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The classification of various organisms according to the free amino acid composition change as the result of biological evolution

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Summary. The free amino acid compositions in archaeobacteria, eubacteria, protozoa, blue-green alga, green alga, slime mold, plants and mammalian cells were analyzed, to investigate whether changes in their free amino acid compositions reflect biological evolution. Cell homogenates were treated with 80–90% ethanol to separate cellular proteins and free amino acids contained in the cells. Different patterns of the free amino acid compositions were observed in the various organisms. Characteristic differences were observed between plant and mammalian cells, and between archaeobacteria and eubacteria. The patterns of the free amino acid composition in blue-green alga, green alga, protozoa and slime mold differed from each other and from those of eubacteria and archaeobacteria. Rat hepatoma cells (R-Y121B) were cultured in Eagle's minimum essential medium (MEM) containing 5% serum or in a modified MEM lacking arginine, tyrosine and glutamine. No significant difference in the free amino acid composition was observed between the two cell groups cultured under two different conditions. It is suggested that the free amino acid composition reflects apparent biological changes as the result of evolution.

Keywords: Amino acids – Free amino acid composition – Nutrition – Archaea – Eubacteria – Eukaryotes

Introduction

Nutritional changes could alter cellular and free amino acid compositions. This nutritional effect could be observed by the comparison of the free amino acid compositions of cells cultured under different conditions. Rat hepatoma cells (R-Y121B) (Niwa et al., 1980) exhibiting ornithine carbamoyltransferase (OCT) activity, can grow in the absence of arginine in serum-free culture media. In general, arginine is an important amino acid in mammalian cell cultures in vitro due to a lack of OCT activity (Schimke, 1964). Whereas other cells need this amino acid to grow continuously in vitro, this cell line can grow

even in the absence of tyrosine and glutamine. As the R-Y121B cell line is a good model system to investigate whether culture conditions induce changes in free amino acid compositions, it has been used in the present study.

Our previous studies (Okayasu et al., 1997; Sorimachi, 1999) have shown that changes in cellular amino acid compositions reflect biological evolution. In these studies, cell homogenates were directly used for the analysis of cellular amino acid compositions, as the amount of free amino acids contained in the cells was negligible compared with total cellular amino acids (Okayasu et al., 1997). However, as the free amino acid composition of cells is strictly controlled under specific conditions, such as amino acid metabolism and amino acid transport by the plasma membrane, changes in the free amino acid composition may partially reflect certain changes in cells as a result of biological evolution. Therefore, the present study has been designed to investigate whether differences in the free amino acid composition of cells are related to biological changes as the result of evolution.

Materials and methods

Chemicals

L-amino acids for cell cultures were purchased from Ajinomoto-Takara Corp. (Tokyo, Japan). Seventeen authentic amino acids for the amino acid analysis (H type) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Other chemicals were commercial products of reagent grade.

Cells

Monkey kidney cells (VERO) (Yasumura et al., 1987), monkey hepatocarcinoma cells (NCLP-6E) (Dawe et al., 1968), rat hepatoma cells (R-Y121B) (Niwa et al., 1980), fish scale cells (GAKS) (Akimoto et al., 2000), human urinary bladder carcinoma cells (HUB) (Kakuya et al., 1983), and human fetal liver cells (HuL) (Katsuta et al., 1980), were cultured in plastic culture flasks at 37°C. NCLP-6E cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 0.5% fetal bovine serum. R-Y121B cells were cultured in MEM supplemented with 5% fetal bovine serum, or in a modified MEM without serum, in which arginine, tyrosine and glutamine were removed from the MEM (Niwa et al., 1980). GAKS cells, HUB cells and *Chlorella* were cultured in DM-160 (Katsuta and Takaoka, 1976) supplemented with 10% fetal bovine serum. VERO and HuL cells were cultured in a modified MEM in which glutamine was replaced with glutamic acid in the absence of serum (Yasumura et al., 1987).

The following samples were kindly supplied: Slime mold (*Dictyostelium discoideum*); Dr. Saburo Uchiyama of Dokkyo University School of Medicine, Protozoa (*Tetrahymena*); Prof. Koei Hamana of Gunma University, blue-green alga (*Chroococi diopsis*); Prof. Sunao Yamazaki of Tokyo University.

Plants

Callus of carrot (*Daucus carota*), *Torenia fournieri* and the protocomb-like body of *Cymbidium*, s.p., which were cultured on a Murashige & Skoog medium, were kindly supplied by ASAHI TECHNO GLASS CORPORATION.

Bacteria

Eubacteria were cultured on 1.5% agar containing Bouillon and washed twice with saline by centrifugation at 5,000 rpm for 10 min. Archaeobacteria, *Archaeoglobus flugidus* (Stetter et al., 1998; Stetter, 1988), *Pyrococcus horikoshii* (Gonzalez et al., 1988), *Methanobactrium thermoautotrophicum* (Zeikus and Wolfe, 1972), and *Methanococcus jannashii* (Jones et al., 1983), were cultured according to the methods described in their references. Cells were washed three times with saline by centrifugation at 7,000 rpm for 10 min. Bacterial cells were homogenized using a VP-5S sonicator, TAITEK (Koshigaya, Japan), at 20 Khz for 30 sec in H_2O.

