ARTIFACTS IN PROTEIN SYNTHESIS BY MITOCHONDRIA *IN VITRO*

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1. Introduction

Protein synthesis by mitochondria *in vivo* and *in vitro* has been the subject of an increasing number of publications in recent years [1, 2]. The importance of the mechanism of mitochondrial biogenesis shifted most investigations from the more complex *in vivo* studies to isolated *in vitro* systems. One of the major disadvantages of using isolated mitochondria is the very low incorporation rate of amino acids into the few mitochondria proteins which can be coded for by mitochondrial DNA. As we will show in this paper, there is striking evidence that despite an *apparent* regulated protein synthesis (temperature-, concentration-, and time-dependency) the "incorporation" of certain amino acids into mitochondrial proteins *in vitro* reflects rather specific binding to, or chemical interactions with, the mitochondrial protein–lipid structures. Our amino acid "incorporation" rates are quantitatively comparable to others published previously from various laboratories as reflecting true mitochondrial protein synthesis. The chemical basis of the described phenomena will be discussed.

2. Materials and methods

Female rats (200–250 g) from Badger Research Corp. (Sprague-Dawley strain) or from Sprague-Dawley Inc. were used for the experiments.

L-[Me-^{14}C]methionine (11 mCi/mM), L-[1-Et-^{14}C]ethionine (3 mCi/mM), L-tryptophan[3-^{14}C] (23 mCi/mM) and L-glutamine[UL-^{14}C] (100 mCi/mM) were obtained from New England Nuclear. L-serine [UL-^{14}C] (120 mCi/mM), L-threonine[UL-^{14}C] (140 mCi/mM), L-proline [UL-^{14}C] (200 mCi/mM), L-phenylalanine[UL-^{14}C] (455 mCi/mM), L-glycine[UL-^{14}C] (80 mCi/mM), L-glutamic[2-^{14}C] (1.47 mCi/mM) and L-isoleucine[UL-^{14}C] (240 mCi/mM) were obtained from International Chemical and Nuclear Corp. and L-leucine[UL-^{14}C] (310 mCi/mM) from Schwarz/Mann.

Mitochondria were prepared from rat livers which were homogenized with 5 vol of either 0.25 M sucrose, 1 mM EDTA (pH 7.4) as described by Sottocasa et al. [3] or 10 mM bicine, 2 mM EDTA, 0.3 M sucrose, pH 7.4, Malkin [6], as described by Beattie [4].

Mitochondria were incubated with labeled amino acids at 37° in reaction mixtures which contained all other unlabeled amino acids according to either Kroon [5] or Malkin [6] for the time interval described in the table and figure legends.

Mitochondrial proteins were denatured by trichloroacetic acid (TCA), perchloric acid (PCA) or heat as described by Hochberg et al. [7].

Isolation of radioactive proteins was as described in Hochberg et al. [8]. This involved TCA precipitation, cold and hot TCA washings, cold and hot ethanol:ether washings, ether washing, dissolving in 1 N NaOH:Triton X-100, reprecipitation with TCA and finally dissolving in 1 N NaOH for determination of radioactivity. In some experiments, as noted in legends, nonradioactive amino acids (0.2 mg/ml) were added to the initial and final 10% TCA.

Protein determination and radioisotope counting was according to Hochberg et al. [8]. Counting efficiency on paper was 60%.

The data of figs. 1, 2 and 3 were obtained from a single massive experiment in which all reactions were

