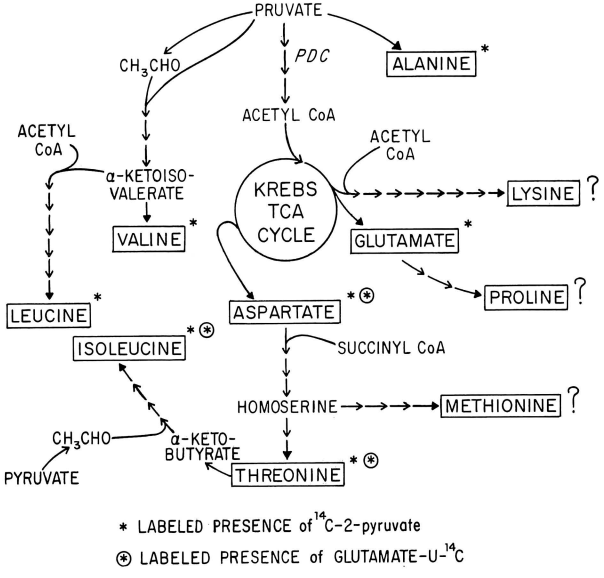

FIGURE 1. The pattern of synthesis of amino acids via pyruvate and the Krebs tricarboxylic acid cycle. Those amino acids marked with an asterisk are labeled after isolated Neurospora mitochondria are incubated in vitro with labeled pyruvate. PDC indicates the pyruvate dehydrogenase complex which is also present in the mitochondria.

Since these are isolated mitochondria we must assume either that the enzymes carrying out these syntheses are integral parts of the mitochondrial structure or that they normally function in the cytosol and stick to the outer surface of the mitochondria when they are being isolated. There are reasons for assuming that the first possibility is the correct one.

THE LOCALIZATION OF ISOLEUCINE-VALINE ENZYMES IN NEUROSPORA MITOCHONDRIA

For the past ten years we have been studying the enzymes involved in the biosynthesis of valine and isoleucine. It will be noted (Figure 1) that both have a two carbon aldehyde derived from pyruvate as precursor. The aldehyde joins with pyruvate to form the valine precursor, α-acetolactate, and with α-ketobutyrate to form the isoleucine precursor α-aceto-α-hydroxybutyrate. The same enzyme,

α-acetohydroxyacid synthetase, catalyzes both steps (GLATZER et al. unpubl.). The succeeding three steps to the amino acids (Figure 2) are also catalyzed jointly by three more enzymes, the reductoisomerase (KIRITANI et al. 1966), the dihydroxy acid dehydratase (ALTMILLER and WAGNER 1970a) and a branched chain amino acid aminotransferase (COLLINS 1971).

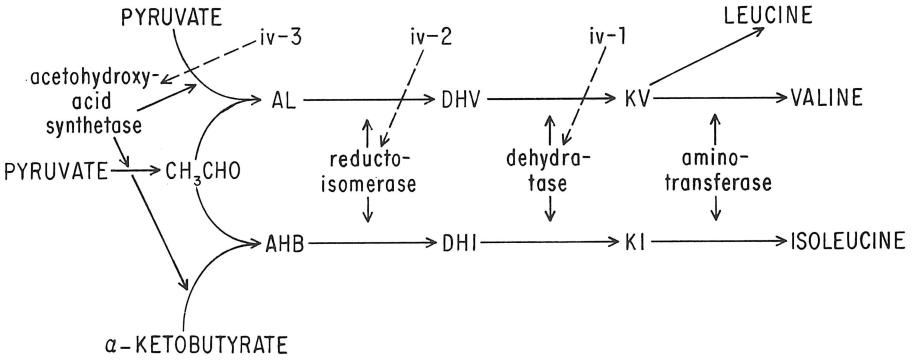

FIGURE 2. The steps in the biosynthesis of valine and isoleucine. The three genetic loci which apparently control three of the steps are indicated.

