A novel 6Fe (2 × [3Fe–4S]) ferredoxin from *Mycobacterium smegmatis*

Takeo Imai[a,*], Akio Urushiyama[a], Hiroshi Saito[a], Yuki Sakamoto[a], Katsuya Ota[a], Daijiro Ohmori[b]

[a]*Department of Chemistry, Rikkyo (St. Paul's) University, 3-34-1 Nishi-Ikebukuro, Toshima-ku, Tokyo 171, Japan*
[b]*Department of Chemistry, Juntendo University, 1-1 Hiragagakuendai, Inba, Chiba 270-16, Japan*

Received 11 April 1995; revised version received 23 May 1995

Abstract A novel ferredoxin was purified from *Mycobacterium smegmatis* by a series of hydrophobic chromatographies in the presence of high concentrations of ammonium sulfate and sodium chloride. The ferredoxin exhibited the same peptide map and N-terminal amino acid sequence as the known 7Fe ferredoxin from the same bacterium. On the other hand, this ferredoxin was found to contain ~6 Fe/mol ferredoxin and was also shown to contain only [3Fe–4S] clusters by resonance Raman spectroscopy, indicating that it is a novel 6Fe ferredoxin which contains two [3Fe–4S] clusters.

Key words: 6Fe ferredoxin; *Mycobacterium smegmatis*; [3Fe–4S]; Raman

1. Introduction

Ferredoxins are usually small acidic proteins which contain one or two iron–sulfur clusters. Iron–sulfur clusters, prosthetic groups of ferredoxins, such as [2Fe–2S] or [4Fe–4S], act mainly as electron carriers in redox reactions. In addition, some [3Fe–4S] clusters have recently been shown to participate in non-redox reactions in several enzymes [1–3].

A *Mycobacterium smegmatis* ferredoxin has been identified as a 7Fe ferredoxin containing both a [3Fe–4S] cluster and a [4Fe–4S] cluster per molecule by several physicochemical techniques such as ^{1}H NMR, resonance Raman, and EPR spectroscopies [4–6], though its physiological role is still unknown. The similar 7Fe ferredoxins have also been isolated from several bacteria such as *Azotobacter vinelandii* [7], *Pseudomonas ovalis* [8] and *Thermus thermophilus* [9], and *Streptomyces griseus* [10].

In this paper, we report the purification and characterization of a second ferredoxin from *M. smegmatis* crude extract. This novel ferredoxin, which was characterized as a 6Fe (2 × [3Fe–4S]) ferredoxin by the physicochemical techniques such as iron content estimation and resonance Raman spectroscopy, was obtained by purification only in the presence of high concentrations of ammonium sulfate and sodium chloride.

2. Materials and methods

2.1. Materials

DE32 was obtained from Whatman Chemical Separation Ltd. DEAE-TOYOPEARL and TOYOPEARL HW65C were purchased from TOSOH and bovine serum albumin was obtained from Sigma. *Staphylococcus aureus* V8 protease [EC 3.4.21.19], trifluoroacetic acid, and 4-vinylpyridine were purchased from Wako Pure Chemicals. Tri-*n*-butylphosphine was obtained from Tokyo Kasei. All other reagents were of the highest grade available.

*Corresponding author. Fax: (81) (3) 5992 3434.

All operations described below were conducted at 0–5°C unless otherwise indicated.

2.2. Cultivation of Mycobacterium smegmatis

The bacterium was cultivated in static glycerol-pepton-bouillon medium as previously described [11].

2.3. Purification of ferredoxins

M. smegmatis cells (~500 g) were harvested on a Büchner funnel and washed with 1 l of 2.5 M ammonium sulfate/1 M NaCl/50 mM Tris-HCl (pH 8.0) (2.5 M AST buffer). Each 250 g portion of the washed cells was ground with quartz sand (500 g) for 2 hours and after the addition of 1.5 l of 2.5 M AST buffer, the cell-free extract was prepared by centrifugation at 12,500 × *g* for 30 min. DEAE-cellulose (DE32, ~25 ml suspended in 2.5 M AST bufler) was added to the supematant. After gentle stirring for 16 hours, the brown-colored DE32 was recovered by decantation. A column was prepared by packing new DE32 (100 ml) in the lower part and putting the brown DE32 (collected from three cultures) on top.