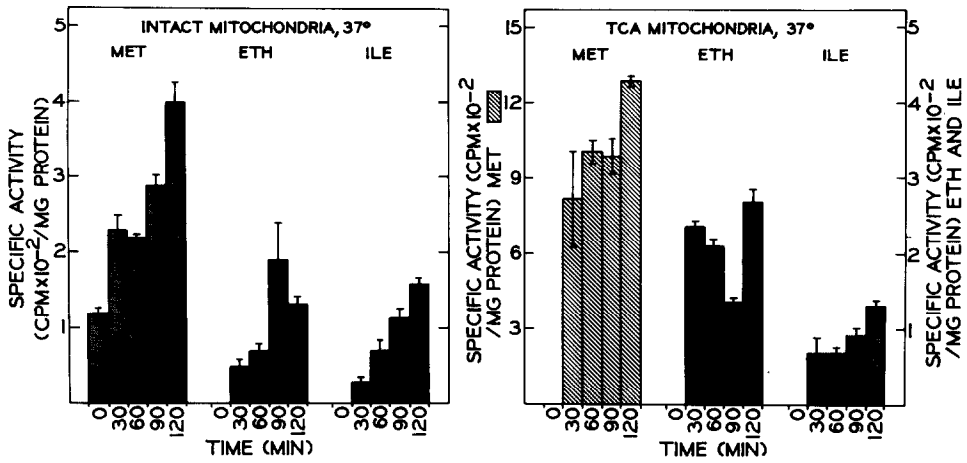

Fig. 1. Time dependent incorporation of methionine, ethionine or isoleucine into intact and denatured mitochondria. Mitochondria were isolated and triplicate reactions were as noted in table 2. Labeled amino acids (1 μCi/0.3 μmole/reaction) were at the same molar and isotopic concentrations. Bars depict standard deviations.

run in triplicate; the control experiments, therefore, apply to all three figures.

3. Results

When labeled Met, Eth or Leu at the same molar and isotopic concentration were incubated with mitochondria in Kroon's [5] medium, which contained all other unlabeled amino acids, the magnitude of "incorporation" was Met > Eth > Leu (table 1). This "incorporation" was both temperature- and time-dependent. Omitting cofactors (ADP, succinate) decreased the "incorporation" by 46% and 44% in the case of Met and Eth, respectively, whereas an increase occurred in the case of Leu. Neither cycloheximide nor chloramphenicol inhibited the "incorporation" of these three amino acids. In fact, "incorporation" of Met and Eth was significantly increased by cycloheximide, and chloramphenicol enhanced "incorporation" of Met. Mitochondria, whose proteins were denatured by TCA, "incorporated" these three amino acids to the same extent as did intact mitochondria.

The "incorporation" of labeled Met, Eth or Ile into mitochondria when incubated at the same molar and isotopic concentration in Bicine buffer containing all other unlabeled amino acids is time-dependent (fig. 1). Eth and Ile are "incorporated" to a lesser extent than Met. However, Met is the only amino acid of the three which is "incorporated" at zero time (mitochondria added and immediately precipitated with TCA). Met and Eth are "incorporated" into denatured mitochondria (TCA, PCA or heat) to a greater extent than into intact mitochondria whereas Ile "incorporation" was not affected significantly. TCA denaturation of mitochondria eliminated the time dependency of "incorporation" of Eth and Ile. However, there was little difference among TCA, PCA or heat denaturation treatments in their effect on amino acid "incorporation".

When all other unlabeled amino acids are present at concentrations used by Malkin [6], "incorporation" of labeled Met, Eth or Ile into intact and TCA denatured mitochondria is concentration-dependent (fig. 2). The "incorporation" of Ile into the proteins of denatured mitochondria is similar to that occurring with intact mitochondria. However, denaturation increased "incorporation" of Met and Eth in a Bicine buffer.

The "incorporation" of labeled Met and Ile but not Eth was decreased in both intact and TCA denatured mitochondria when the temperature was lowered to 0° (fig. 3). The zero time control reaction mixture was incubated with the radioactive amino acid for 120 min, TCA was added, followed by mito-

Table 1
The incorporation of methionine, ethionine and leucine into intact mitochondria and denatured mitochondria[a].

Temperature	Time (min)	Specific activity (cpm/mg protein)					
		Methionine		Ethionine		Leucine	
		$\bar{x}$	r	$\bar{x}$	r	$\bar{x}$	r
Intact							
37°	0	48	5	33	10	0	0
37°	30	140	16	64	2	42	13
37°	60	360	27	81	4	32	9
37°	90	270	38	140	1	62	38
37°	120	380	32	170	15	66	26
37°	120[b]	210	20	94	1	90	12
37°	120[c]	480	34	250	32	53	20
37°	120[d]	420	40	250	5	43	12
0°	120	140	21	52	5	17	4
TCA denatured							
37°	120	440	64	140	11	110	2

[a] Mitochondria were isolated according to Sottocasa [3]. Two μCi of methionine, ethionine or leucine (specific activity 3.3 μCi/μmole) were added to duplicate reaction mixtures of 1 ml (Kroon medium) [5] containing 2 to 3 mg mitochondrial protein and a mixture of nonlabeled amino acids (50 μg/ml) minus the amino acid which was labeled. $\bar{x}$, mean value; r, range.
[b] Cofactors (ADP, succinate) were omitted.
[c] Cycloheximide, 100 μg/ml.
[d] Chloramphenicol, 100 μg/ml.