Eighty to close to one hundred percent of the activity of these four enzymes is found in the mitochondrial fraction of actively growing mycelium. Additionally, the activity for the overall synthesis of valine and isoleucine from pyruvate and α-ketobutyrate is also concentrated in the mitochondrial fraction. This activity remains with the mitochondria through various stages of washing, and purification by centrifugation on a sucrose gradient. Mitochondria so purified are capable of synthesizing valine at the rate of about 140 nanomoles per mg mitochondrial protein per 20 minutes or almost 430 nanomoles per hour (BERGQUIST et al. 1969). In order to obtain this rate of synthesis in "intact" isolated mitochondria it is necessary to add to the incubation mixture: NADP (triphosphopyridine nucleotide), Mg^{2+}, thiamine pyrophosphate, pyridoxal phosphate, ADP (adenosine diphosphate) and inorganic phosphate, and succinate along with an amino acid as an amino donor. The stimulation by the addition of ADP + Pi indicates that the synthesis of valine (and also isoleucine, glutamate and aspartate among others) is in part dependent on respiration and associated oxidative phosphorylation which also occurs in the mitochondria. This conclusion is supported by the finding that the addition of KCN, oligomycin, or 2,4-dinitrophenol causes a suppression of amino acid synthesis. KCN has by far the most drastic effect. It should also be noted that ADP plus Pi or ATP (adenosine triphosphate) stimulate valine synthesis only in the mitochondria and not when the enzymes are solubilized and outside the mitochondria (CASSADY et al. 1972).

More direct evidence that the isoleucine-valine enzymes are located within the mitochondria and associated with the IM or matrix has been obtained by treating highly purified mitochondria with digitonin and subsequently separating the outer and inner membrane and soluble components on a sucrose gradient. By using various concentrations of digitonin ranging from 2 to 6 mg/10 mg mitochondrial protein, patterns for solubilization of the enzymes were obtained and compared to those for three marker enzyme systems: kynurenine hydroxylase, known to be in the outer membrane (CASSADY et al. 1972), succinic acid cytochrome c reductase, an enzyme complex known to be attached to the inner membrane, and mitochondrial malate dehydrogenase attached to the IM-matrix. The pattern obtained for the four isoleucine-valine enzymes was almost identical to that for malate dehydrogenase, and

distinctly different from either the kynurenine hydroxylase or the cytochrome c reductase. The pattern is different from the cytochrome c reductase complex because this is a so-called "insoluble" IM complex in distinction to the "soluble" IM enzymes as described above.

When the mitochondria are disrupted by digitonin, valine synthetic activity from pyruvate is found in all parts of the sucrose gradient after centrifugation (CASSADY et al. 1972). However, the appearance of valine biosynthetic activity above the IM layer in the centrifuge tube was found to be a function of time of incubation in digitonin prior to centrifugation. Digitonin incubation periods for 5, 10 and 15 minutes before centrifugation produced a marked increase in valine synthetic activity with time of incubation in the "light" layers of the gradient whereas the heavy IM layer maintained a constant or slightly reduced activity with time. In this connection it is also interesting to note that only the IM fraction shows an increase in valine synthetic activity in response to ADP. Indeed we have found that the isolated IM-matrix fraction is more active than the isolated mitochondria. This is presumably due to the IM-matrix being exposed and more readily available to substrates.

Table I. Some properties of the acetohydroxy acid synthetase in and out of mitochondria of Neurospora

	"Intact" and in mitochondria	Partially purified and in solution
pH optimum	ca 7.4-8.0	ca 7.0-7.5
inhibition by valine	up to 70%	3%
enhancement by FAD	ca 65%	ca 22%
Km	3.2×10^{-3}M	1.7×10^{-2}M
substrate inhibition	+	-
TPP binding	loose	tight

Another kind of evidence that these enzymes are active in isoleucine-valine synthesis within rather than without the mitochondria was obtained in connection with studies on the acetohydroxy acid synthetase. It has been found that this enzyme has different properties when located in the intact mitochondria from those it has when it is solubilized and partially purified (GLATZER et al. in press). Perhaps the most significant differences to be emphasized here are those relating to substrate inhibition and affinity, and end product inhibition. It will be noted that the Km value obtained for the enzyme with pyruvate in situ is lower than for the solubilized enzyme, and that there is also positive evidence for substrate inhibition in situ. Even more important perhaps is the fact that there is a significant effect of valine as a feedback inhibitor when the enzyme is in the mitochondria, and an insignificant effect when it is solubilized. These data indicate that the enzyme is more responsive to the substrate, pyruvate, inside the mitochondria, and also responds like an allosteric enzyme in response to one of the end products at the end of the pathway. At best this is no more than indirect evidence but it fits in with the notions of metabolic control which have been derived from studies with bacteria (COHEN 1968).