The constructed column (2.5 × 30 cm) was washed with 2 l of 2.0 M AST buffer. After elution of a yellow fluorescent substance, ferredoxin was eluted with 1.4 M AST buffer. The brown fractions were combined and after the concentration of ammonium sulfate was increased to 3.0 M by the addition of solid ammonium sulfate, the eluate was applied to a TOYOPEARL HW65C column (3.0 × 35 cm) which had been equilibrated with 3 M AST buffer. The column was washed with 500 ml of the same buffer and then eluted with 2.2 M AST buffer. Two brown fractions were collected. Each brown fraction was pooled and after increasing the ammonium sulfate concentration to 3 M, each combined fraction was applied separately to a DEAE-TOYOPEARL column (2.5 × 20 cm) which had been equilibrated with 3 M AST buffer. The column was washed with the same buffer and each ferredoxin was then eluted with 2.0 M AST buffer. The purified ferredoxins were concentrated by ultrafiltration using Amicon YM5 membranes and their media were replaced by 0.05 M Tris-HCl (pH 8.0)/0.15 M NaCl.

2.4. Purity of ferredoxins

The purified ferredoxins were analyzed by 30% polyacrylamide gel electrophoresis [12] and SDS-polyacrylamide gel electrophoresis [13].

2.5. Amino-terminal analysis

Automatic Edman degradation of the ferredoxins were accomplished on a gas vapor sequencer (Applied Biosystems model 470A). This instrument was interfaced with an on-line phenylthiohydantoin (PTH)-amino acid analysis system (Applied Biosystems model 120A). All sequencing and analysis programs were used as specified by the manufacturer.

2.6. Peptide map

Each ferredoxin was reduced with tri-*n*-butylphosphine and pyridylethylated with 4-vinylpyridine in 0.5 M Tris-HCl (pH 8.5) containing 7 M guanidine hydrochloride and 10 mM EDTA. Digestion with *S. aureus* protease V8 was performed in ammonium bicarbonate buffer, pH 7.8, with an enzyme/substrate ratio of 1:50. Cleavage time was 24 h at 37°C . The resulting peptides were separated by reversed-phase-HPLC on a TSK ODS-120T column (particle size 5 μm, 4.6 × 250 mm) with a linear gradient of acetonitrile. Elution profiles were obtained by monitoring the absorbance at 210 nm.

2.7. Iron content

Iron content estimation of the ferredoxins was performed by coloration of ferrous iron with *o*-phenanthroline [14] using a standard solution

SSDI 0014-5793(95)00601-X

prepared by dissolving metallic iron in diluting hydrochloric acid and diluted with 5% trichloroacetic acid. Each purified ferredoxin was precipitated by incubation at 0°C for 60 min with 5% trichloroacetic acid. After centrifugation for 10 min at 12,500 × *g*, the resultant supernatant solution was used for iron estimation.

2.8. Raman spectrum

Resonance Raman spectra were recorded by the use of an Ar^+ ion laser (457.9 nm) as described previously [6] except that the measurements were conducted at 77 K: the sample was immersed into a liquid nitrogen reservoir made up of quartz glass and the scattered light near 90° to the incident beam was collected. The spectral slit width was 6 cm^{-1} and a multiscan averaging technique was employed. Each spectrum shown is the sum of five scans.

2.9. EPR spectrum

EPR spectra were measured at 10K on a JEOL FE3XG spectrometer with a variable temperature cryostat (Air Products model LTR-3–110) for controling the sample temperature. Instrumental conditions were as follows: microwave frequency was 9.0 GHz, microwave power was 1 mW, and modulation amplitude was 0.25 mT. Spin concentrations were estimated by double integration of EPR signals using Cu-EDTA as a standard.

