IN VIVO INCORPORATION OF DIFFERENT AMINO ACIDS INTO ELECTROPHORETICALLY CHARACTERISTIC POLYPEPTIDES SYNTHESIZED BY HELA CELL MITOCHONDRIA

EDWIN CHING, PAOLO COSTANTINO and GIUSEPPE ATTARDI

Division of Biology, California Institute of Technology

Pasadena, California 91125

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SUMMARY - The *in vivo* incorporation of each of the twenty common amino acids into electrophoretically characteristic polypeptides synthesized by HeLa cell mitochondria has been investigated. Under labeling conditions which allow translation only on mitochondrial ribosomes, incorporation of all the amino acids, except aspartic acid, cysteine, glutamic acid and glycine, has been detected. These exceptions are probably due to problems related to amino acid pool size and/or equilibration.

Several recent investigations have suggested that the tRNA species coded for by HeLa cell mitochondrial DNA are far fewer than those required to read all the 61 amino acid specifying codons, and may not even represent a full complement for the 20 common amino acids (1-4). Furthermore, in a study of the incorporation of different amino acids into acid-precipitable and alkali-resistant material (assumed to represent mitochondrial protein synthesis on the basis of its cycloheximide resistance and chloramphenicol sensitivity), eight amino acids were found not to be incorporated to a significant extent (5). However, these apparently "negative" amino acids did not correspond in any meaningful way to those for which no mitochondrial DNA-coded tRNA had been detected (3). The above mentioned amino acid incorporation work was mostly performed *in vitro* in order to circumvent problems of amino acid equilibration with the cytoplasmic pools. However, the possible role of the mitochondrial pools in affecting the observed labeling patterns could not be excluded, nor were the mitochondrially synthesized products characterized as polypeptides.

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TABLE I

Amino Acids and Labeling Conditions

Amino Acid[+]	Labeling Time[‡] (minutes)
*[^{3}H]alanine (51)	30 (15)
[^{3}H]arginine (12,10)	30 (15), 60
*[^{3}H]asparagine (12)	30 (15), 120
*[^{3}H]aspartic acid (13)	30 (15), 120
[^{3}H]cysteine (2.6)	120, 240
*[^{3}H]glutamic acid (13.5)	30 (15), 120
[^{3}H]glutamine (23)	30 (15), 120
*[^{3}H]glycine (33, 43)	30 (15), 120
[^{3}H]histidine (22)	30 (15), 60
[^{3}H]isoleucine (30)	30 (15), 30 (30), 60
[^{3}H]leucine (57, 64)	30, 30 (15)
[^{3}H]lysine (50)	30 (30)
[^{35}S]methionine (64)	30 (15)
[^{3}H]phenylalanine (50)	30 (15)
*[^{3}H]proline (17, 46)	120, 240
[^{3}H]serine (1.6, 17, 17)	60, 120, 240
[^{3}H]threonine (1.4)	120
[^{3}H]tryptophan (1.5)	60
[^{3}H]tyrosine (46)	30 (15), 120
[^{3}H]valine (16)	30 (15)

All labeling experiments were carried out using medium which lacked the particular amino acid utilized, except in the case of amino acids marked with an asterisk (*), where complete medium was utilized.

[+]In parentheses, specific activity in Ci/mmole of the amino acid in the experiments reported in the table.

[‡]All the amino acids were tested in several experiments; the numbers reported indicate the labeling times utilized (those underlined refer to the experiments illustrated in Figure 1). The numbers in parentheses indicate the length of the chase period, performed by addition of an excess (0.01 M) of unlabeled amino acid.

In the present paper, we have examined the *in vivo* utilization of different amino acids for mitochondrial protein synthesis in HeLa cells using labeling

conditions designed to minimize possible pool effects. In addition, the radioactive products were analyzed by sodium dodecyl SO_4-polyacrylamide gel electrophoresis.

With the exception of aspartic acid, cysteine, glutamic acid and glycine, all of the common amino acids exhibited emetine-resistant and chloramphenicol-sensitive incorporation into polypeptides having the characteristic electrophoretic behavior of the products of mitochondrial protein synthesis.