Sample preparation

Cultured cells were washed twice with phosphate buffered saline (PBS), harvested by silicone-rubber policeman in PBS, and then centrifuged at 1,000 rpm for 10 min. The cells were resuspended in PBS and centrifuged at 1,000 rpm for 10 min. This procedure was carried out twice. The cells were kept at $-20°C$ and then homogenized in H_2O with a Physcotron, NICHI-ONI Rikagaku Seisakusho (Tokyo, Japan), at 20,000 rpm for 20 sec. Cell homogenates (100–200 μl) were mixed with ethanol (0.9 ml) and centrifuged at 14,000 rpm for 5 min to separate protein precipitates from free amino acids. The precipitates were washed again in 1 ml of ethanol and centrifuged using the same conditions. The pooled supernatants were dried in vacuo for amino acid analysis. The precipitates were resuspended in H_2O (200 μl) and homogenized with a Physcotron to make a homogeneous suspension. An aliquot (20–100 μl) of the homogenate was used for the amino acid analysis.

Amino acid analysis

An aliquot of the homogenate was dried under reduced pressure, and hydrolyzed at 110°C in 6 N HCl (200 μl) for 24 hr. After hydrolysis, the sample was dried under reduced pressure at room temperature, and then dissolved in 200 μl of 0.2 N HCl. The sample solution was passed through a membrane filter of 0.45 μm pore size before application on the amino acid analyzer, HITACH L-8500A.

Results

Effect of ethanol precipitation

In a previous study (Okayasu et al., 1997), no significant effect was observed for the ethanol precipitation of cell homogenates of rat hepatoma cells (R-Y121B). To confirm this result, another cell line, monkey hepatocarcinoma cells (NCLP-6E), was examined. The amino acid compositions are shown in Table 1. The radar graphs for amino acid compositions are identical for the ethanol-untreated and ethanol-treated samples, as shown in Fig. 1A. Thus, ethanol treatment had no significant effect on cellular amino acid compositions.

The free amino acid composition of NCLP-6E cells is shown in Table 1 and Fig. 1B. The free amino acid composition differs from the cellular amino acid composition. The absolute amount of free amino acids was 2.0% of the total cellular amino acids.

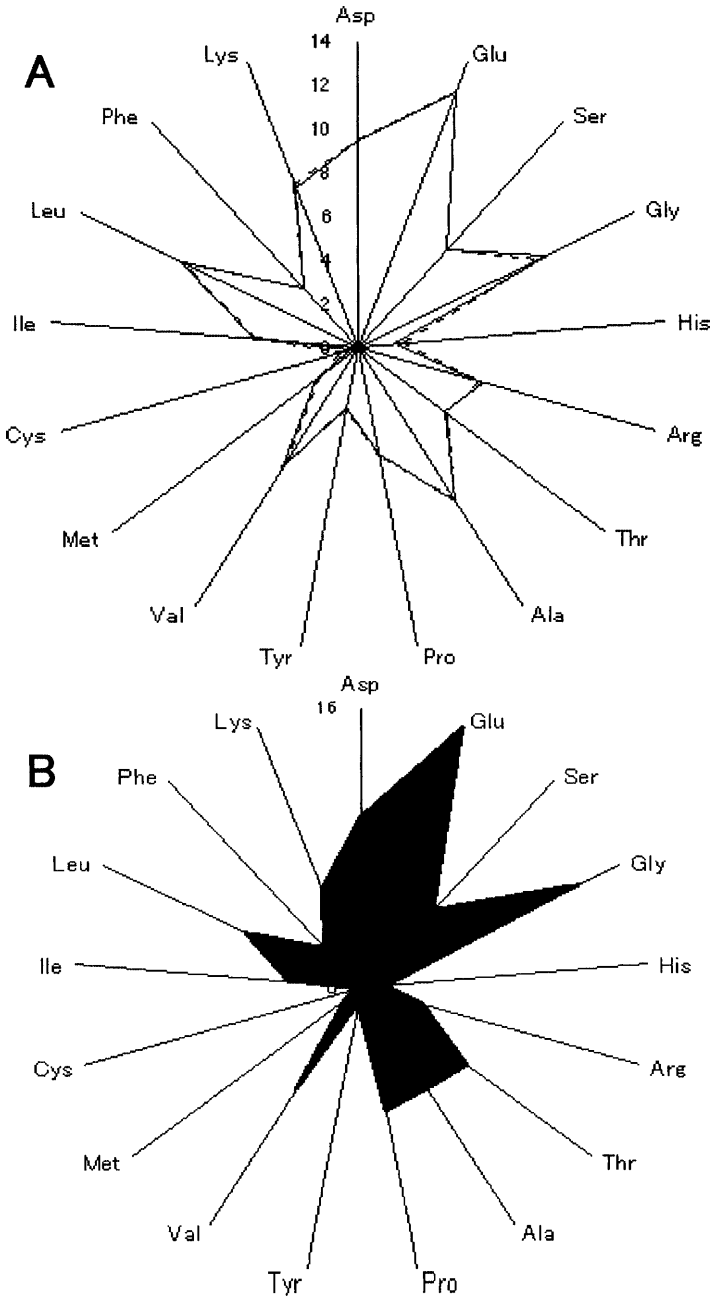

Fig. 1. Radar graphs of cellular and free amino acid compositions of NCLP-6E cells. Solid lines represent the cellular amino acid composition of the sample not treated with ethanol and the dotted line represents the sample treated with ethanol (**A**). Each line was drawn with the mean of triplicate analyses. The free amino acid composition of NCLP-6E cells (**B**)