2.10. Others

The absorption spectra were recorded with a Hitachi 3600 recording spectrophotometer at ambient temperature. Estimation of protein concentration was done with Folin–Ciocalteu reagent [15] with bovine serum albumin as a standard: the protein precipitated with 5% trichloroacetic acid described in the 'Iron Content' section was dissolved in 10 mM potassium phosphate, pH 7.0, and used for the protein assay.

3. Results

3.1. Purification of M. smegmatis ferredoxins

Ferredoxins were purified according to the methods described in section 2 using three hydrophobic column chromatographies incorporating DEAE-cellulose (DE-32), TOYOPEARL, and DEAE-TOYOPEARL. Fig. 1 shows a typical separation profile of two *M. smegmatis* ferredoxins by the TOYOPEARL column. Two brown peaks (designated as Fd I′ and Fd I as shown in Fig. 1) were detected in the elution with decreasing ammonium sulfate concentration. Each peak exhibited UV-visible absorption spectra characteristic to bacterial-type ferredoxin. Each brown fraction was further purified by DEAE-TOYOPEARL column chromatography. The ferredoxins obtained by the chromatography were found to be

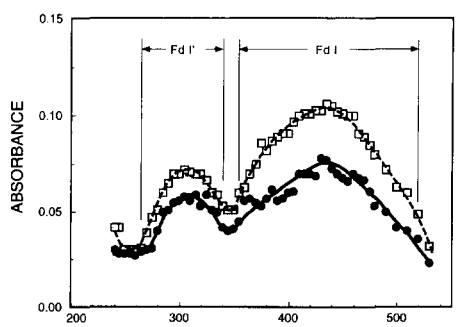

Fig. 1. Elution profile of column chromatography on a TOYOPEARL HW65C column in the presence of high concentrations of ammonium sulfate and sodium chloride. Column size was 3 × 35 cm. Two ferredoxins were eluted with 50 mM Tris-HCl, pH 8.0 containing 2.2 M ammonium sulfate/1 M NaCl. □, absorbance at 280 nm; ●, absorbance at 400 nm.

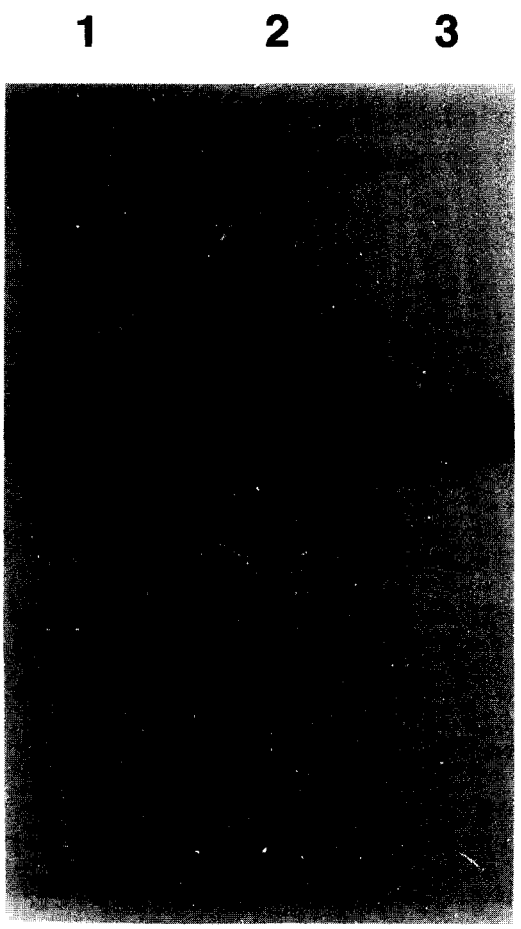

Fig. 2. Polyacrylamide gel electrophoresis of the isolated ferredoxins without SDS. The concentration of the gel was 30% acrylamide. Two μg of each protein were applied to the gel. After electrophoresis proteins were stained with Coomassie brilliant blue R-250. Lane 1; Fd I′, lane 2; Fd I′+ Fd I, lane 3; Fd I.