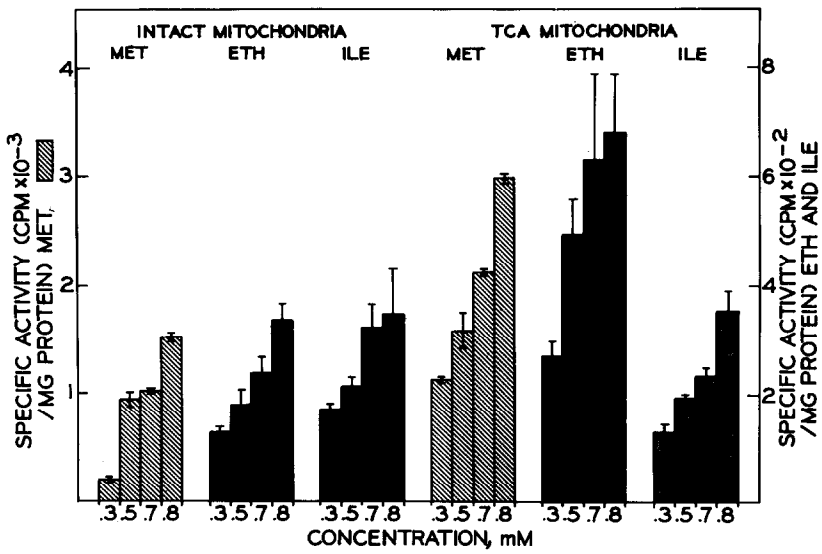

Fig. 2. Concentration dependent incorporation of methionine, ethionine or isoleucine into intact and denatured mitochondria. All conditions identical to fig. 1.

Table 2
The effect of dithiothreitol on the incorporation of methionine and ethionine into mitochondrial and denatured mitochondrial proteins[a].

Mitochondria	Specific activity (cpm/mg protein)									
	Methionine		Ethionine		Methionine +DTT			Ethionine +DTT		
	$\bar{x}$	r	$\bar{x}$	r	$\bar{x}$	r	%I	$\bar{x}$	r	%I
Intact	460	174	240	122	51	16	89	16	9	93
TCA denatured	1220	336	580	224	500	46	59	62	18	90
Heat denatured	1260	90	700	164	90	123	61	68	27	90

[a] Mitochondria were isolated according to Beattie [4], using Bicine buffer (Malkin [6]). Triplicate reaction mixtures containing 2–3 mg mitochondrial protein, a mixture of unlabeled amino acids (2 μg/reaction) minus the amino acid which was labeled, only labeled methionine and ethionine (1 μCi/0.3 μmole) in the same molar and isotopic concentration and cofactors (ATP, PEP, pyruvate kinase) were incubated at 37° for 60 min (Malkin [6]). Dithiothreitol (DTT) (5 mM) was included in the reaction mixture where noted. $\bar{x}$, mean value; r, range; %I, percent inhibition.

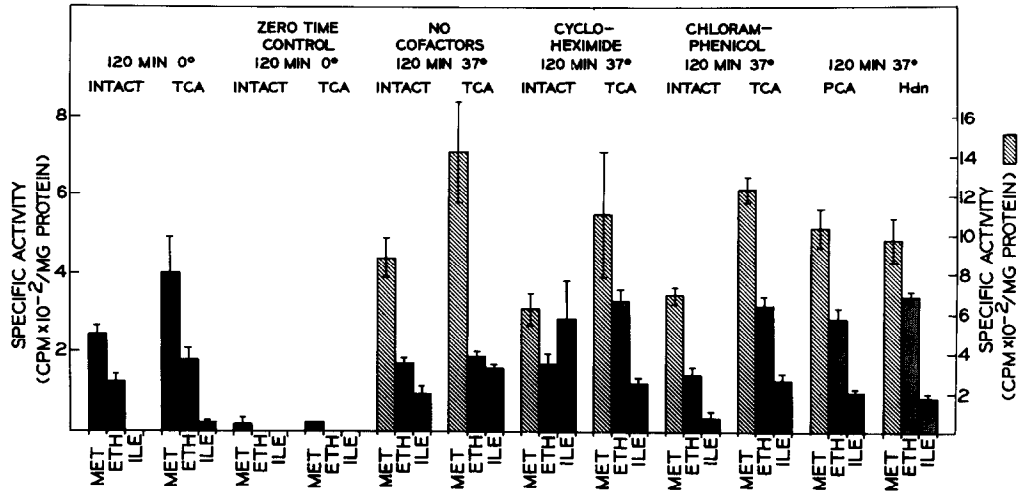

Fig. 3. The effect of temperature, cofactors and inhibitors on the incorporation of methionine, ethionine or isoleucine into intact and denatured mitochondria. All conditions identical to fig. 1, except the temperature in some cases was 0°, cofactors (ATP, PEP, pyruvate kinase) were omitted, and inhibitors (cycloheximide, 500 μg/ml; chloramphenicol, 50 μg/ml) were added when stated.

