Amino Acid Production in Isolated Rat Liver Mitochondria

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Amino acid analyses of mitochondrial membranes are compared with the amino acid composition of whole mitochondria (Alberti, 1964) and found to be very similar except in the cystine content. The composition of the endogenous amino acids found in freshly prepared mitochondria has been established and shown to differ considerably from the amino acid composition of membranes or whole mitochondria. The amino acids produced during anaerobic incubation of mitochondria at pH 7.4, on the other hand, resemble the membrane in composition, supporting the view that neutral proteinase activity is responsible for their appearance. Aerobic incubation produces a similar pattern of amino acids except that amino acids such as proline, serine, asparagine, glutamic acid and glutamine, which can be metabolically utilized under aerobic conditions, are present to a smaller extent. The presence of large relative concentrations of endogenous taurine, cysteic acid and oxidized glutathione and the accumulation of taurine during incubation is found. The selective retention of taurine and cysteic acid within the mitochondria is established. It is proposed that the first step in the degeneration of isolated mitochondria results from lipid hydroperoxide accumulation caused by the lack of glutathione reductase in isolated mitochondria.

In a series of papers Alberti & Bartley (1963, 1965, 1969) have demonstrated that mitochondria isolated from rat liver contain low endogenous concentrations of amino acids but that the amino acid content increases either on storage at 0°C or during incubation under a variety of conditions. The effect of anaerobic and aerobic incubation, pH and buffer ion, the presence of oxidizable substrate, and of various inhibitors of metabolism or inhibitors of proteolytic activity were all studied. The broad conclusions from that work were that neutral proteinase activity residing within the mitochondria could account for the observations. More than one neutral proteinase is probably implicated, and the physical state of the mitochondria, whether swollen or not, their age and type, light or heavy, all influence the degree of proteolysis that occurs. Under aerobic conditions metabolic utilization of some of the amino acids may occur. A major question raised by the work of Alberti & Bartley concerns the mechanism by which proteolytic activity is suppressed in functioning mitochondria *in vivo*, where the turnover of protein is relatively slow (half-life of about 10 days; Bailey *et al.*, 1967).

In all the previous work, the production of total amino acids by mitochondria was measured by the ninhydrin method without determining the contributions of different amino acids to the total. Preliminary work by Alberti (1964) on the amino acid composition of mitochondrial 'membrane protein' has been extended in the present work and, in addition, the amino acid composition of the material produced

by incubation under various conditions has been studied.

Materials and Methods

The animals used and the preparation and incubation of mitochondria and extraction of amino acids have already been described (Alberti & Bartley, 1969).

Mitochondrial 'membranes'

These were prepared by sonication (4×15 s) of mitochondria suspended in 0.25 M-sucrose, followed by centrifugation for 1 h at r_{av} 100 000g. The pellet was taken for acid hydrolysis. In one case 12.4 mg was hydrolysed with 2 ml of 6.0 M-HCl for 24 h under vacuum and in the other 25.9 mg was hydrolysed for 48 h under identical conditions.

Preparation of samples for amino acid analysis

Hydrolysates were rotary-evaporated to dryness at 40°C under vacuum and redissolved in 0.21 M-sodium citrate buffer, pH 2.15 (Atkin & Ferdinand, 1970). Extracts of mitochondria, prepared and deproteinized as described previously (Alberti & Bartley, 1969) were freeze-dried and redissolved in the same sodium citrate buffer, pH 2.15.

In either case the solution of amino acids in sodium citrate buffer, pH 2.15, was filtered through a Millipore HAWP filter (0.45 μ m pore size) before applying a sample to the amino acid analyser.

Amino acid analysis

This was carried out on an EEL 199 amino acid analyser by the method of Atkin & Ferdinand (1970, 1971).

Results

Amino acid composition of mitochondrial 'membranes'

After sonic disruption of freshly prepared mitochondria the particulate fraction, designated the mitochondrial 'membrane', was hydrolysed with 6M-HCl and the amino acid composition of the hydrolysate determined on the amino acid analyser. The results are expressed as mol% in column 1 of Table 1. The major amino acid is glutamic acid (which includes glutamine originally present before hydrolysis), whereas leucine is the second most abundant amino acid. Alanine, aspartic acid plus asparagine, and glycine are equal third and valine lies fourth. Only trace amounts of cystine were detected.

