ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 12 (2001) 109–113

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

D-Amino acid contents of mitochondria and some purple bacteria

Yoko Nagata [a,*], Atsushi Fukuda [a], Masaki Sakai [a], Teruhito Iida [a], Kumiko Kawaguchi-Nagata [b]

[a] *Department of Life Science, Himeji Institute of Technology, Himeji, Hyogo 671-2201, Japan*
[b] *Department of Bacteriology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8131, Japan*

Received 23 July 1999; received in revised form 11 November 1999; accepted 11 November 1999

Abstract

The endosymbiont most likely to have given rise to mitochondria is an aerobic bacterium belonging to the α subdivision of the so-called purple bacteria such as *Rickettsia*, *Bradythizobium* and *Agrobacterium* [1,2]. Contents of the D-enantiomers of serine, alanine, proline, glutamate and aspartate in rat liver whole mitochondria, mitochondrial outer membranes, inner membranes and matrix, soluble proteins and free amino acids were detected. These values for D-amino acid content were compared with those in soluble proteins and free amino acids from the purple bacteria *Paracoccus denitrificans*, *Pseudomonas aeruginosa* and *Escherichia coli*, members, respectively of the α, β, and γ subdivisions, to find any similarity between mitochondria and these purple bacteria. A similarity was observed in protein D-amino acid contents which were low (<1.5%, D-type/D-type + L-type) both in the membrane and soluble protein fractions from mitochondria and in soluble protein from bacteria. Oddly, substantial amounts of free D-serine and free D-aspartate (around 2%) were found for the first time in mitochondria. The contents of D-serine and D-aspartate were higher than those of D-alanine, D-proline and D-glutamate. In purple bacteria, the concentration of D-serine (<2%) was the lowest of the five amino acids examined, and those of D-alanine (27–32%) and D-glutamate (7–26%) were high. Therefore, no similarity was shown in the free D-amino acid content between mitochondria and any of the three purple bacteria.

Keywords: D-Amino acid; Mitochondria; Symbiosis; *Paracoccus denitrificans*; *Pseudomonas aeruginosa*; *Escherichia coli*

1. Introduction

Mitochondria arose as bacterial endosymbionts within some ancestral type of archaeum or eukaryotic cell [3]. Mitochondria possess their own DNA, which does not have introns like nuclear DNA. The entire base sequence of human mitochondrial DNA has been determined [4], and it was found that mitochondrial DNA encodes only 13 mitochondrial proteins, such as components of respiratory complexes I, II, III, and IV (cytochrome c oxidase) and ATPase, and also ribosomal RNA and transfer RNA that function in the mitochondria. Protein synthesis and RNA polymerase in the mitochondria are sensitive to Kanamycin and Rifampicin, respectively, as are bacteria. Comparison of the amino acid sequences of cytochrome c indicated a close relationship between the mitochondrial protein and those of purple bacteria [5]. Since the mitochondrial cytochrome c is encoded by a nuclear gene, Yang et al. [1] compared mitochondrial ribosomal RNA gene sequences of various organisms with each other. It was found that wheat mitochondria is closer to *Agrobacterium tumefaciens* (α subdivision) than to *Pseudomonas*

* Corresponding author. Tel.: +81-792-67-4939; fax: +81-792-66-8868.
E-mail address: nagata@sci.himeji-tech.ac.jp (Y. Nagata).

1381-1177/01/$ – see front matter
PII: S1381-1177(00)00210-1

testosteroni (β subdivision) and *Escherichia coli* (γ subdivision).

Proteins or peptides in nature are comprised solely of L-enantiomers of amino acids in most cases, with exceptions such as bacterial cell wall peptidoglycan and antibiotics. It is generally thought that bacteria contain large amounts of D-amino acids while eukaryotic cells do not, since peptidoglycan in the bacterial cell wall contains D-alanine and D-glutamate as components of its pentapeptide [6]. In recent years, however, free D-serine [7–9] and D-aspartate [10,11] have been found in mammal brain tissues, etc. at high concentrations (up to 30% of the total free amino acids). These amino acids seem to be endogenous, as supported by the finding of serine racemase in rat brain [12].