If we grant that these enzymes are indeed active in the synthesis of these two amino acids within the deep recesses of the mitochondria, a next logical question to ask is where do they come from? Three of the enzymes are either altered or rendered inactive by mutations at three identified gene loci in the nucleus: the iv-1 and iv-2 loci which apparently code for the dehydratase and the reductoisomerase respectively, and the iv-3 locus which presumably codes for the synthetase. The amino-transferase has not yet been identified with a gene. The reason is probably because there are two different branched-chain aminotransferases, one in the mitochondria and the other in the cytosol. Hence two mutations in two different genes may be necessary to get a block at this step.

The identification of these three genes would lend credence to the idea that three enzymes at least are coded for in the nucleus and synthesized in the cytosol. This assumes these genes are structural genes which is probable, but not yet proved. However, even if they are structural genes, the possibility remains that any messenger transcribed by them is translated on the mitochondrial ribosomes and not the cytosol ribosomes. This is probably not the case as the following observations show. Studies have been made with dormant and germinating conidia of wild type Neurospora. Dormant conidia are characterized by a 30 fold lower activity of the acetohydroxy acid synthetase and the dehydratase than the actively growing mycelium (JOBBAGY 1971). The aminotransferase is at least 5-10 fold lower, and the overall activity for the synthesis of valine ten to one hundred-fold lower. Furthermore the distribution of these activities is different in the conidia than in the mycelium insofar as the supernatant fraction has in general equal or significantly higher activity than the mitochondria. As germination proceeds the activities in the mitochondria increase more rapidly than in the cytosol to reach levels comparable to the mature mycelial levels about 8 hours after the onset of germination. Since cycloheximide stops the increase in the activity of the enzymes in the mitochondria whereas chloramphenicol does not, it can be concluded that they must be synthesized in the cytosol and thence transported into the mitochondria.

The introduction of enzyme or other protein into whole mitochondria from the cytosol is supported by evidence from a number of sources. Cytochrome c, which in yeast has been proved to be coded for in the nucleus (SHERMAN et al. 1966), apparently enters yeast mitochondria readily and assumes its role as an active element of the electron transport system. Mitochondria of yeast mutants deficient in cytochrome c are obviously deficient in electron transport, but these deficiencies can be rectified by supplying exogenous cytochrome c from either wild type or the horse (MATTOON and SHERMAN 1966). The reconstituted mitochondria have been shown to bind the cytochrome c quite tightly so that it is retained during the isolation and purification of the mitochondria. Similar data have also been found for rat mitochondria (KADENBACH 1970). But it also has been found that the cytochrome c synthesized in the cytosol is probably modified after it enters the mitochondria and then assumes its assigned function (KADENBACH 1970, SCOTT and MITCHELL 1969).

It should be noted that added cytochrome c probably enters the mitochondria and penetrates into the inner membrane where the electron transport system is located rather than merely being adsorbed to the outer membrane. It would be difficult to see how it would function in this highly integrated system unless it was located within it.

We have been able to do comparable experiments with the Neurospora dehydratase enzyme which may be obtained in a highly purified state from wild type. When purified wild type enzyme is supplied to the isolated mitochondria of a mutant deficient in dehydratase they appear to take up the enzyme and are able to synthesize much more valine from pyruvate than would be the case otherwise (LEITER et al. 1971). This mutant (330) has mitochondria

which produce only 4% as much valine as the wild type (ALTMILLER and WAGNER 1970b). However, when wild type dehydratase is added valine begins to be formed at the rate of 93% of wild type after a five minute lag. Washing the mitochondria in a sucrose gradient reduces this activity to 29%. The reason why we are not able to elevate the rate to 100% may be that the dehydratase sites within the mutant derived mitochondria may be occupied by mutant dehydratase and these sites are given up slowly to be reoccupied by the wild type form of the enzyme. Substantive support for this argument is given by the observation that the untreated mutant mitochondria possess a CRM (cross reacting material) to wild type dehydratase which appears to be about as active as that in the wild type mitochondria. The 330 mutant, like all dehydratase mutants, possesses a considerable amount of dehydratase activity in the cytosol fraction, but not in its mitochondria. We suspect that this is because the mutant dehydratase, although quite active when in free solution, is relatively inactive when in the mitochondrion. There is no evidence currently at hand to show that the mutant dehydratases have altered amino acid composition from wild type, but they do show definite differences from wild type, i.e. they are highly unstable during purification procedures in contrast to the wild type. Some of them also show a much reduced activity with the dihydroxy acid analogue of isoleucine as compared to the analogous valine precursor.