Endogenous amino acids

The amino acids present in freshly prepared mitochondria are shown in column 2 of Table 1, expressed as mol%, together with the total amount (μmol) present per g dry weight of mitochondria. Only low amounts were found, as expected, but of the total a remarkably high proportion (49.6%) consisted of the oxidized sulphur-containing compounds taurine, glutathione (GSSG) and cysteic acid. On the other hand, aspartic acid, asparagine, proline, lysine and arginine were undetectable. The proportions of the amino acids are quite different from those found for mitochondrial 'membranes'.

Amino acids produced by incubation of mitochondria

Mitochondria were incubated for 1 h under either anaerobic or aerobic conditions at pH 7.4 in either Tris-HCl or potassium phosphate buffer. Alberti & Bartley (1963, 1965, 1969) showed that the production of amino acids depended on the incubation conditions and also on whether 'heavy' or 'light' mitochondria were used. Preliminary work by Alberti (1964) showed, in addition, that there were differences in the patterns of amino acids produced. Light mitochondria were therefore treated separately from heavy ones. The amino acids present at the end of the period were analysed and the results are expressed as mol% in columns 3-10 of Table 1, together with the total amount (μmol) found per g dry weight of mitochondria.

On the whole the patterns seen in heavy mitochondria resemble those found in light ones. In all cases the total amount of amino acid has increased relative to the endogenous amount, but is greater for light mitochondria than for heavy ones, greater in Tris-HCl than in phosphate buffer and greater

anaerobically than aerobically, as found by Alberti & Bartley (1963). The proportion of the sulphur compounds taurine plus GSSG plus cysteic acid is much lower than in the endogenous analysis; the absolute amount, however, of the sum of these compounds does not change very much, being sometimes higher and sometimes lower than in the endogenous case. Within this group of three, on the other hand, the absolute amount of taurine is always approximately double the endogenous amount, that of cysteic acid is lower in the anaerobic situations or roughly the same under aerobic conditions, whereas GSSG disappears completely in the incubations in Tris-HCl buffer but remains in the incubations in phosphate buffer, being lower than the endogenous value with light mitochondria and higher with heavy ones. As with GSSG, aspartic acid and asparagine are never found after incubation in Tris-HCl buffer. Proline, on the other hand, is not found after aerobic incubation. Arginine was not detected after any of the incubations. An unexpected result was the detection of a substance eluting from the amino acid analyser at the same position as 5-aminopentanoic acid, after incubations under anaerobic conditions in phosphate buffer with both light and heavy mitochondria, and under aerobic conditions as well in the case of light mitochondria. It was not detected after incubations in Tris-HCl buffer. Tryptophan appears after incubation of the heavy mitochondria in all but aerobic Tris-HCl buffer, whereas it appears with the light mitochondria only after anaerobic incubation in Tris-HCl buffer. 4-Amino-*n*-butyric acid only appeared after Tris-HCl buffer incubation; 1-methylhistidine and 3-methylhistidine were found only after incubation of heavy mitochondria in Tris-HCl buffer, the former aerobically and the latter anaerobically. Small amounts were found of various unknown ninhydrin-positive compounds which may have been small peptides but which individually constituted only small proportions of the whole and collectively never exceeded 12% of the total. The proportion of ammonia was found to be lower with Tris-HCl than with phosphate buffer, and lower anaerobically than aerobically.

Serine, asparagine, glutamic acid, glutamine, glycine and ornithine were always present at a lower percentage of the total aerobically than anaerobically, as was alanine in all but the case of heavy mitochondria in Tris-HCl buffer. The essential amino acids show no appreciable difference between aerobic and anaerobic conditions.

Comparison of the internal and external amino acids produced by incubation of mitochondria in 0.25M-sucrose

Alberti & Bartley (1969) observed a high ratio of internal to external total amino acid concentration

Table 1. *Amino acid analyses (mol%) of mitochondrial 'membranes' and mitochondrial extracts under various conditions*

The values given are the means of either two or three separate analyses. The 'membrane' hydrolysate was prepared as described in the Materials and Methods section. The endogenous amino acids were measured immediately after preparing mitochondria. Incubations were carried out in 25mm buffers, pH7.4, for 1h at 40°C. Anaerobic incubations were carried out under N₂.