Mitochondria are enclosed within double layered membranes; the inner membrane is derived from the bacterial symbiont and the outer one is from the ancestral cell. In the present study, we compared the D-amino acid contents of the inner and outer membranes of rat mitochondria to those in *Paracoccus denitrificans*, *Pseudomona aeruginosa* and *E. coli* as, α, β and γ purple bacteria, respectively, to detect any similarities in D-amino acid content between them.

2. Materials and methods

2.1. Mitochondria

All procedures for mitochondria preparation were done at 0–4°C. Livers from female Wistar rats (200–250 g) were minced with scissors in a medium containing 0.25 M sucrose, 10 mM HEPES (pH 7.5) and 1 mM EDTA (Sol A), and then homogenized in a glass homogenizer at 1100 rpm for 3 min. After filtration with nylon cloth, the homogenate was centrifuged at 5600 × *g* for 20 min. The resultant pellet was suspended in 10 volume of Sol A followed by centrifugation at 8000 × *g* for 20 min. The pellet was used as the mitochondrial fraction after washing with Sol A at 8000 × *g* for 20 min. The mitochondria were suspended in a small amount of 20 mM K-phosphate buffer (pH 7.5), and were broken with a mortar and a pestle with the aid of quartz sand. The mitochondrial inner membrane with matrix fraction was obtained by centrifugation of the homogenate at 2000 × *g* for 10 min. The supernatant was further centrifuged to sediment the outer membrane fraction at 35,000 × *g* for 30 min. The inner membrane with matrix fraction and the outer membrane fraction thus obtained were dried under reduced pressure, after washing with acetone and diethyl ether two and three times, respectively, to remove lipids.

The preparation of soluble proteins and free amino acids from mitochondria were done as described below. The supernatant solution of the homogenate of mitochondria centrifuged at 600 × *g* for 5 min, was subsequently centrifuged at 100,000 × *g* for 30 min. To the resultant supernatant, 10% trichloroacetic acid (TCA) solution was added to yield a final concentration of 5% (w/v), and then centrifuged to separate the soluble protein fraction as the pellet. The pellet was then washed with acetone and diethyl ether as mentioned above. The supernatant of the TCA-treated sample was passed through a Dowex 50W × 8 (H^+-form) column, and eluted with 2 M NH_4OH after washing with distilled water, to obtain purified free amino acids. The eluate was evaporated to dryness in vacuo in a centrifugal evaporator (Taitec, Saitama, Japan) below 40°C.

2.2. Bacteria

P. denitrificans (DSM 65) cells were grown in medium composed of 1.0% Nutrient broth (Difco Laboratories, Detroit, Mich., USA), 1.0% glucose and 0.5% NaCl (pH 7.2) for 15 h at 37°C with shaking. Cells of *P. aeruginosa* and *E. coli* strain K12 were cultured in medium containing 1% L-broth peptone, 0.5% yeast extract, 0.1% glucose and 0.5% NaCl (pH 7.2) for 18 h at 37°C with shaking.

Bacterial cells were harvested at mid-to post-logarithmic phase, and washed with 50 mM K-phosphate buffer, pH 7.5, twice to completely remove the culture medium. They were disrupted by sonication with a sonic oscillator (Model UR-200P, Tomy, Tokyo, Japan) at 20 kHz and 90 W for 3 min. The resultant cell homogenate was centrifuged at 1000 × *g* for 10 min at 4°C followed by 100,000 × *g* for 30 min at 4°C. The sediment produced from the supernatant by adding 10% TCA solution to yield a final concentration of 5% (w/v) was used as the soluble protein fraction. The sediment was washed twice with acetone followed by three times with diethyl ether to remove

TCA and other small molecules such as free amino acids. The supernatant of the TCA-treated sample was passed through the Dowex 50W × 8 column to purify the free amino acid fraction, as mentioned above.

2.3. Hydrolysis of proteins

Protein samples were hydrolyzed to liberate free amino acids in the same manner as described in [13]. Briefly, dried whole mitochondria, the inner membrane with matrix, the outer membrane and the mitochondrial and bacterial soluble protein fractions were suspended in 6 M HCl and hydrolyzed for 3, 6, 16 and 24 h at 110 ± 0.5°C under reduced pressure in an aluminum block in a Dry Thermo Unit (Taitec, Saitama, Japan).