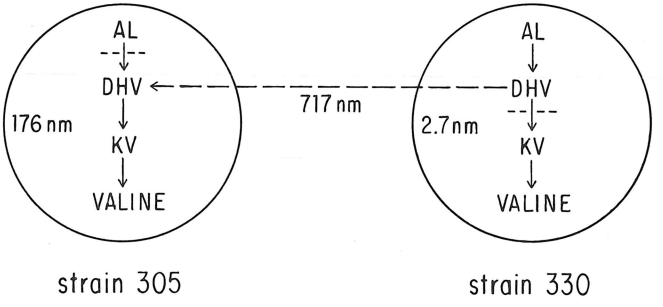

FIGURE 3. Biochemical complementation of 330 and 305 strains of Neurospora. Mitochondria from each strain were sequestered in separate dialysis bags. The broken lines indicate the step at which each strain is blocked. The figures give the nanomoles of valine accumulated in each of the two bags and the incubation medium outside in four hours. (Data from LEITER et al. 1971)

All of the evidence given above supports the conclusion that although the isoleucine-valine enzymes are synthesized in the cytosol by its protein synthesizing machinery, they are active in the biosynthesis of isoleucine and valine in the mitochondria. However, even though the enzymes are compartmentalized, the intermediates in the pathways are not, at least for mitochondria, observed in vitro. Experiments have been done which demonstrate this quite conclusively. If mitochondria from the mutant, iv-1 (330) which is deficient in dehydratase are placed together with mitochondria from mutant, iv-2 (305) which is deficient in the reductoisomerase, valine is synthesized from pyruvate at a rate some 15 times above the rate obtained with either mitochondrial type alone (LEITER et al. 1971). Thus there is some sort of complementation. The evidence is that this is the result of leakage of intermediates rather than any exchange of enzyme. When the mitochondria are placed in separate dialysis sacs suspended in the proper assay medium valine again is produced at a high rate and is to be found primarily in the suspending incubation medium outside the bags. However, it is interesting to note that the valine concentration in the bag containing the 305 mitochondria was

45 times higher than in the bag containing the 330 mitochondria. Presumably then the dihydroxy acid precursor (DHV) of valine readily diffused from the 330 sac into the 305 sac where it was efficiently converted to valine (Figure 3). As will be seen from Figure 3 most of the valine synthesized is to be found in the medium between the dialysis sacs. Presumably most of this was the product from the 305 mitochondria which diffused out of the 305 sac. These data show that the intermediate dihydroxy acid readily diffused out of the 330 mitochondria, and that once in the 305 mitochondria it is converted to valine with little loss of the keto acid (KV) derivative. From these data it is reasonable also to conclude that probably there is little leakage of intermediates in the wild type mitochondria. Their concentration is probably low under the steady state conditions in the wild type and only the end product amino acids diffuse out into the cytosol. When a block is present, however, their concentrations build up and diffusion occurs rapidly.

THE MALATE DEHYDROGENASE OF MITOCHONDRIA

Malate dehydrogenase exists in two forms in the eucaryotes, a mitochondrially bound form, and a cytosol form which apparently is involved in the glyoxylate cycle since it is found in the glyoxysomes (BENEVISTE and MUNKRES 1970). The mitochondrial form (M-MDH) is almost certainly part of the Krebs cycle within the mitochondria. The two enzymes are related in structure since they are apparently controlled by the same nuclear genes, but they are packaged into different compartments. They carry out the same kind of reaction, malate $\longrightarrow$ oxaloacetate, but in two quite different biochemical systems and cellular locations.

Our interest here is mainly in the mitochondrial MDH (M-MDH) (MUNKRES 1970) which, like the isoleucine-valine enzymes, appears to be located within the IM-matrix of the intact mitochondria. It is readily solubilized like the isoleucine-valine enzymes, and presumably acts in close association with them.