chondria [6]. Such samples "incorporated" negligible amounts or none of these three amino acids in either intact or denatured mitochondria. A higher "incorporation" of Met was observed in intact mitochondria when cofactors (ATP, PEP, pyruvate kinase) were omitted; however, Eth and Ile "incorporation" was not altered significantly. The omission of cofactors (ATP, PEP, pyruvate kinase) had no significant effect on the "incorporation" of the three amino acids into TCA denatured mitochondria. Cycloheximide increased the "incorporation" of Met and Eth and did not alter Ile "incorporation" in intact mitochondria. Chloramphenicol increased the "incorporation" of Met, failed to affect Eth, and reduced that of Ile in intact mitochondria. However, neither cycloheximide nor chloramphenicol had any significant effect on "incorporation" of these amino acids into TCA denatured mitochondria.

Labeled Met and Eth were "incorporated" into both intact and denatured mitochondria (TCA or heat)

Table 3
The effect of dithiothreitol on the incorporation of amino acids into mitochondria and heat denatured mitochondrial proteins[a].

Amino acid	Specific activity (cpm/mg protein)									
	Intact mitochondria		Hdn. mitochondria		Intact mitochondria+DTT			Hdn. mitochondria+DTT		
	$\bar{x}$	r	$\bar{x}$	r	$\bar{x}$	r	%I	$\bar{x}$	r	%I
Met	890	13	1800	161	98	28	89	240	1	86
Gly	52	3	87	12	0	0	100	0	0	100
Ser	14	2	94	0	0	0	100	44	7	53
Thr	0	0	9	1	0	0	–	0	0	–
Pro	340	1	420	4	120	24	64	91	21	78
Phe	190	36	310	16	100	20	46	180	76	42
Trp	970	101	800	169	300	24	69	160	9	80
Glu	0	0	0	0	0	0	–	0	0	–
Gln	6	4	16	10	0	0	100	0	0	100
Leu	10	2	110	10	0	0	100	34	13	70

[a] Mitochondria were isolated and duplicate reactions were as noted in table 2. The labeled amino acids (1 μCi/0.3 μmole/tubes) were at the same molar and isotopic concentration, except for glutamic acid (1 μCi/0.7 μmole/tube). Hdn, heat denatured.

(table 2) as was shown in figs. 1 and 2. Dithiothreitol (DTT) included in the reaction mixture inhibited the "incorporation" of Met and Eth into intact mitochondria by 89 and 93%, respectively. DTT caused a similar inhibition in the "incorporation" of Eth into TCA or heat denatured mitochondria (90%) as into intact mitochondria; however, it was less effective in the case of Met (60%).

When each of ten labeled amino acids, in presence of all other unlabeled amino acids [Malkin, 6], were compared in a similar molar and isotopic concentration range the magnitude of "incorporation" into intact mitochondria was: Trp > Met > Pro > Phe ≫ Gly > Ser > Leu > Gln. Thr and Glu were not "incorporated" into intact mitochondria (table 3). Heat denaturation of mitochondria increased the "incorporation" of labeled Met, Gly, Ser, Pro, Phe, Gln and Leu. There was little or no change of "incorporation" in the case of Trp, Glu, Gln or Thr.

The inclusion of DTT (5 mM final concentration) in the reaction mixture inhibited the "incorporation" of labeled Met (88%), Gly (100%), Pro (71%), Phe (44%), Trp (75%) and Gln (100%) to the same extent in either intact or heat denatured mitochondria. Ser and Leu "incorporation" into heat denatured mitochondria (53%, 70%) were inhibited to a lesser extent by the inclusion of DTT than in intact mitochondria (100%, 100%). In experiments similar to those described in table 3, dithiothreitol (5 mM) included with the first TCA wash, or DTT or histidine included with the TCA in the precipitation of the protein which had been dissolved in 1 N NaOH: Triton X-100 and neutralized with 3 N HCl, did not change the "incorporation" of Met, Pro, Trp, or Ser into intact or heat denatured mitochondria. All specific activity values were similar to those in table 3.