Table 1. Cellular and free amino acid composition of NCLP-6E cells

Amino acid	Total	Cellular	Free
Asp	9.41 ± 0.02	9.43 ± 0.00	9.74 ± 0.17
Glu	12.57 ± 0.08	12.47 ± 0.10	15.92 ± 0.36
Ser	6.04 ± 0.02	6.01 ± 0.03	5.93 ± 0.11
Gly	9.08 ± 0.02	9.57 ± 0.07	13.33 ± 0.23
His	1.95 ± 0.01	1.61 ± 0.03	1.36 ± 0.10
Arg	5.63 ± 0.04	5.78 ± 0.04	3.52 ± 0.08
Thr	5.04 ± 0.02	4.87 ± 0.04	7.36 ± 0.14
Ala	8.14 ± 0.06	8.29 ± 0.25	6.75 ± 0.10
Pro	4.99 ± 0.12	4.94 ± 0.17	7.13 ± 0.08
Tyr	2.92 ± 0.04	2.84 ± 0.03	0.84 ± 0.15
Val	6.22 ± 0.02	6.44 ± 0.02	6.71 ± 0.26
Met	2.22 ± 0.01	2.38 ± 0.14	1.10 ± 0.46
Cys	0.76 ± 0.01	0.33 ± 0.03	0.15 ± 0.27
Ile	4.55 ± 0.02	4.69 ± 0.04	4.05 ± 0.22
Leu	8.86 ± 0.06	8.87 ± 0.09	7.03 ± 0.16
Phe	3.61 ± 0.03	3.64 ± 0.04	2.98 ± 0.11
Lys	8.00 ± 0.15	7.84 ± 0.13	6.08 ± 0.19

NCLP-6E cell homogenates were treated or untreated with ethanol as described in the Materials and methods. Each value is a percentage of total amino acids and the mean ± S.D. of three samples.

Effect of different media on free amino acid composition

To determine whether a culture medium affects the free amino acid composition, the R-Y121B cell line was used. When R-Y121B cells are cultured under two different conditions, the effect of a culture medium on the free amino acid composition can be evaluated.

The free amino acid composition of R-Y121B cells cultured in the modified serum-free MEM lacking arginine, tyrosine and glutamine was almost identical with R-Y121B cells cultured in MEM supplemented with 5% serum, as shown in Table 2 and Fig. 2A and 2B.

No significant difference in the cellular amino acid composition was observed between R-Y121B cells cultured in MEM supplemented with 5% serum and R-Y121B cells cultured in the modified medium (data not shown).

Vertebrate cells

VERO, HuL and HUB cells were examined as mammalian cells. GAKS fish cells were also examined as another vertebrate. The main free amino acids were glutamic acid, glycine and alanine in these cells, and the concentration of serine varied among vertebrate cell as shown in Table 2 and Fig. 3C–F.

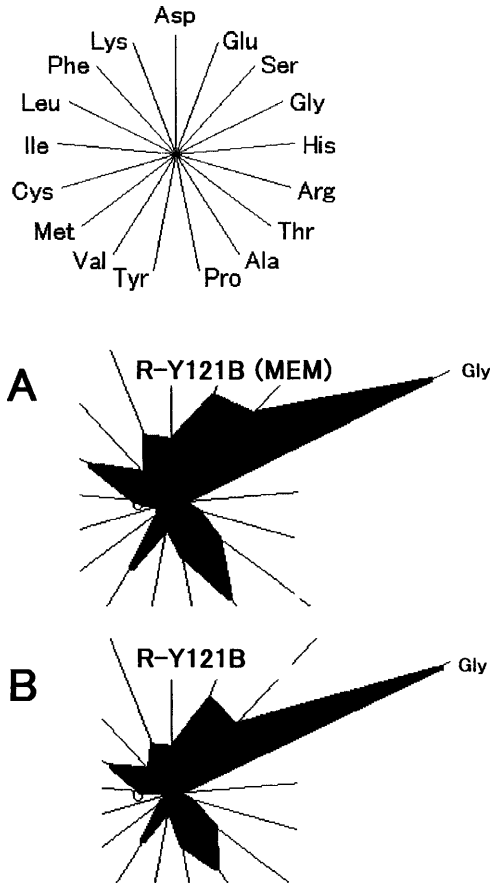

Fig. 2. Radar graphs of the free amino acid composition of vertebrate cells. (**A**); R-Y121B cells cultured in MEM supplemented with 5% serum (**B**); R-Y121B cells cultured in a modified MEM

Prokaryotes and primitive eukaryotes

The free amino acid compositions of blue-green alga, protozoa, slime mold and green alga are shown in Table 3 and Fig. 4. In blue-green alga, a very high concentration of glutamic acid was observed together with a relatively high proportion of lysine, glycine, threonine, alanine, proline and valine compared with other amino acids (Fig. 4A). Free amino acid composition in *Tetrahymena* was similar to that of human urinary bladder carcinoma (HUB) cells, except a higher concentration of alanine in *Tetrahymena* (Fig. 4B). In slime mold, the concentrations of aspartic acid, serine, glycine, threonine, alanine, proline, valine, isoleucine and leucine were high, forming a characteristic pattern of free amino acids which was different to that of other organisms (Fig. 4C). The high concentrations of lysine and threonine in the free amino acid composition in *Chlorella* were unique (Fig. 4D). Evidently, the pattern of the free amino acids in *Chlorella* differed markedly from those in other organisms.