MUNKRES and his coworkers have done extensive work with the M-MDH of Neurospora, and among other things discovered two classes of mutants in which the M-MDH structure is altered (MUNKRES et al. 1970). In the C-mutant class the M-MDH is malfunctional when it attaches to its position in the mitochondria, although it may be quite active when free in solution. A second class, the K-mutants, produces a type of M-MDH which apparently does not attach into the mitochondria and hence is nonfunctional in the Krebs cycle. The two different enzymic forms, C and K, are the result of mutations of the same genes and may be considered isozymes. Logically one might expect intermediate forms in some mutants. These indeed are found forming a spectrum of extreme C through to extreme K.

These findings are important because they show that the conformation of a mitochondrial enzyme is important in determining whether this enzyme will attach to its proper place for active participation, and if it does attach or locate in its proper position whether it will in fact be active in that position. From this it is also logical to conclude that in the case of M-MDH the conformation of the IM-matrix complex is also important, because if it does not have the proper conformation not even the wild type M-MDH will be active, and the same applies presumably to other enzymes which are associated with this complex. Furthermore, Neurospora cytoplasmic mutants exist which are the result of mitochondrial DNA mutations which seemingly result in an altered mitochondrial inner membrane(MUNKRES and WOODWARD 1966, WOODWARD et al. 1970). This alteration(s) is probably in a protein component, but its exact nature is not known even though it has been shown that there are changes in the cytochrome a and b complexes in these mutants (EAKIN and MITCHELL 1970, WOODWARD et al. 1970). M-MDH associates with this protein IM fraction in an unknown

fashion. Wild type M-MDH has the same Michealis constant with or without the presence of the wild type IM protein. But the mutant enzymes have for the most part quite different properties in the presence as compared to the absence of this membrane fraction.

These observations on the mitochondrial malate dehydrogenase in Neurospora make it apparent that both the configuration of the enzyme and its presumed site in the mitochondria are of overriding importance in determining its activity within the mitochondrion. It is logical to assume that the same is probably true of other enzymes of the IM-matrix region of the mitochondria including the isoleucine-valine enzymes discussed previously. The conclusion is obvious, if the above is true, that a close interaction must exist between the genetic systems of nucleus and mitochondrion (WOODWARD et al. 1970). This is apparent even though practically nothing is known about the effects of mutation in mitochondrial DNA on the composition or organization of the mitochondria.

It is clear that the mitochondrial DNA is partly transcribed to form mitochondrial ribosomal RNA and transfer RNA, and that these are involved in the synthesis of protein. But it is not known whether any of the messenger RNA translated in the mitochondria is of mitochondrial origin. It could conceivably all be of nuclear origin. The question then becomes what is the genetic significance of this DNA? To attempt to answer this, evolutionary considerations are best resorted to.

It is postulated that the enzymes within the mitochondrial IM-matrix are there in the functional state because of historical reasons. The role of the mitochondrial DNA relative to these enzymes is a supportive one, and far from being accidental or superficial.

THE ORIGIN OF MITOCHONDRIA

MARGULIS (1970) has recently revived a rather ancient hypothesis dating back to the last century (ALTMANN 1890), and vigorously supported by a number of the workers on mitochondria and chloroplasts early in this century (see WILSON 1925) who posited that these organelles are descendants of formerly free living organisms which infected and became symbiotic with the original nucleated cells. The invaders found their new environment sufficiently compatible to remain and gradually become transformed into dependent mitochondria and chloroplasts containing relatively small amounts of DNA presumably representing a fragment of what they formerly had.

This hypothesis has no proof and probably never will, but it is supported by reasonable circumstantial evidence, and lends itself admirably to teleological rationalization. It is by no means universally accepted by all evolutionists (ALLSOPP 1969), but the prevalence of symbiosis even now among organisms makes it mandatory that the suggestion be considered seriously (RAVEN 1970).

Following the usual line of reasoning prevalent among speculators on the origin of life, we start by assuming that the original organism(s) was a completely heterotrophic anaerobe. It used the amino acids, carbohydrates, etc. which had been produced in the seas by the action of radiation and other physical factors during the chemosynthetic period that preceded the origin of life (KENYON and STEINMAN 1969). The atmosphere lacked free oxygen and was reducing in nature. Since there was no substantial supply of hydrogen acceptors readily available, we assume that this primordial cell-type (which may have become nucleated) was capable only of glycolysis, and produced two or three carbon products and ATP as the sole ends to the energy transforming pathway. Its protein synthesizing machinery utilized only the amino acids available from the supporting medium, since it was incapable of synthesizing its own.