4. Discussion

Mitochondrial protein synthesis *in vitro* was first reported by McLean et al. [9]; however, their mitochondrial preparation was most likely not acceptable in terms of purity nor did they give proof that the amino acid was incorporated into the interior of the polypeptide chain. Bacterial contamination of mitochondrial preparations was suggested as being responsible for protein synthesis, but was excluded by Beattie et al. [10]. The requirements reported as necessary for efficient incorporation of amino acids into mitochondria proteins are ATP as an energy source, Beattie et al. [10] and a relatively high magnesium (10 mM) and potassium (60–100 mM) ion concentration. Bicine buffer was described as being

superior to Tris buffer, and Pro, Ser and Met are the possible rate-limiting amino acids in mitochondrial protein synthesis, Beattie et al. [11].

The complete exclusion of microsomal protein synthesis as a contaminant in the mitochondrial protein synthesizing system by use of cycloheximide [11] could not be shown in our experiments with different amino acids. Also, the expected inhibition of mitochondrial protein synthesis with chloramphenicol [11, 2, 5] was not convincing. In fact, cycloheximide stimulated the apparent "incorporation" of Met, Eth and Ile in Bicine buffer, Met and Eth in Tris buffer, and had no effect on Leu "incorporation" in Tris buffer. Cycloheximide reduced the apparent "incorporation" of Met into TCA denatured mitochondria in Bicine buffer but had little effect on the "incorporation" of Eth or Ile. Chloramphenicol inhibited the "incorporation" of Ile into intact mitochondria in Bicine buffer. Leu "incorporation" into intact mitochondria in Tris buffer was inhibited by chloramphenicol, whereas the "incorporation" of Met and Eth were *stimulated* in both buffers. In Bicine buffer, the apparent "incorporation" of Met, Eth and Ile into TCA denatured mitochondria was unchanged by chloramphenicol. The inconsistency in the effect of cycloheximide and especially chloramphenicol on the "incorporation" of several amino acids into mitochondria, under conditions implied by leading investigators [2, 5, 6] in the field of mitochondrial protein synthesis as being ideal, suggests that further investigations are necessary before one can unequivocally state that mitochondria synthesize protein *in vitro* from all amino acids.

Furthermore, the basic assumption of a true biosynthetic mechanism becomes rather doubtful when denatured mitochondrial preparations "incorporate" amino acids in quantitatively similar or higher amounts than are incorporated by intact mitochondria.

The possibility of non-ionic adsorption of radioactive amino acids or of ionic interaction with mitochondrial constituents seems to be ruled out since several TCA washings (pH $<$ 1) containing an excess of the corresponding unlabeled amino acid as well as reprecipitation after dissolving with NaOH (pH $>$ 10) did not release the amino acids tightly bound to mitochondrial proteins. It appears likely that only a relatively stable covalent bond would withstand the described isolation procedures. It has not been possible to deduce the nature of the amino acid–mitochondria interaction by comparing the binding of a number of different amino acids.

Met, Ser, Phe, and Leu "incorporation" was increased by denaturation of mitochondrial proteins, but these amino acids have no common functional groups apart from the amino and carboxyl groups. The amino acids with the highest degree of "incorporation" are Met, Pro and Trp. The last two amino acids mentioned above have a ring structure which contains an –NH group; Met under oxidizing conditions may also form a ring structure enclosing an –NH group [12]. The reducing agent dithiothreitol (DTT) strongly decreased and in some cases completely abolished the "incorporation" of the amino acids into intact as well as denatured mitochondria. This inhibitory action of DTT suggests that redox mechanisms are involved in the interaction between mitochondrial proteins and free amino acids. A satisfactory explanation for the observed phenomena cannot be presented without further investigations.

The incorporation of less than one percent of the radioactivity added as labeled amino acid into protein of mitochondria was suggested earlier by Peters and Greenburg [13] and Rutman et al. [14] to represent adsorption or coupling of the amino acids or their metabolic products by bonds other than peptide, e.g., S–S bonds. Performic acid [13], ninhydrin [14], mercaptoethanol [13] and thioglycolic acid [15] have been used to release adsorbed or non-peptide bound amino acids. Thus, a careful reevaluation of the methodological aspects of mitochondrial protein synthesis *in vitro* must be considered.

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