2.4. Determination of D- and L-amino acids

The determination of the enantiomeric concentrations of each amino acid was performed as described previously [14]. Free amino acids and hydrolysates prepared as above were treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide [15] (FDAA or Marfey's reagent, Pierce, Rockford, IL, USA) to form diastereomers. The FDAA-derivatives were separated on a Silica Gel 60 plate (Merck, Darmstadt, Germany) by two-dimensional thin-layer chromatography. FDAA-amino acids recovered from the plate were analyzed by high-performance liquid chromatography (HPLC) for the resolution of D- and L-enantiomers, using a reversed-phase column, Nova-Pak C_{18} (150 mm × 3.9 mm i.d., Waters, Milford, MA, USA), and a Tosoh (Tokyo, Japan) or Jasco (Tokyo) gradient HPLC system. The amounts of D- and L-enantiomers of amino acids were calculated based on the peak areas of the elution patterns obtained by a Chromato-Integrator (D-2500, Hitachi, Tokyo). As standards, known amounts of D- and L-enantiomers of the amino acids were derivatized with FDAA and purified by two-dimensional thin-layer chromatography as above, before HPLC.

Calculation of D-isomer contents of peptidyl amino acids was done as described elsewhere [13]. Briefly, to obtain the true value by subtracting the rate of chemical racemization caused during acid hydrolysis, each sample was hydrolyzed for four different time periods, 3, 6, 16 and 24 h. The %D-type value (100 × D-type/D-type + L-type, the ratio in molar concentration of a D-amino acid to the sum of concentrations of the D-amino acid and the corresponding L-isomer) was plotted against the length of hydrolysis time. The linear regression line of the four plots was extrapolated to the *y*-axis, i.e. 0 h. The %D-type value for the 0 h hydrolysis was employed as the value showing the 'true' content of a D-amino acid residue.

Reagents used were of analytical grade obtained from Sigma (St. Louis, MO, USA), Aldrich (Tokyo, Japan), Nacalai (Kyoto, Japan) and Wako (Osaka, Japan).

3. Results

The purity of the whole mitochondria, the inner membrane with matrix, and the outer membrane fractions was determined by light absorbance of cytochromes (Fig. 1). The absorption at 553, 560 and 605 nm shown by the inner membrane with matrix fraction appeared due to cytochrome c_1, cytochrome b and cytochrome c oxidase, respectively (Fig. 1B). These components of the electron transfer system are characteristic of the inner membrane of mitochondria. In the outer membrane fraction, there was no peak at 605 nm (which is an indicator of the presence of inner membrane) (Fig. 1C). The absorption spectrum of the whole mitochondria fraction showed the sum of that of the inner membrane with matrix and outer membrane fractions (Fig. 1A).

The whole mitochondria, the inner membrane with matrix and outer membrane fractions, and mitochondrial soluble protein fraction as well as free amino acids were assayed for contents of D-enantiomers of serine, alanine, proline, glutamate (glutamine) and aspartate (asparagine). The reason for analysis of these five amino acids was that only the D-enantiomers of these five amino acids have been shown to occur generally in organisms [13]. As shown in Table 1, the D-amino acid contents were low, at most 1.5%, in these proteins (Table 1). The occurrence of D-amino acid was also low in the soluble protein fractions from *P. denitrificans*, *P. aeruginosa* and *E. coli* (Table 2). As for free amino acids, it was found that mitochondria contain not high but substantial amounts of D-enantiomers of serine and aspartate (Table 1). In the three purple bacteria, the contents of free D-alanine