Following the establishment of this kind of primordial cell there arose a second kind capable of utilizing CO_2, light and a hydrogen donor to produce carbohydrate. With water as the hydrogen donor free O_2 was produced as the result of this photosynthesis. As the atmosphere became increasingly oxidizing due to the presence of the oxygen a new electron acceptor became available in abundance.

The appearance of oxygen presaged the beginnings of aerobic organisms which may have been somewhat similar to the present day *Escherichia coli* in their ability to synthesize all their molecular needs except for a source of high energy carbon. The essential biochemical apparatus for these organisms that we envisage differentiated them from all others was the possession of a Krebs TCA cycle and the electron transport system which utilized O_2 as an acceptor, and produced ATP with higher efficiency than the glycolytic pathway. They may also have had an amino acid synthetic capability associated with the Krebs cycle.

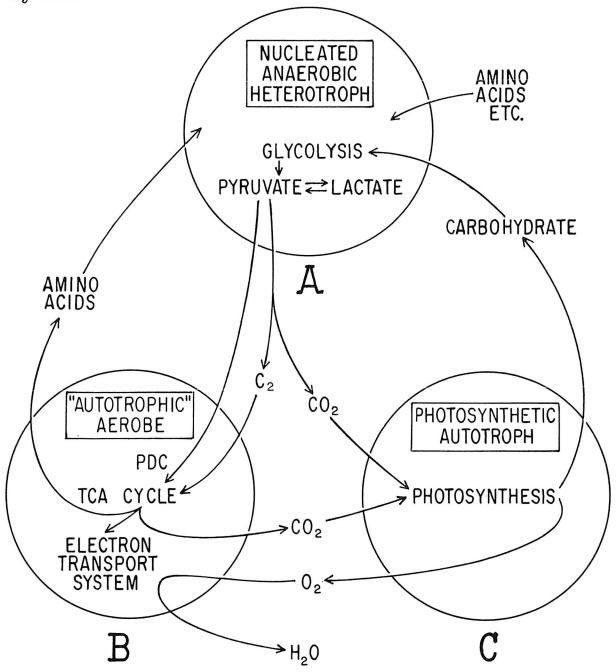

FIGURE 4. Hypothetical original three cell types. A. the original heterotrophic anaerobe, B. a procaryote able to carry out the dissimilation of 2 and 3-carbon compounds with the production of ATP and the utilization of O_2, C. a procaryote capable of carrying out photosynthesis.

These three types of organisms obviously would be expected to form the happy types of associations shown in Figure 4 which are too well known by all biologists to dwell on here, but several points need to be emphasized, since they are important to our arguments. It is logical to assume that the original anaerobic organism(s) had a system for fermentation by a glycolytic pathway with the production of pyruvate and its products: CO_2 and CH_3CHO or ethanol. The pyruvate might be expected to be readily utilized by cell type B through the pyruvate dehydrogenase (PDC) complex which we postulate was present to produce acetyl CoA which was readily taken up by the Krebs TCA cycle. The two carbon compounds would also be utilized through acetyl CoA.

The resultant CO_2 was readily utilized by C cells and the electron transport system took care of the hydrogens which are combined with the O_2 from C cells to produce H_2O. The "autotrophy" of the B cells was of course not complete unless they were chemosynthetic. We assume, however, that they could make everything else needed including amino acids and proteins. The C-type cells we also assume had considerable synthetic powers in addition to photosynthetic ones not possessed by the A cells.