Table 2. Free amino acid composition in mammalian cells

A. A.	R-Y121B MEM[1]	R-Y121B[2]	HUB-15	HuL	VERO	GAKS
Asp	9.58 ± 0.10	9.69 ± 0.04	8.23 ± 0.07	0.79 ± 1.11	7.10 ± 0.18	9.79 ± 0.26
Glu	12.01 ± 0.16	11.96 ± 0.11	19.44 ± 0.01	23.69 ± 0.30	19.25 ± 0.61	35.42 ± 0.62
Ser	6.19 ± 0.07	6.02 ± 0.03	13.48 ± 0.03	5.13 ± 0.13	8.85 ± 0.14	6.25 ± 0.08
Gly	9.61 ± 0.12	9.33 ± 0.02	28.62 ± 0.45	38.14 ± 0.67	23.74 ± 0.57	21.22 ± 0.38
His	1.81 ± 0.03	1.85 ± 0.01	0.23 ± 0.20	0.61 ± 0.13	0.78 ± 0.12	0.14 ± 0.23
Arg	5.51 ± 0.05	5.42 ± 0.01	0.63 ± 0.05	1.59 ± 1.25	4.13 ± 0.07	0.54 ± 0.51
Thr	5.46 ± 0.05	5.54 ± 0.02	2.77 ± 0.02	6.42 ± 0.48	4.88 ± 0.11	1.90 ± 0.65
Ala	8.74 ± 0.22	8.84 ± 0.11	8.24 ± 0.14	10.61 ± 0.21	11.27 ± 0.20	13.71 ± 0.32
Pro	5.40 ± 0.91	5.56 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	4.40 ± 0.35	0.00 ± 0.00
Tyr	2.84 ± 0.01	2.51 ± 0.02	0.95 ± 0.02	1.15 ± 0.11	1.08 ± 0.12	0.45 ± 0.39
Val	6.96 ± 0.09	6.92 ± 0.11	2.62 ± 0.41	3.42 ± 1.11	2.98 ± 0.30	1.93 ± 0.45
Met	0.00 ± 0.00	0.03 ± 0.04	2.15 ± 0.13	1.38 ± 0.22	1.58 ± 0.95	1.39 ± 0.35
Cys	0.02 ± 0.03	0.09 ± 0.09	2.60 ± 0.31	0.54 ± 0.47	0.14 ± 0.25	1.51 ± 0.43
Ile	4.91 ± 0.03	5.04 ± 0.01	1.87 ± 0.02	1.77 ± 0.22	1.70 ± 0.57	1.00 ± 0.05
Leu	9.25 ± 0.09	9.41 ± 0.03	4.99 ± 0.03	2.32 ± 0.19	3.21 ± 0.17	2.30 ± 0.13
Phe	3.98 ± 0.05	4.05 ± 0.02	1.72 ± 0.09	1.25 ± 0.03	1.56 ± 0.02	0.85 ± 0.08
Lys	7.75 ± 0.09	7.73 ± 0.03	1.47 ± 0.04	1.19 ± 0.04	3.32 ± 0.13	1.61 ± 0.11

[1] R-Y121B cells cultured in MEM suplemented with 5% serum.
[2] Cultured in the modified MEM in which arginine, tyrosine and glutaimine were removed and no serum was added. See the legends for Table 1.

Table 3. Free amino acid composition of prokaryote and primitive eukaryotes

Amino acids	Cyanobacteria	*Tetrahymena*	Slime mold	*Chlorella*
Asp	9.15 ± 0.20	5.95 ± 0.07	7.96 ± 0.07	12.42 ± 0.15
Glu	23.94 ± 0.54	12.26 ± 0.16	4.48 ± 0.04	18.61 ± 0.18
Ser	4.85 ± 0.16	6.52 ± 0.08	12.23 ± 0.02	3.92 ± 0.03
Gly	9.27 ± 0.16	20.08 ± 0.28	16.02 ± 0.19	5.45 ± 0.02
His	0.06 ± 0.03	0.43 ± 0.38	0.46 ± 0.01	4.21 ± 0.08
Arg	3.66 ± 0.15	2.58 ± 0.04	0.42 ± 0.00	13.41 ± 0.09
Thr	7.47 ± 0.23	3.74 ± 0.04	12.15 ± 0.04	2.30 ± 0.02
Ala	8.06 ± 0.08	17.93 ± 0.29	6.82 ± 0.04	8.01 ± 0.09
Pro	6.64 ± 0.28	3.33 ± 0.19	11.56 ± 0.30	2.34 ± 0.07
Tyr	0.51 ± 0.05	1.82 ± 0.04	0.35 ± 0.00	0.30 ± 0.04
Val	6.06 ± 0.72	5.45 ± 0.03	12.44 ± 0.12	1.29 ± 0.02
Met	0.28 ± 0.49	1.29 ± 0.26	0.11 ± 0.00	0.32 ± 0.15
Cys	0.00 ± 0.00	0.41 ± 0.09	0.21 ± 0.01	0.16 ± 0.01
Ile	2.92 ± 0.31	3.83 ± 0.08	9.10 ± 0.13	0.45 ± 0.05
Leu	4.79 ± 0.16	6.62 ± 0.04	4.68 ± 0.09	0.97 ± 0.06
Phe	1.56 ± 0.02	2.61 ± 0.04	0.39 ± 0.00	0.40 ± 0.01
Lys	10.18 ± 0.28	5.15 ± 0.08	0.62 ± 0.01	25.45 ± 0.09

Each value is a percentage of total amino acids and the mean ± S.D. of three samples.