highly purified as judged by 30% native polyacrylamide gel electrophoresis (Fig. 2). In the electrophoresis, Fd I′ migrated slightly faster than Fd I: the Rf values of Fd I and Fd I′ were 0.43 and 0.46, respectively. A faint brown band appeared in the upper part in lane 1 and 2 disappeared by incubating the ferredoxin fraction with 5 mM dithiothreitol for 60 min at 20°C, indicating that this faint band may be a dimer linked with disulfide bridge. We also electrophoresed the purified ferredoxins in the presence of SDS and obtained the result that the two ferredoxins co-migrated with each other and also co-migrated with the known 7Fe ferredoxin which was described in the previous paper [4] (data not shown). The results of the electrophoresis indicate that Fd I′ has a different net charge or different molecular shape than Fd I. It was also concluded that Fd I is the known 7Fe ferredoxin from the above results, together with the several experimental data shown below.

3.2. Several differences among the two ferredoxins

The UV-visible absorption spectra of Fd I and Fd I′ are shown in Fig. 3. As well as both exhibited two peaks around 280 and 400 nm, some differences in spectral shape were observed: in Fd I′ two shoulders around 340 and 470 nm were clear, while in Fd I only a shoulder around 310 nm was distinct.

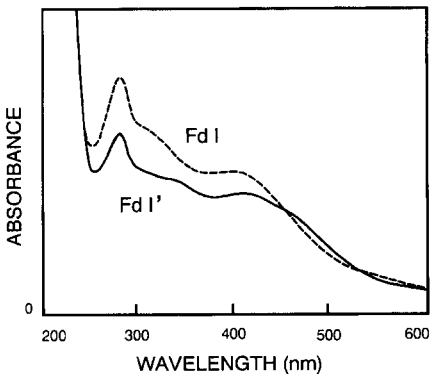

Fig. 3. UV-visible absorption spectra of the two purified ferredoxins. The ferredoxins were dissolved in 50 mM Tris-HCl, pH 8.0/0.15 M NaCl. The solid line indicates the spectrum of Fd I′ and the broken line indicates that of Fd I.

The latter spectral feature surely accords with that of the 7Fe ferredoxin [4]. This spectral difference indicates that there are some differences in the cluster-type and/or the microenvironment around the Fe–S clusters of these two ferredoxins.

We then investigated the resonance Raman spectra at 77 K to characterize the Fe–S clusters (Fig. 4). The spectrum of Fd I was quite similar to that of the 7Fe ferredoxin reported earlier [6]. That is, in the region of 300–400 cm^{-1}, five bands at 336, 347, 358, 368, and 388 cm^{-1} were observed in Fd I. However, only three bands at 348, 368 and 390 cm^{-1} were detected in Fd I′. According to the assignments of Johnson et al. [16], the bands at 336 and 358 cm^{-1} are those characteristic to 4Fe cluster even though these bands are observed commonly to be very weak. On the basis of this Raman spectroscopic evidence, it can be concluded that Fd I′ contains only the 3Fe cluster type, whereas Fd I contains both a 3Fe and a 4Fe clusters.

To identify the number of the iron-sulfur clusters in the isolated ferredoxins, iron and protein estimations were done by the methods described in section 2. The Fe/ferredoxin molar ratios measured were 6.2 and 7.4 for Fd I′ and Fd I, respectively. This result suggests that Fd I is a 7Fe ferredoxin and Fd I′ is a 6Fe ferredoxin, respectively.

The EPR spectra of Fd I′ exhibited a typical [3Fe–4S] cluster signal around $g = 2.01$. The temperature dependence and the power saturation profiles of Fd I′ were quite similar to those of the co-purified 7Fe ferredoxin, Fd I [17]. The spin concentrations of Fd I and Fd I′ were estimated to be 0.84 and 1.62, respectively. These EPR results also support that Fd I′ is a two [3Fe–4S] cluster-containing ferredoxin.