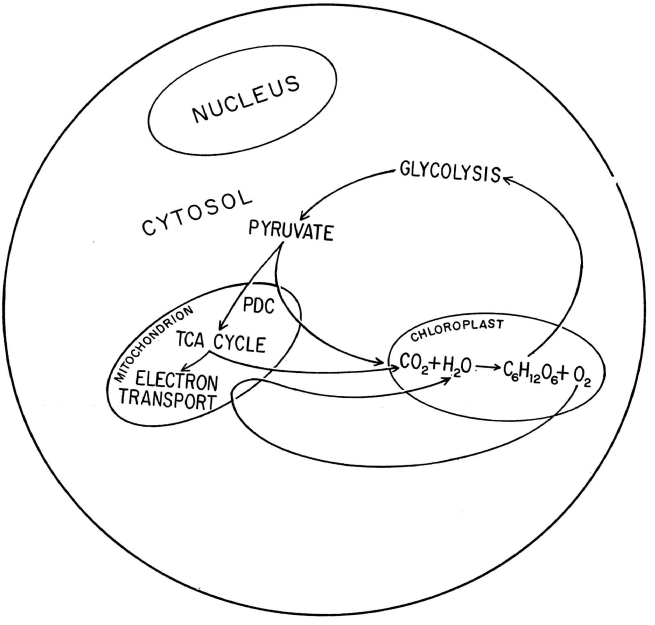

FIGURE 5. The composite eucaryote cell resulting from the infection of A with B and C and the subsequent modification of B to mitochondria and C to chloroplasts. Animal cells were derived from this by loss of chloroplasts.

The association of these three cell types by endosymbiotic invasion of the C and B cells into the original anaerobic heterotroph's "cytoplasm" is a logical expectation because of their obvious interdependence (Figure 5). The assumption is made that the bacterial-like B-type invader became the mitochondrion and the blue-green algal-like C-type became the chloroplast (MARGULIS 1970). That bacteria and blue green algae are the procaryotic progenitors of the mitochondria and chloroplasts of the eucaryotes is supported by more direct evidence than mutual interdependence (see FLAVELL 1972). Most important is the fact that the procaryote circular chromosome, without evident protein associated with it, resembles the DNA found in these organelles. Secondly, the elements of the protein synthesizing systems of the chloroplasts and mitochondria more resemble those of present day procaryote bacteria than they do those of the eucaryotic cytoplasmic system associated with the endoplasmic reticulum. The ribosomes, tRNA's, synthetases, polymerases, etc. involved in transcription and translation are different from the corresponding elements in the cytosol. The organelles also replicate themselves like bacteria, but they are far from being independent, autonomous entities. About eighty percent of the mitochondrial protein is synthesized outside of it making it very dependent on nuclear and cytosol activity. Indeed yeast cells make mitochondria-like structures in the absence of detectable mitochondrial DNA (mDNA) (GOLDRING et al. 1970); and again the question of the significance of this DNA is raised. If the mitochondrion is indeed a descendent of a free

living procaryote, then it must have lost a great deal of mDNA in the process of evolution. We rephrase the question to ask: why does it have any DNA left at all? Part of the answer is that without the mDNA present the mitochondrion cannot synthesize protein (KUZELA and GRECNA 1969).

HYPOTHESIS CONCERNING THE INNER MEMBRANE AND MITOCHONDRIAL DNA

It is the IM-matrix and the DNA and RNA of the mitochondrion which represent the remains of its procaryote ancestry. The outer membrane is completely of cytosol origin from the standpoint of its evolution.

The original procaryote promitochondrion had a membrane-associated electron transport system which in turn had associated with it a Krebs TCA cycle. Some of the intermediates of this cycle were used as a source for synthesizing certain amino acids, such as indicated in Figure 1. Also associated with the original IM was a system for generating acetyl CoA from pyruvate, the pyruvate dehydrogenase complex (PDC). This pyruvate in turn was also a precursor for certain amino acids (Figure 1). All of these metabolic systems were dependent on the production of a non fermentable carbon source, and were further interrelated by being membrane associated.

The original promitochondrion had present within it all the necessary genetic material necessary to duplicate itself as a free-living organism, but soon after its establishment as an endosymbiont it began to lose those parts no longer needed in a protected environment, such as a cell wall, and those functions which it originally shared in common with its host. (An analogous type evolution has presumably occurred more recently among the tapeworms.) During this preliminary evolution, which was accompanied by a certain loss of promitochondrial DNA no longer needed, since it duplicated homologous DNA in the host nucleus, the promitochondrion was presumably partly integrated into the host cell and covered with a host-generated outer membrane. The next evolutionary step we assume was the transfer of the genetic material necessary for the synthesis of certain of the enzymes syntehsized originally only in the promitochondrion to the host nucleus. Messenger RNA transcribed from this transferred DNA was translated either in the cytosol or in the promitochondrion now become mitochondrion. (The feasibility of this latter possibility is supported by the recent finding that mitochondria are capable of taking up high molecular weight ribopolynucleotides (SWANSON 1971).