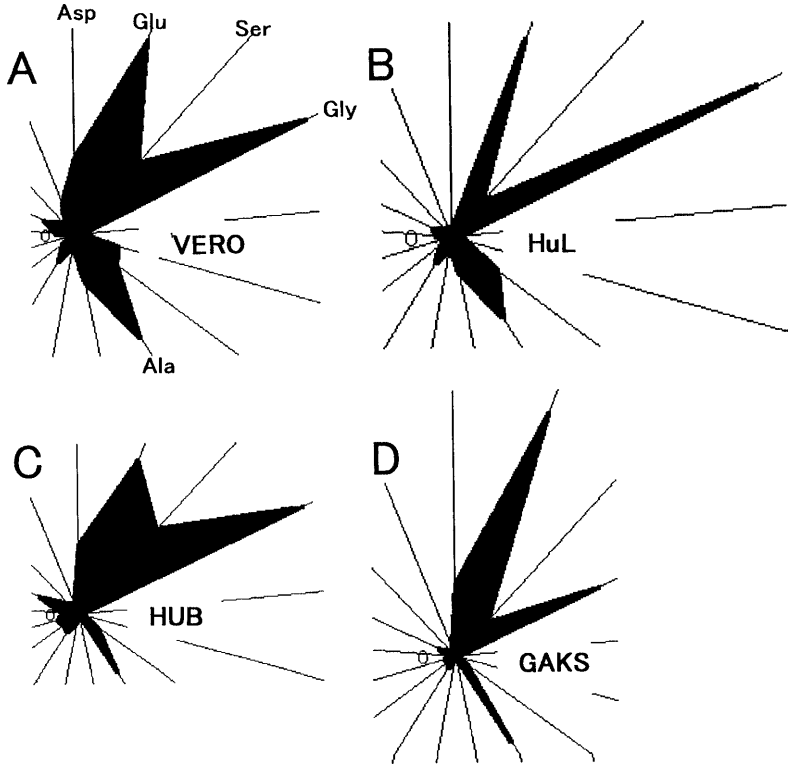

Fig. 3. Radar graphs of the free amino acid composition of vertebrate cells. (**A**); VERO cells, (**B**); HuL cells, (**C**); HUB cells, (**D**); GAKS cells

Eubacteria

The three species of Gram-negative bacteria (*E. coli*, *Proteus* and *V. alginoliticus*) showed interspecies differences in the free amino acid composition, although glutamic acid and glycine were the main amino acids in all of them (Table 4 and Fig. 5A–C). The serine concentration was very low in *V. alginoliticus* (Fig. 5C). The free amino acid compositions of two species of Gram-positive bacteria (*S. aureus* and *B. subtilis*) markedly differed from each other (Fig. 5D and 5E) and from Gram-negative bacteria (Fig. 5A–C). The main amino acids were glutamic acid, aspartic acid and lysine in *S. aureus* (Fig. 5D) and glutamic acid in *B. subtilis* (Fig. 5E). An extremely high concentration of glycine was observed in the cellular amino acid composition of *S. aureus* (Okayasu et al., 1997; Sorimachi, 1999), whereas the concentration of glycine in the free amino acid composition was very low compared with aspartic acid, glutamic acid and lysine (Table 4 and Fig. 5E). As these eubacteria were cultured under the same conditions, the differences in the free amino acid composition are based on the cellular characteristics. Similarly, great differences in the cellular amino acid composition between Gram-positive and Gram-negative bacteria were also observed in the amino acid analysis of whole cells, however the cellular amino acid compositions of

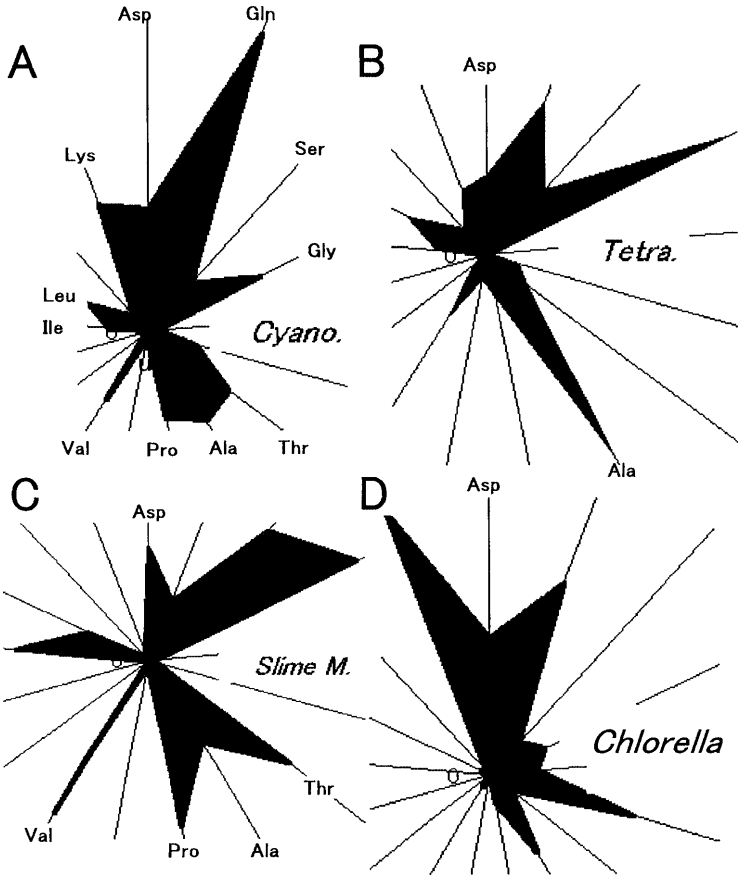

Fig. 4. Radar graphs of the free amino acid composition of prokaryotic and primitive eukaryotic cells, (**A**); Cyanobacteria, (**B**); *Tetrahymena*, (**C**); Slime mold, (**D**); *Chlorella*

Gram-negative bacteria (*E. coli*, *Proteus*, *V. alginoliticus* and *Klebsiella pneumoniae*) were almost identical (Sorimachi, 1999).