MATERIALS AND METHODS

HeLa cells were grown in suspension as previously described (6). Modified Eagle's medium (7) supplemented with 5% dialyzed calf serum was used. Exponentially growing cells were collected by centrifugation, resuspended in the appropriate medium [whenever possible, the particular amino acid being studied was omitted (Table I)] at 5×10^6 cells/ml and labeled as previously described (6). The isotope concentration used varied between 7.0 and 11.6 μCi/ml. Table I lists the specific activity of the amino acids and the labeling conditions used. For each amino acid two identical cultures were labeled, one in the presence of 100 μg/ml emetine [an effective inhibitor of cytoplasmic protein synthesis (8)], and the other in the presence of both emetine and 100 μg/ml chloramphenicol [the latter, a specific inhibitor of mitochondrial protein synthesis (9-11)]. The preparation of the 5000 g_{av} crude mitochondrial fraction, its disruption by sonication, and the electrophoresis of the sodium dodecyl SO_4 solubilized samples through 12.5% polyacrylamide cylindrical gels were carried out as previously described (6). In some experiments, the labeled sample was precipitated with trichloroacetic acid in order to remove labeled non-protein contaminants (see below), dissolved in 100 μl of sample buffer, and neutralized by careful addition of NaOH (using bromphenol blue as an indicator) before being applied to the gel. Peak assignments in the electrophoretic patterns were made by comparison with the typical profile of [^{3}H]isoleucine- or [^{35}S]methionine-labeled mitochondrial protein products run

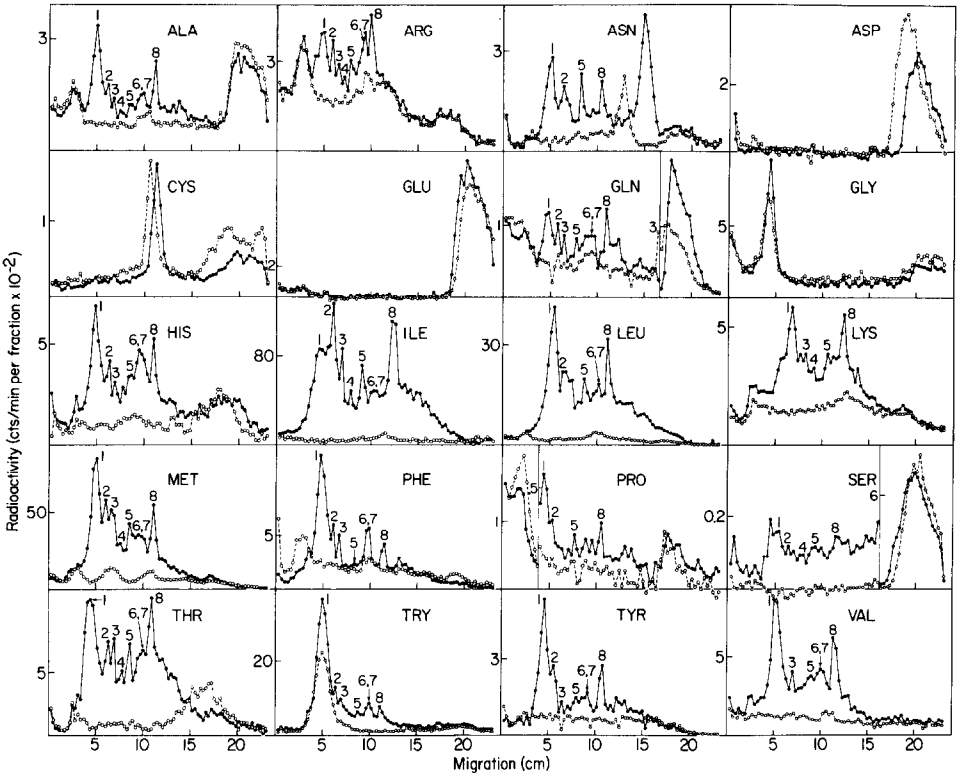

Fig. 1 Electrophoretic distribution (after 24-hr runs) of the products of HeLa cell mitochondrial protein synthesis labeled in vivo with different amino acids. Cell populations were labeled with different amino acids in the presence of 100 µg/ml of emetine and in the absence or presence of 100 µg/ml of chloramphenicol. The counts/min per fraction are normalized to 1 mg protein of the mitochondrial fraction applied to the gel. Peak assignments were made on the basis of the comparison with an internal [^{35}S]methionine-labeled standard or with the profile of a [^{3}H]isoleucine- or [^{35}S]methionine-labeled sample run in a parallel gel. ●---●, radioactivity in the presence of emetine; O----O, radioactivity in the presence of emetine and chloramphenicol.

on the same gel as an internal standard or in a parallel gel. Correction of ^{35}S spillover into the ^{3}H channel was made when appropriate.