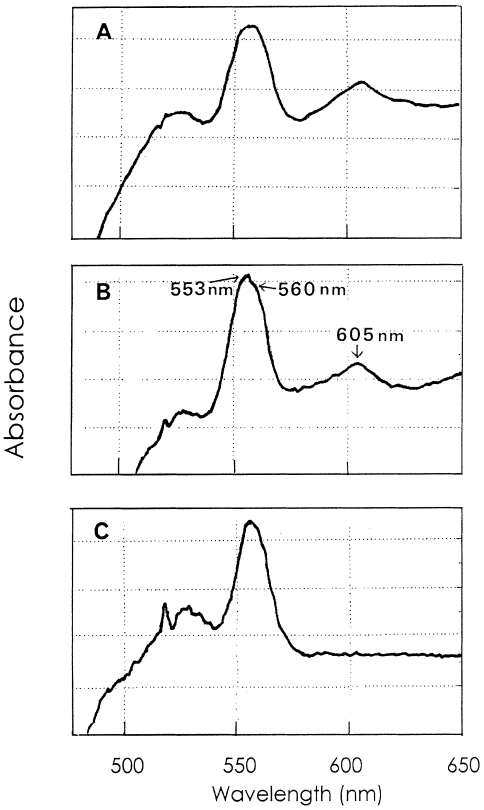

Fig. 1. Absorption spectra of mitochondria (A); inner membrane/matrix fraction (B); and outer membrane fraction (C). Difference spectra (reduced form minus oxidized form) are shown in arbitrary scale. Specimens were dissolved in 10 mM Na-phosphate buffer (pH 6.5) containing 0.1% Triton X-100, and were reduced with a small amount of solid $Na_2S_2O_4$ or oxidized with a small amount of solid $K_3F(CN)_6$.

(27–32%) and D-glutamate (7–26%) were the highest among the five amino acids examined, and that of D-serine was lowest (Table 2). In addition, high contents of D-proline (42%) and D-aspartate (25%) were observed in *P. denitrificans* and *P. aeruginosa*, respectively.

4. Discussion

The absorption spectra showed the presence of cytochrome c_1, cytochrome b and cytochrome c oxidase in the inner membrane with matrix fraction, and the absence of cytochrome c oxidase in the outer membrane fraction. The spectrum of whole mitochondria coincided with the sum spectra of the inner membrane with matrix fraction and outer membrane fraction. Therefore, the mitochondria, the inner membrane with matrix and outer membrane samples used in the present experiment seem suitable for the experimental purpose although, they were not checked for contamination with peroxisomes. In a separate study, we have confirmed that peroxisomes contained no D-amino acids at an appreciable level.

In this study, we did not investigate the bacterial membrane fraction and the cell walls, since it is known that they contain D-alanine and D-glutamate in the pentapeptide of peptidoglycan or its precursor molecule [6]. The D-amino acid contents were low both in the mitochondrial membrane and soluble protein and in the purple bacteria soluble proteins. Regarding free amino acids, we expected a closer relationship between mitochondria and *P. denitrificans*, which is an α purple bacterium, than the other two bacteria which belong to β and γ subdivisions. The rate of free D-amino acids, however, showed no similarity between the mitochondria and the bacteria examined: the most prevalent D-amino acids detected were serine and aspartate in the mitochondria,

Table 1
D-Amino acid contents of mitochondria[a]

	%D-type (100 × D-type/D-type + L-type)				
	Ser	Ala	Pro	Glu	Asp
Mitcohodria	0.25 ± 0.14	0.71 ± 0.10	0.68 ± 0.08	0.99 ± 0.08	1.23 ± 0.17
IMM[b]	0.29 ± 0.19	0.66 ± 0.10	0.55 ± 0.06	0.98 ± 0.15	1.18 ± 0.23
OM[c]	0.72 ± 0.20	0.65 ± 0.08	0.60 ± 0.08	1.07 ± 0.18	1.52 ± 0.21
Soluble protein	0.91 ± 0.17	0.37 ± 0.06	0.57 ± 0.06	0.69 ± 0.070	0.43 ± 0.12
Free amino acid	2.25 ± 0.38	0.62 ± 0.08	0.77 ± 0.23	0.74 ± 0.14	1.90 ± 0.50

[a] Mean ± S.D. values of three to five independent experiments are shown.
[b] Inner membrane and matrix fraction.
[c] Outer membrane fraction.

Table 2
D-Amino acid contents in soluble protein and free amino acids of some purple bacteria[a]

	%D-type (100 × D-type/D-type + L-type)				
	Ser	Ala	Pro	Glu	Asp
Soluble protein					
P. denitrificans	0.73 ± 0.32	0.65 ± 0.15	0.70 ± 0.37	1.22 ± 0.15	1.08 ± 0.44
P. aeruginosa	0.78 ± 0.22	0.29 ± 0.06	0.61 ± 0.19	0.68 ± 0.37	1.70 ± 0.32
E. coli	1.95 ± 0.51	0.72 ± 0.25	0.69 ± 0.12	1.01 ± 0.09	1.57 ± 0.14
Free amino acid					
P. denitrificans	1.63 ± 0.51	32.05 ± 5.61	42.49 ± 15.70	11.66 ± 0.74	4.99 ± 2.10
P. aeruginosa	0.94 ± 0.32	27.40 ± 0.84	3.38 ± 0.11	26.18 ± 5.90	24.61 ± 0.59
E. coli	1.23 ± 0.38	31.78 ± 2.90	2.22 ± 1.19	7.57 ± 2.65	2.94 ± 0.31