Archaea

In addition, the free amino acid composition was analyzed in archaeobacteria. In archaeobacteria, the concentration of glycine was very high, and the concentrations of glutamine, alanine, valine, isoleucine and leucine were relatively higher than those of other amino acids (Table 5 and Fig. 6A–D). The concentration of serine in *Methanococcus jannaschii* was higher than that of other archaeobacteria. The cellular amino acid composition of the 4 species of archaeobacteria differs from each other, but a similar basic pattern could be observed (Sorimachi et al., 2001).

Table 4. Free amino acid composition of eubacteria

Amino acid	*E. coli*	*Proteus*	*V. algino.*	*S. aureus*	*B. subtilis*
Asp	9.01 ± 0.04	8.00 ± 0.05	3.76 ± 0.08	15.81 ± 0.20	10.62 ± 0.13
Glu	25.84 ± 0.66	19.80 ± 0.12	38.62 ± 0.23	27.46 ± 0.22	45.61 ± 0.37
Ser	6.22 ± 0.17	9.52 ± 0.03	3.47 ± 0.02	1.77 ± 0.02	3.66 ± 0.02
Gly	20.35 ± 0.30	21.19 ± 0.16	23.17 ± 0.13	3.37 ± 0.04	13.28 ± 0.11
His	1.49 ± 0.04	0.58 ± 0.01	0.85 ± 0.03	0.23 ± 0.04	13.28 ± 0.11
Arg	1.22 ± 0.02	1.19 ± 0.01	0.64 ± 0.01	2.72 ± 0.00	0.98 ± 0.03
Thr	3.14 ± 0.08	4.30 ± 0.02	1.84 ± 0.02	0.90 ± 0.01	2.41 ± 0.02
Ala	6.54 ± 0.10	12.71 ± 0.04	5.41 ± 0.13	6.93 ± 0.02	6.71 ± 0.05
Pro	3.19 ± 0.08	4.34 ± 0.01	1.74 ± 0.05	0.96 ± 0.02	2.41 ± 0.07
Tyr	0.80 ± 0.08	0.57 ± 0.01	0.37 ± 0.01	0.13 ± 0.00	0.35 ± 0.03
Val	6.17 ± 1.11	3.57 ± 0.23	2.49 ± 0.05	2.06 ± 0.02	3.26 ± 0.01
Met	0.83 ± 0.03	0.76 ± 0.07	0.63 ± 0.11	0.15 ± 0.00	0.25 ± 0.01
Cys	0.43 ± 0.01	0.14 ± 0.01	0.00 ± 0.00	0.09 ± 0.00	0.11 ± 0.01
Ile	1.59 ± 0.04	2.16 ± 0.02	1.32 ± 0.02	0.87 ± 0.01	1.60 ± 0.02
Leu	2.59 ± 0.55	2.93 ± 0.04	3.48 ± 0.05	1.47 ± 0.02	2.21 ± 0.03
Phe	0.92 ± 0.07	0.85 ± 0.02	1.26 ± 0.02	0.55 ± 0.01	0.89 ± 0.08
Lys	9.66 ± 0.13	7.39 ± 0.04	10.96 ± 0.02	34.53 ± 0.15	5.17 ± 0.03

Each value is a percentage of total amino acids and the mean ± S.D. of three samples.

Table 5. Free amino acid composition of archaea bacteria

Amino acids	*A. fulgidus*	*P. horikoshii*	*M. thermo*	*M. jannashii*
Asp	6.57	6.18	7.50	8.38
Glu	12.68	10.41	14.58	12.69
Ser	10.02	5.84	6.24	14.12
Gly	25.94	23.46	23.09	19.62
His	1.92	0.00	0.00	0.00
Arg	6.30	3.55	1.49	2.38
Thr	4.95	3.66	4.48	3.83
Ala	7.55	8.80	11.79	8.96
Pro	0.00	0.00	0.00	0.00
Tyr	0.55	2.05	0.00	0.87
Val	5.04	8.19	10.77	10.40
Met	0.32	8.13	2.41	1.00
Cys	0.00	0.00	0.00	0.00
Ile	3.10	5.59	5.57	4.64
Leu	4.08	7.35	6.69	6.21
Phe	3.25	2.59	1.59	2.43
Lys	7.74	4.19	3.80	4.47

Each value is a percentage of total amino acids and the mean of two samples.