RESULTS

The conditions for labeling the products of mitochondrial protein synthesis were chosen so as to minimize problems connected with pool dilution of

the label and to increase the sensitivity of detection of amino acid incorporation. High isotope concentrations and, in some cases, long labeling times were used; furthermore, in some experiments, a chase period was introduced after the pulse, in order to reduce the heterogeneous background in the electrophoretic profiles and to make the polypeptide peaks more evident (12). Figure 1 shows the patterns from 24-hr electrophoretic runs of mitochondrial translation products labeled with different amino acids in the absence or presence of chloramphenicol. The well characterized profile of the mitochondrially synthesized polypeptides labeled with [^{3}H]isoleucine (6) or [^{35}S]methionine was used as the standard for peak assignment. All the amino acids, with the exception of aspartic acid, cysteine, glutamic acid and glycine, are incorporated into the characteristic electrophoretic components, though to different overall extents. The relative labeling of the individual peaks also varies with different amino acids: for example, phenylalanine gives an unusually high labeling of components 6 and 7.

For some of the amino acids (i.e., alanine, arginine, glutamine, phenylalanine and proline), the electrophoretic distribution of the labeled polypeptides exhibited a relatively high background of chloramphenicol-resistant heterogeneous material (Fig. 1); furthermore, glutamine, proline and serine showed very low absolute levels of incorporation, even under the conditions of most extensive labeling used. However, in all cases, the chloramphenicol-sensitive products showed an electrophoretic profile with the same identifiable peaks as in the profile of the [^{3}H]isoleucine labeled proteins.

In some cases, the analysis was complicated by the presence, in the electrophoretic profile, of peaks of emetine- and chloramphenicol-resistant labeled material. Such peaks were found in the low molecular weight region of the gel in the case of the products labeled with [^{3}H]aspartic acid, [^{3}H]glutamic acid or [^{3}H]serine, and in the middle or high molecular weight region, in the case of [^{3}H]cysteine or [^{3}H]glycine (Fig. 1). A broad peak of emetine- and chloramphenicol-resistant material had been previously observed after [^{3}H]isoleu-

cine incorporation (6). Characteristic of this material were its lack of reproducibility in different experiments and its lability, i.e., its tendency to decrease in amount or to change in electrophoretic mobility after storing or freezing and thawing. This lability may explain the lack of correspondence in position and the difference in size of these peaks in the patterns of material labeled in the presence and absence of chloramphenicol (see, for example, the profiles of the products labeled with [^{3}H]asparagine). In several instances, as in the case of [^{3}H]serine or [^{3}H]tyrosine labeled products, it was possible to eliminate this chloramphenicol-resistant material forming discrete peaks by precipitating the labeled sample with trichloroacetic acid and redissolving it in the electrophoresis buffer. The nature of this material is not known; it is possible that it is some kind of amino acid-containing lipid which can be labeled with different amino acids. In any case, the presence of these spurious peaks in the electrophoretic profiles did not affect the recognition of the peaks characteristic of the mitochondrially synthesized polypeptides.

Figure 2 shows the patterns obtained, after 42 hr of electrophoresis, for the mitochondrial translation products labeled with some of the amino acids; the better resolution of the discrete components reveals more clearly the basic similarity among the various electrophoretic profiles.

DISCUSSION

The present study has extended earlier observations on the utilization of different amino acids for mitochondrial protein synthesis in HeLa cells (5). In particular, all the amino acids previously reported to be incorporated into chloramphenicol-sensitive products were shown here, under more stringent criteria, to be incorporated into mitochondrially synthesized, electrophoretically characteristic polypeptides. More significantly, however, four of the eight amino acids previously reported as apparently non-incorporated, i.e., alanine, arginine, glutamine and lysine, were found in the present work to be actually

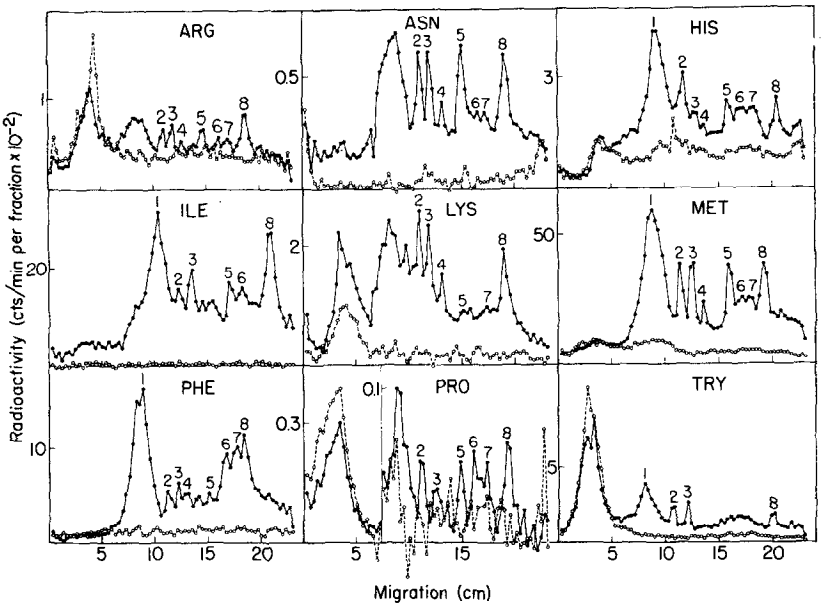

Fig. 2 Electrophoretic profiles, after 42-hr runs, of the products of mitochondrial protein synthesis labeled with selected amino acids. For details see legend for Figure 1.