	'Membrane' hydrolysate	Endogenous content	Content after incubation (mol%)									
			Light mitochondria					Heavy mitochondria				
			Phosphate buffer		Tris-HCl buffer			Phosphate buffer		Tris-HCl buffer		
			Anaerobic	Aerobic	Anaerobic	Aerobic		Anaerobic	Aerobic	Anaerobic	Aerobic	
Cysteic acid		9.7	—	1.5	0.3	0.7		1.3	4.8	1.0	2.3	
Taurine		25.3	3.3	5.3	5.3	6.0		9.4	16.1	7.2	8.9	
Phosphoethanolamine		1.7	~0.01	~0.03	0.4	0.2		0.3	0.2	0.8	0.7	
Aspartic acid	{ 8.9	—	1.9	2.0	—	—		2.6	0.9	—	—	
Asparagine		—	4.5	3.6	—	—		5.4	2.3	—	—	
Threonine	4.8	0.9	4.1	4.2	3.7	3.4		3.0	3.1	2.6	1.9	
Serine	5.1	2.1	2.4	0.8	2.6	1.9		1.5	0.8	2.8	2.2	
Glutamic acid	{ 11.4	4.0	5.3	0.5	6.9	4.1		2.6	1.6	10.5	3.6	
Glutamine		1.0	1.7	0.7	2.9	1.2		1.0	0.1	2.3	0.9	
Sarcosine	—	0.3	—	~0.06	0.9	0.9		—	0.1	0.9	0.7	
Proline	4.8	—	3.2	—	3.3	—		2.8	—	2.0	—	
Glycine	8.9	2.5	9.1	6.2	9.1	7.6		7.4	3.3	9.1	4.1	
Alanine	8.9	1.0	6.3	1.5	8.7	5.2		5.2	0.8	5.1	5.6	
Glutathione	—	14.6	0.4	1.4	—	—		4.4	5.7	—	—	
Valine	7.6	6.3	6.2	6.9	9.4	10.2		4.9	5.4	8.3	12.8	
Cystine	Trace	0.2	0.2	~0.04	0.4	0.3		~0.04	0.3	0.5	0.2	
Methionine	2.7	0.6	1.9	2.2	2.3	2.5		1.5	1.3	1.6	1.4	
Isoleucine	5.7	0.6	4.1	4.5	4.3	4.7		3.0	3.3	2.6	2.4	
Leucine	9.9	1.1	7.9	9.2	8.6	9.4		6.0	7.4	5.1	4.5	
Tyrosine	2.8	0.8	3.4	3.8	3.4	3.8		2.2	2.4	2.4	1.8	
Phenylalanine	4.4	0.3	3.5	4.3	4.3	4.9		2.4	3.0	2.5	2.0	
3-Aminoisobutyric acid	—	—	—	—	0.7	0.8		—	—	1.3	1.3	
Hydroxylysine	—	0.1	—	—	—	0.6		—	1.3	—	—	
Ornithine	—	0.2	3.3	0.3	4.8	1.0		3.6	0.3	3.1	2.5	
Ethanolamine	—	0.3	0.4	0.5	0.5	1.0		1.0	1.1	2.4	2.5	
Ammonia	—	16.6	18.7	30.8	4.2	10.8		19.9	25.9	7.6	12.9	
Lysine	6.8	—	5.5	6.9	5.0	9.2		6.1	6.3	4.3	5.4	
1-Methylhistidine	—	0.7	—	—	—	—		—	—	—	3.4	
Histidine	2.7	1.1	0.8	0.7	2.4	2.1		0.8	0.7	0.6	1.8	
3-Methylhistidine	—	—	—	—	—	—		—	—	1.0	—	
Arginine	4.4	—	—	—	—	—		—	—	—	—	
5-Aminopentanoic acid	—	—	1.3	1.7	—	—		1.4	—	—	—	
4-Aminobutyric acid	—	—	—	—	0.2	~0.03		—	—	0.2	0.1	
Tryptophan	—	—	—	—	0.9	—		0.2	0.3	0.1	—	
Unknown	—	8.5	0.6	0.8	1.0	6.7		1.3	1.7	12.0	13.5	
Total	99.8	100.5	99.0	100.4	100.5	99.2		101.2	100.5	99.9	99.4	
(μmol/g dry wt.)	11 260	10.8	115.0	75.5	127.2	97.8		54.0	32.8	60.9	56.7	

when mitochondria were incubated aerobically in 0.25M-sucrose. It was of interest to see whether this gradient was maintained uniformly for all the amino acids, or whether some were retained to a greater extent than others within the mitochondria.