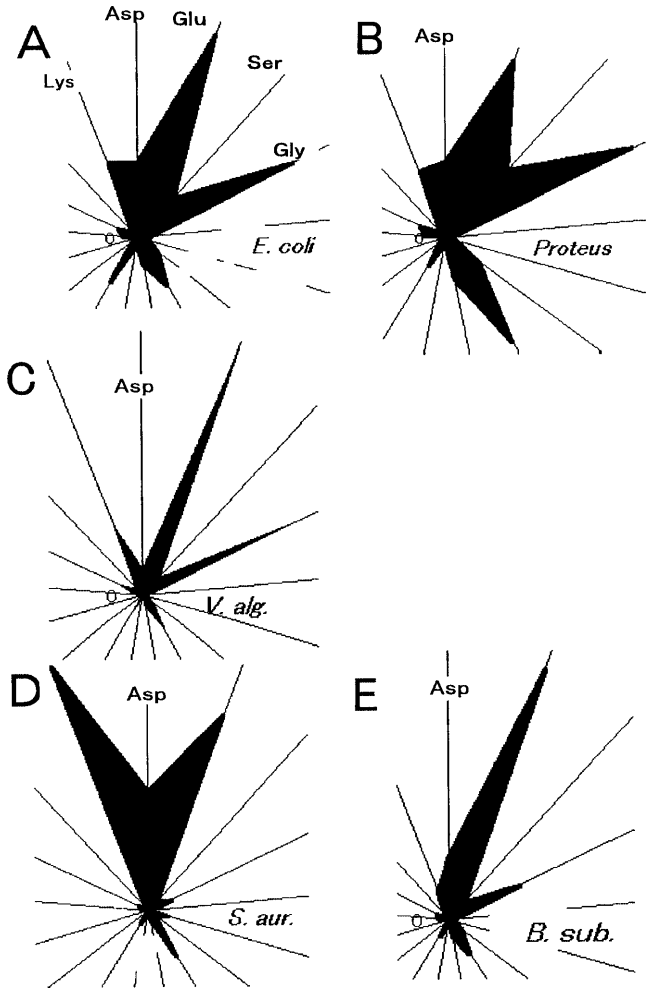

Fig. 5. Radar graphs of the free amino acid composition of eubacteria. (**A**); *E. coli*, (**B**); *Proteus*, (**C**); *V. alginolyticus*, (**D**); *S. aureus*, (**E**); *B. subtilis*

Plants

Three species of plant, carrot, *Torenia fournieri* and *Cymbidium* were examined. Their free amino acid compositions are quite different (Table 6 and Fig. 7). In addition, also the cellular amino acid composition differs from each other, but the basic pattern was again similar (Sorimachi et al., 2000).

Discussion

The present study together with a previous one (Okayasu et al., 1997) shows that the concentration of free amino acids contained in the cells is negligible compared to the concentration of cellular amino acids when whole cells are used for amino acid analysis. The free amino acids account for less than 10% of the total cellular amino acids. As this value is distributed among 17 amino

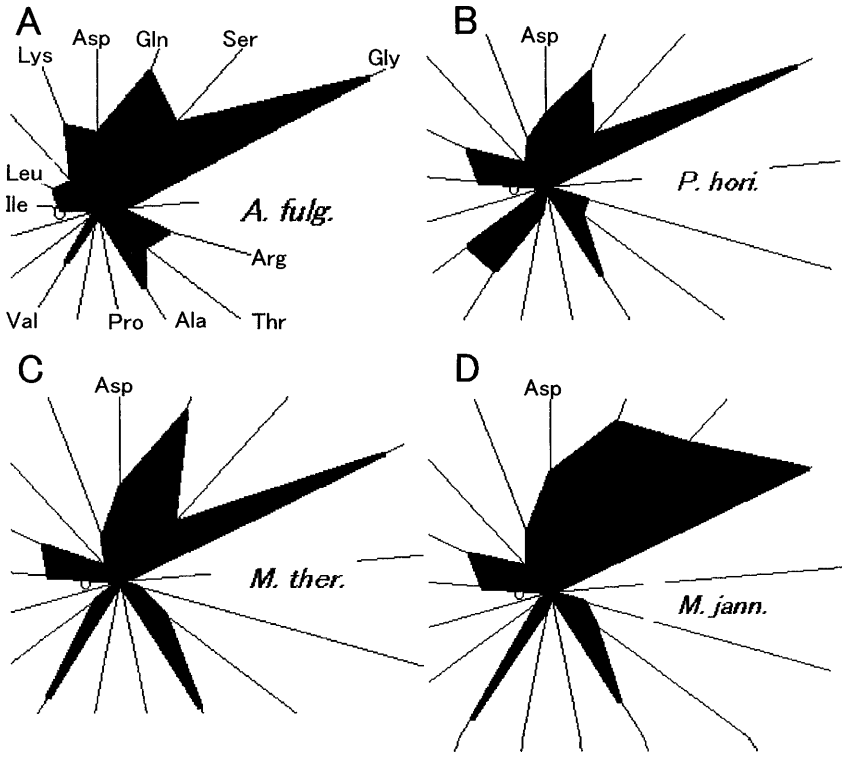

Fig. 6. Radar graphs of the free amino acid composition of archaea. (**A**); *Archaeoglobus flugidus*, (**B**); *Pyrococcus horikoshii*, (**C**); *Methanobactrium thermoautotrophicum*, (**D**); *Methanococcus jannashii*

Table 6. Free amino acid composition of plant cells

Amino acids	Carrot	*Torenia fournieri*	*Cymbidium*
Asp	8.60 ± 0.07	4.24 ± 0.07	61.24 ± 0.11
Glu	42.99 ± 0.19	81.85 ± 0.33	19.39 ± 0.22
Ser	4.29 ± 0.03	0.97 ± 0.01	3.28 ± 0.02
Gly	4.34 ± 0.05	1.63 ± 0.02	3.91 ± 0.03
His	10.57 ± 0.25	0.88 ± 0.01	0.21 ± 0.00
Arg	10.62 ± 0.04	3.92 ± 0.06	0.74 ± 0.00
Thr	1.46 ± 0.02	0.72 ± 0.02	1.07 ± 0.00
Ala	8.85 ± 0.09	1.27 ± 0.03	4.70 ± 0.01
Pro	1.36 ± 0.12	0.62 ± 0.18	0.92 ± 0.16
Tyr	0.00 ± 0.00	0.23 ± 0.01	0.00 ± 0.00
Val	2.98 ± 0.15	0.71 ± 0.01	1.62 ± 0.02
Met	0.64 ± 0.03	0.29 ± 0.10	0.00 ± 0.00
Cys	0.14 ± 0.13	0.12 ± 0.01	0.00 ± 0.00
Ile	0.72 ± 0.01	0.51 ± 0.01	0.61 ± 0.01
Leu	1.07 ± 0.01	0.78 ± 0.01	0.77 ± 0.01
Phe	0.17 ± 0.02	0.44 ± 0.04	0.48 ± 0.00
Lys	1.20 ± 0.02	0.82 ± 0.00	1.08 ± 0.01

Each value is a percentage of total amino acids and the mean ± S.D. of three samples.