utilized for protein synthesis. It seems likely that pool phenomena accounted for the very low incorporation obtained with these amino acids in the early experiments. It should be recalled that in vivo labeling of the mitochondrially synthesized polypeptides probably involves equilibration of the radioactive amino acid with both the cytoplasmic and the mitochondrial amino acid pools. The higher isotope concentrations and the longer labeling times used in the present work have reduced these pool effects. This approach, together with the electrophoretic analysis of the products, have increased the sensitivity and reliability of detection of the amino acids utilized by the mitochondrial translation apparatus.

There are reasons to believe that the failure to detect incorporation for aspartic acid, cysteine, glutamic acid and glycine in the present work could still be due to pool phenomena. Aspartic acid and glutamic acid are in fact known to be synthesized within the mitochondria (13), suggesting the

possibility of large mitochondrial pools or slow transport through the mitochondrial membrane via the aspartate-glutamate shuttle. Glycine and cysteine are both components of glutathione, which may enlarge the available pools because of its fairly rapid turnover (14); about six times as much glycine is present in glutathione as in the free pool (15). That glutamic acid and glycine have large cytoplasmic pools in HeLa cells is suggested by the very low levels of incorporation observed for these amino acids, relative to others, in an in vitro system measuring protein synthesis by endoplasmic reticulum-bound polysomes in the presence of the 105,000 g_{av} supernatant (5).

The interpretation that large intramitochondrial and/or extramitochondrial pools or uptake restrictions may be responsible for the results obtained with aspartic acid, cysteine, glutamic acid and glycine would also be consistent with other observations. In particular, each of these amino acids has been shown to be present in at least some of the mitochondrially synthesized polypeptide components of the cytochrome c oxidase from yeast (16) and Neurospora crassa (17) and of cytochrome b from the latter organism (18,19), and of the oligomycin-sensitive ATPase from yeast (20), and in the presumptive subunits of mitochondrial origin of the cytochrome c oxidase from beef heart (21,22). It should also be recalled that mitochondrial DNA-coded tRNAs specific for these amino acids have been shown to exist in HeLa cell mitochondria (3).

Metabolic conversion from one amino acid to another could have conceivably led to some spurious positive results in the present work. However, the well-known conversions of glutamic or aspartic acid to other amino acids (23) presumably did not play a role in producing spurious labeling data, since, as discussed above, these amino acids were not themselves incorporated to any detectable extent. As concerns other possible relevant interconversions known in animal cells, that of phenylalanine to tyrosine (23) seems to be excluded by the difference in the observed labeling profiles.

The pool phenomena discussed above and the difference in the specific activities of the amino acids and in the labeling conditions make it impossible

to interpret the data of relative incorporation of different amino acids into a given mitochondrially synthesized polypeptide as reflecting directly its amino acid composition. However, the relative incorporation of a given amino acid into different polypeptides is probably a measure of its relative abundance in the various mitochondrial products. The labeling data obtained in the present work will therefore be useful for the selection of the appropriate amino acid likely to give the maximum specific labeling of individual mitochondrial polypeptides in future work.

In a previous study on the specificity of HeLa cell mitochondrial tRNAs, at least 17 species coded for by mitochondrial DNA, which are specific for 16 different amino acids, were detected (3). No tRNAs specific for asparagine, glutamine, histidine or proline were found to hybridize with mitochondrial DNA. These amino acids have been shown in the present work to be incorporated into characteristic mitochondrially synthesized polypeptides. Therefore, if the observations in the tRNA study are not due to experimental difficulties, they would imply an import into the mitochondria of tRNAs coded for by the nuclear genome, which are specific for the four amino acids. Such a possibility is open to direct experimental test. Evidence suggesting a transport of some tRNA species from the nucleo-cytoplasmic compartment has already been presented for Tetrahymena pyriformis mitochondria (24,25).

On the basis of the evidence presented in this work and of the tRNA specificity data discussed above, it seems reasonable to conclude that all amino acids are very probably utilized for mitochondrial protein synthesis in HeLa cells.

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