[a] Mean ± S.D. values of four or five independent experiments are shown.

whereas they were alanine and glutamate in the bacteria, in which free D-alanine and D-glutamate are necessary for peptidoglycan synthesis. This pattern of D-amino acid distribution among free amino acids of the mitochondria is rather similar to that of some archaea [16].

Regarding the endosymbiosis model of mitochondrial origin, it is less clear whether the host was a nucleus-containing eukaryote or an archaebacterium [2]. However, the nuclear genome has been shown to be an evolutionary chimera that incorporates substantial contributions from both archaebacterial and eubacterial progenitors [17]. The present findings show similarity in the distribution pattern of free D-amino acids between mitochondria and some archaea, suggesting that the gene encoding a D-amino acid producing enzyme (racemase or transaminase) is in the nucleus, descended from the ancestral archaeum. This postulation is consistent with the fact that no gene for racemase or transaminase is present in the mitochondrial DNA genome [4].

Endogenous free D-serine, it has been strongly suggested, is produced in the brain by serine racemase [12]. The present finding that liver mitochondria contain free D-serine and D-aspartate may provide insight into a source, other than brain serine racemase, of these endogenous free D-amino acids. To our knowledge, this is the first finding of D-amino acids in mitochondria.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Cooperative Research (A) (No. 07308048) to YN from the Ministry of Education, Science and Culture of Japan.

References

[1] D. Yang, Y. Oyaizu, H. Oyaizu, G.J. Olsen, C.R. Woese, Proc. Natl. Acad. Sci. U. S. A. 82 (1985) 4443–4447.
[2] M.W. Gray, C. Burger, B.F. Lang, Science 283 (1999) 1476–1481.
[3] M.W. Gray, W.F. Doolittle, Microbiol. Rev. 46 (1982) 1–42.
[4] S. Anderson, A.T. Bankier, B.G. Barrell, M.H.L. de Bruijin, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, P.H. Schreier, A.J.H. Smith, R. Staden, I.G. Young, Nature 290 (1981) 457–465.
[5] R.E. Dickerson, Nature (London) 283 (1980) 210–212.
[6] M. Osborn, Ann. Rev. Biochem. 38 (1969) 501–538.
[7] A. Hashimoto, T. Nishikawa, T. Hayashi, N. Fujii, K. Harada, T. Oka, K. Takahashi, FEBS Lett. 296 (1992) 33–36.
[8] Y. Nagata, Experientia 48 (1992) 753–755.
[9] Y. Nagata, K. Horiike, T. Maeda, Brain Res. 634 (1994) 291–295.
[10] D.S. Dunlop, A. Neidle, D. McHale, D.M. Dunlop, A. Lajtha, Biochem. Biophys. Res. Commun. 141 (1986) 27–32.
[11] J. Lee, H. Homma, K. Sakai, T. Fukushima, T. Santa, K. Tashiro, T. Iwatsubo, M. Yoshikawa, K. Imai, Biochem. Biophys. Res. Commun. 231 (1997) 505–508.
[12] H. Wolosker, K.N. Sheth, M. Takahashi, J.P. Mothet, R.O. Brady Jr., C.D. Ferris, S.H. Snyder, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 721–723.
[13] Y. Nagata, T. Fujiwara, K. Kawaguchi-Nagata, Y. Fukumori, T. Yamanaka, Biochim. Biophys. Acta 1379 (1998) 76–82.
[14] Y. Nagata, K. Yamamoto, T. Shimojo, J. Chromatogr. 575 (1992) 147–152.
[15] P. Marfey, Carlsberg Res. Commun. 49 (1984) 591–596.
[16] Y. Nagata, K. Tanaka, T. Iida, Y. Kera, R. Yamada, Y. Nakajima, Y. Koga, S. Tsuji, K. Kawaguchi-Nagata, Biochim. Biophys. Acta 1435 (1999) 160–166.
[17] D.-F. Feng, G. Cho, R.F. Doolittle, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 13028–13033.