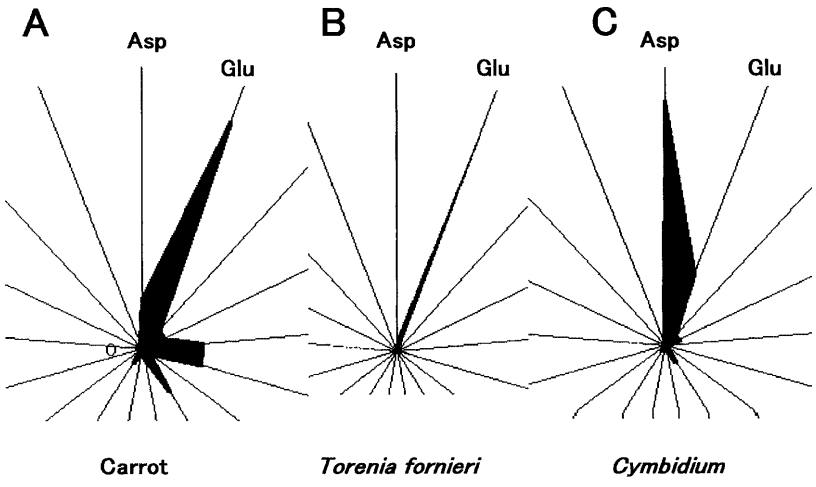

Fig. 7. Radar graphs of the free amino acid composition of plants. (**A**); carrot, (**B**); *Toreani fournieri*, (**C**); *Symbidium* sp.

acids, the contribution of the free amino acids to each cellular amino acid becomes virtually negligible. Thus, the amino acid composition of the whole cells represents the cellular amino acid composition based on cellular proteins. Therefore, our previous conclusions obtained from amino acid analysis of whole cells are verified.

However, the effect of free amino acids on cellular amino acid composition should be considered, when the concentration of a limited number of free amino acid is extremely high. For example, only glutamic acid or aspartic acid was observed in the plants, carrot, *Torenia fournieri* and *Cymbidium*. In these samples, cellular proteins and free amino acids should be separated before analyzing cellular amino acids in order to reduce the effect of free amino acids.

Using R-Y121B cells, we have shown that different media did not alter the free amino acid composition (Table 2). Thus, the acute medium change does not affect the free amino acid composition in cultured cells. This means that cell functions controlling free amino acid composition does not change within a short period.

The free amino acid compositions in R-Y121B, HUB, HuL and VERO cells differed from each other (Tables 1–2 and Figs. 1–3), although the cellular amino acid compositions of whole cells were quite similar (Okayasu et al., 1997; Sorimachi, 1999). In addition, although HuL and VERO cells were cultured in the same medium, the free amino acid compositions were different. It is likely that the free amino acid composition is tightly controlled by the cell function and is therefore more sensitive to biological changes as the result of evolution than the cellular amino acid composition of proteins. This may be an explanation for different free amino acid compositions among mammalian cells analyzed in the present study. In fish cells (GAKS), not only the free amino acid composition (present study), but also the cellular amino acid composition (Sorimachi, 1999) differed slightly from those of mammalian

cells. Futhermore, in addition to different cellular amino acid composition in primitive eukaryotes such as Tetrahymena, Chlorella and slime mold (Sorimachi, 1999), great differences in the free amino acid composition were also detected in these organisms in the present study. Thus, it seems that changes in amino acid composition are more pronounced in free amino acids than in protein amino acids.

Prokaryotes appear more closely related to primitive forms of life and might retain cell functions strongly linked to environmental conditions. Comparing free amino acid compositions between archaeobacteria and eubacteria, an important finding is that concentrations of hydrophobic amino acids such as alanine, valine, isoleucine and leucine were much lower in eubacteria (Fig. 5A–E). Archaeobacteria might conserve their characteristics due to hydrophobic proteins suitable for self-aggregation by hydrophobic force at low concentrations in a primitive ocean. These hydrophobic amino acids may need to be preserved in primitive life forms. This characteristic of archaeobacteria is also conserved in codon usages for leucine, isoluecine, valine and alanine (Klenk et al., 1997; Kawarabayasi et al., 1998; Smith et al., 1997; Bult et al., 1996). The present study shows that eubacteria and archaeobacteria are classified into two different groups based on the free amino acid composition.

The free amino acid compositions of plant cells (carrot, *Torenia fournieri* and *Symbidium*), differed markedly from those of other organisms. Glutamic acid accounted for more than 80% of free amino acids in *Torenia fournieri*. Extremely high concentrations of glutamic acid or aspartic acid characterize plant cells.

The results of the present study indicate that glutamic acid is commonly one of the main amino acids among the free amino acids of archaeobacteria, eubacteria and plants, and that hydrophobic amino acids such as alanine, valine, leucine and isoleucine are relatively high only in archaeobacteria. The free amino acid compositions of plants differ markedly, not only from each other, but also from the mammalian cells. In particular, the free amino acid compositions differ greatly between plant and mammalian cells, and between eubacteria and archaeobacteria. Thus, the free amino acid composition seems to reflect changes in biological evolution.

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