Mitochondria were incubated aerobically for 24h in 0.25M-sucrose at either 0° or 25°C. The amino

acid composition of the external medium was then analysed, together with that of the internal medium released after breaking the mitochondria. The results are given in Table 2, expressed as mol%, together with the total (μmol) found per g dry weight of mitochondria and the internal and external concentrations (total).

Table 2. *Amino acid analysis (mol%) of the internal and external media of mitochondria incubated aerobically in 0.25M-sucrose at 0°C and 25°C for 24h*

The values given are the means of two separate analyses. For details see the text.

	Content (mol%)			
	Incubation at 0°C		Incubation at 25°C	
	Internal	External	Internal	External
Ninhydrin-positive substance				
Cysteic Acid	8.0	0.4	3.0	0.2
Taurine	13.8	7.6	0.5	0.3
Phosphoethanolamine	0.5	0.8	0.2	0.07
Aspartic acid	1.3	1.6	—	—
Asparagine	1.5	1.5	—	2.5
Threonine	3.2	3.5	2.4	2.5
Serine	1.3	1.0	0.3	0.5
Glutamic acid	2.0	5.3	6.3	5.6
Glutamine	1.3	2.0	—	—
Sarcosine	—	0.5	1.6	2.1
2-Aminoadipic acid	—	—	0.3	—
Proline	—	—	2.3	2.6
Glycine	7.1	9.9	9.1	7.6
Alanine	3.4	6.0	12.8	11.4
2-Aminobutyric acid	—	—	0.2	—
Glutathione	3.5	2.8	—	—
Valine	3.3	4.5	8.3	7.6
Cystine	0.3	0.1	—	—
Methionine	0.7	2.3	2.6	4.3
Isoleucine	2.8	4.3	5.7	6.5
Leucine	7.5	8.6	12.4	13.4
Tyrosine	2.1	3.0	3.9	4.1
Phenylalanine	2.9	4.5	6.0	6.6
3-Aminoisobutyric acid	—	—	0.4	—
Hydroxylysine	—	0.03	0.2	0.4
Ornithine	0.5	1.0	0.5	0.3
Ethanolamine	0.6	0.3	2.7	2.6
Ammonia	17.7	12.8	8.7	6.9
Lysine	6.3	6.2	5.0	5.2
Histidine	0.3	0.5	2.2	2.3
Arginine	0.3	0.07	—	0.4
5-Aminopentanoic acid	0.6	2.3	—	0.2
4-Aminobutyric acid	0.2	0.03	0.5	0.4
Tryptophan	0.2	0.1	—	1.0
Unknown	6.5	6.5	2.0	2.1
Total	100.5	100.0	100.1	99.7
($\mu\text{mol/g dry wt.}$)	7.48	22.7	64.1	509.0
Concentration (mM)	2.60	0.67	11.10	13.10

At 0°C a concentration ratio, P/S [internal/external as defined by Alberti & Bartley (1969)], of 3.88 was found, whereas the total amino acids had increased to three times the endogenous amount. Cysteic acid and taurine were retained inside the mitochondria in a highly selective manner, whereas glutamic acid, sarcosine, methionine, 5-aminopentanoic acid, and to a lesser extent glutamine, alanine, isoleucine, phenylalanine and ornithine tended to be relatively excluded from the mitochondria, although in all cases the internal concentration of these amino acids was higher inside than outside, except for 5-aminopentanoic acid where the absolute internal and external concentrations were the same.

At 25°C, on the other hand, the P/S ratio was approximately 1, whereas the total amino acids had increased to 53 times the endogenous amount. Cysteic acid was still concentrated selectively inside the mitochondria but in all other cases the amount (mol%) inside was very similar to the value outside. In this case very little 5-aminopentanoic acid was found and all of it was outside the mitochondria.

Discussion

Columns 1 and 2 of Table 1 reveal that the endogenous amino acids did not exhibit the same amino acid composition as the 'membrane' protein suggesting that proteolytic activity is relatively unimportant in functioning mitochondria before isolation. Incubation of the isolated mitochondria at pH 7.4 leads to the accumulation of amino acids and, if the conclusions of Alberti & Bartley (1963, 1965, 1969) are correct in that this accumulation depends on neutral proteinase activity, then one might expect the composition of the amino acid mixture produced to resemble that of membrane protein.