This transfer did not, however, result in the obliteration of mitochondria which still remain with their IM, associated enzymes and DNA. It may, of course, be answered that we are witnessing a phase in the evolution of mitochondria, and that they are in transition to complete obliteration. The evidence seems to indicate the contrary especially if we consider the metazoic animals all of which seem to have mitochondria with similar important functions and generally no less than ca 5μm of DNA. It would appear that the animal line arose with the minimal needed amount of DNA, and this has not been reduced over a period of several hundred million years.

It is possible that the explanation for this minimal need is really quite simple. The inner membrane proteins and some of the enzyme complexes associated with them such as cytochrome oxidase and cytochrome b are, in fact, largely hydrophobic and do not readily go into solution in water or the usual buffers. They can be coaxed into solution only by the use of detergents or at very alkaline pH. Obviously the insoluble parts of such protein complexes would not be expected to be synthesized by the endoplasmic reticulum and then moved into the inside of the mitochondrion to become inner membrane. It is logical to assume that they are synthesized in situ by the mitochondrial protein synthesizing system. It is therefore suggested then that this system exists, for the purpose of carrying out the

synthesis of insoluble proteins, and must itself depend on the minimal DNA present with it. As mentioned above the mitochondrial ribosomal RNA and tRNA are apparently transcribed from this DNA. There is no reason whatever, of course, why the messenger RNA utilized by this system cannot all be coded for in the nucleus. It is only required that the translation of this message be done is situ.

It may also be asked, of course, why the DNA must be within the mitochondrion. Why cannot it translate the proper mitochondrial RNA's within the nucleus, and these be transmitted to the mitochondria? The answer here is less clear. But it has been reported that the cytosol tRNA's and ribosomal RNA enters Xenopus mitochondria only with difficulty (SWANSON 1971). Thus there may be a possible reason for keeping the mitochondrial and cytosol rRNA's and tRNA's separate. One way to maintain them separately would be to have them synthesized in different compartments which appears to be the case. A further reason may be that some of the protein synthesized in the mitochondria must, for some unexplained reason, be translated from mRNA while the mRNA is still being transcribed from DNA. This, of course, is the way synthesis apparently occurs for proteins in bacteria (STENT 1971). If this is the case in mitochondria too, it is obvious that the DNA must be in situ for proper conditions of protein synthesis.

From these considerations we draw the conclusion that the animal 5µm piece of DNA is the minimum necessary for the synthesis of the insoluble parts of the IM of the mitochondria. In fungi and plants the DNA is longer, and possibly more different types of proteins are synthesized, although the evidence as presented above on page 41 indicates that animals may synthesize more insoluble protein than the fungi.

It remains now to rationalize the presence of all the soluble components of the system associated with the inner membrane. It is assumed here that these all were first introduced as components of the promitochondrion and became nuclear and cytosol dependent later. Why do they return to the mitochondria later much like birds returning to their nesting grounds after a winter in warmer climes? One possible reason, and this may be the only one, is because of efficiency. This is quite obvious in the case of cytochrome c, a soluble component of the electron transport system synthesized in the cytosol under control of nuclear genes. There is even good evidence that this cytochrome entered originally with the promitochondrion because the cytochrome c of Neurospora bears a close resemblence in its amino acid sequence to the cytochrome-c 551 of Pseudomonas, a procaryote (CANTOR and JUKES 1966).

The other IM associated soluble proteins include the Krebs TCA cycle enzymes such as malate dehydrogenase, and the enzymes associated with the biosynthesis of certain amino acids all of which have been discussed above. These again may be present in the mitochondria because of efficiency. It is obvious that the TCA cycle feeds directly into the electron transport system, and that the amino acids synthesized in the mitochondria are closely related to TCA cycle intermediates and pyruvate (Figure 1).

As has been also discussed earlier the properties of these enzymes seem to be altered when they are associated with the IM-matrix. This is an important observation, because the data indicate that their properties within the mitochondria are of the type expected for the functions they perform. The IM-matrix complex forms the substratum upon which they attach and its configuration is therefore important to their activity. This configuration may in large part be determined by the mitochondrial DNA. Again we see that though a small piece of DNA exists in the mitochondria it occupies a position of great importance in the functioning of the eucaryote cell. In other words it is highly significant genetically.

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