In Table 3 the amino acid composition of whole mitochondria (Alberti, 1964) and of mitochondrial 'membranes' is compared with the composition of the amino acids released on anaerobic incubation, after correcting for the endogenous amounts present at the beginning of the incubation. There is good agreement between the sets of values but with some anomalies. The absence of aspartic acid and asparagine in Tris-HCl buffer incubations suggests that, if they are

Table 3. *Comparison of the amino acids produced during anaerobic incubation of mitochondria with the amino acid composition of whole mitochondria and mitochondrial 'membranes' (mol%)*

The amino acids are arranged in the order of decreasing content in 'membranes'. The amino acids produced during incubation were corrected for the endogenous amounts before expressing the result as mol%; minor components were omitted. The analysis of whole mitochondria is taken from the data of Alberti (1964).

Amino acid	Content (mol%)					
	Whole mitochondria	'Membrane'	Light mitochondria		Heavy mitochondria	
			Phosphate buffer	Tris-HCl buffer	Phosphate buffer	Tris-HCl buffer
Glutamic acid and glutamine	11.2	11.4	8.8	10.9	4.5	16.5
Leucine	9.9	9.9	10.0	9.9	9.7	7.2
Alanine	9.1	8.9	8.4	10.0	8.4	7.2
Aspartic acid and asparagine	9.4	8.9	8.8	—	10.6	—
Glycine	8.9	8.9	11.9	10.2	11.5	12.4
Valine	8.2	7.6	8.4	10.9	8.2	12.3
Lysine	6.4	6.8	7.5	5.8	10.2	6.4
Isoleucine	6.1	5.7	5.5	5.0	4.8	3.6
Serine	4.9	5.1	2.9	2.7	1.6	3.1
Threonine	5.3	4.8	5.5	4.1	4.7	3.5
Proline	5.4	4.8	4.4	3.8	4.8	3.0
Phenylalanine	4.4	4.4	4.7	5.0	3.9	3.6
Arginine/ornithine	4.1	4.4	4.4	5.5	6.0	4.6
Tyrosine	2.1	2.8	4.5	3.9	3.4	3.1
Histidine	2.1	2.7	0.9	2.6	0.9	1.0
Methionine	1.6	2.7	2.4	2.6	2.4	2.1
Cystine	0.6	Trace	0.2	0.5	0.01	0.7

being released by proteolysis, they must be subsequently metabolized. Conversely, in phosphate buffer aspartic acid and asparagine appear as expected from proteolysis but glutamic acid and glutamine are collectively lower than expected. Glycine is released to a greater extent than expected from the composition of 'membrane' whereas the reverse is true of serine. Arginine, present in the 'membrane' protein to the extent of 4.4mol%, never appears during incubation but ornithine appears instead at about the expected value, suggesting arginase activity and the release of urea. Arginase is known to occur in mitochondria (Rosenthal *et al.*, 1956). The values in Table 3 suggest that proteolysis of mitochondrial protein occurs, followed by specific metabolic conversions of some of the amino acids. One would not expect an exact correlation between the amino acid composition of 'membrane' or mitochondrial protein and that of the amino acids released by proteolysis since some mitochondrial proteins might be more susceptible to proteolysis under one set of conditions and others under different conditions.

Aerobically the obvious amino acids disappear, but apart from these the similar distribution to that found anaerobically bears out the hypothesis that proteolysis of structural proteins is occurring.

It was unexpected that the sulphur compounds taurine, cysteic acid and GSSG contributed nearly 50% of the endogenous total (concentrations of taurine and GSSG in the mitochondrial water approximate to 1.2 and 1.0mM respectively) as was the absence of reduced glutathione, GSH, even though Pinto & Bartley (1969b) found a high [GSH]/[GSSG] ratio in whole rat liver homogenates. Presumably the available glutathione would be converted into GSSG by the lipid hydroperoxides and the mitochondrial glutathione peroxidase.

It is tempting to propose that lipid hydroperoxide accumulation represents the first step in the degeneration of mitochondria after they have been isolated. Hydroperoxide accumulation *in vivo* would

normally be prevented by the glutathione reductase-glutathione peroxidase system (Neubert *et al.*, 1962; Little & O'Brien, 1968; Pinto & Bartley, 1969a) depending on a supply of NADPH. On isolation mitochondria are separated from the cytoplasmic glutathione reductase, glutathione would become completely oxidized and excess of lipid hydroperoxides remaining or accumulating in the mitochondrial membranes would bring about a change in membrane structure leading to mitochondrial swelling as suggested by Neubert *et al.* (1962). This in turn might release the neutral proteinases, or expose membrane protein to their action, leading to still further degradation and swelling with all the observed effects.

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