3.3. Similarity of peptides in Fd I and Fd I′

The N-terminal amino acid sequences were determined by automatic Edman degradation to examine the similarity of the peptide parts of the purified ferredoxins. The results showed that the sequences of the two isolated ferredoxins were the same from the N-terminus to the 13th amino acid except for the 8th amino acid residue which was not detected. In addition, the obtained sequences are the same as that of the known 7Fe ferredoxin from *M. smegmatis* except for the cysteine residue at position 8 from the N-terminus [9]. For further characterization of the peptide parts of Fd I and Fd I′, peptide maps for the two ferredoxins were constructed according to the methods described in section 2 by using the pyridylethylated ferredoxin hydrolyzed by *S. aureus* protease V8. The elution patterns of the digested peptides from the HPLC column indicate that the peptide parts of the two ferredoxins are the same within experimental error (Fig. 5).

4. Discussion

From the evidences described in the preceeding sections, it is clear that a new 6Fe ferredoxin is present in the extract of *M. smegmatis* in the medium with high concentrations of ammonium sulfate and sodium chloride. All spectroscopic data indicated that the present 6Fe ferredoxin contains two [3Fe–4S] clusters unlike the prismatic 6Fe protein from *Desulfovibrio desulfuricans* which contains a single [6Fe–6S] cluster [18]. At the present time, we have no direct evidence that this 6Fe ferredoxin was produced within the cells, though it had the ability to replace spinach ferredoxin in the reduction of cytochrome *c* with spinach ferredoxin-$NADP^+$ reductase and NADPH [19]. In fact, several reports have appeared in which conversion of 4Fe clusters into 3Fe clusters by the action of potassium ferricyanide [20–22] or by exposure in air [23–25] was mentioned. In this study the purification of the ferredoxins were conducted in air. Therefore, it is neccesary to inquire as to whether the conversion of the [4Fe–4S] cluster to a [3Fe–4S] cluster might be involved during the purification. We examined this type of conversion for the 7Fe ferredoxin of *M. smegmatis* in the aqueous medium with high concentrations of ammonium

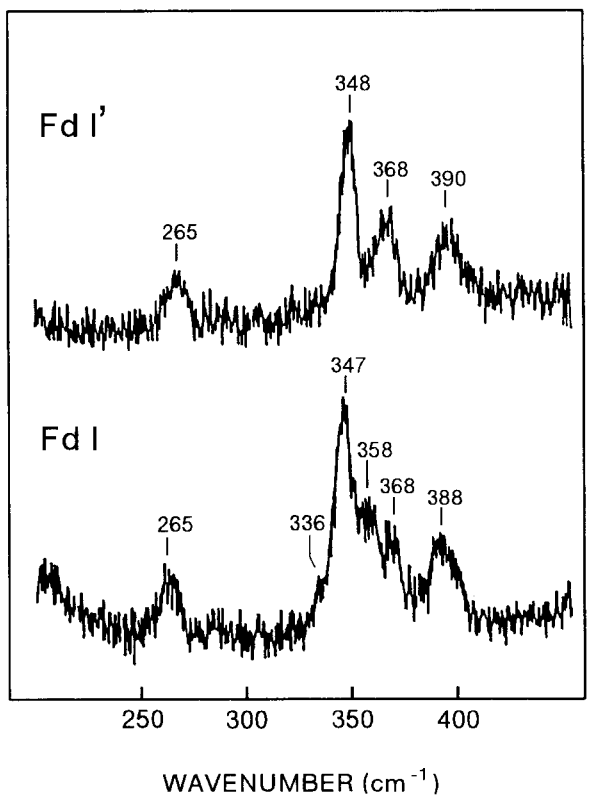

Fig. 4. Resonance Raman spectra of the purified ferredoxins. Spectra were recorded using 457.9 nm Ar^+ ion laser excitation (40 mW). The spectral band width was 6 cm^{-1}. Upper spectrum; Fd I′ and lower spectrum; Fd I. Ferredoxin concentrations were ~10 mM in 50 mM Tris-HCl, pH 8.0/0.15 M NaCl.

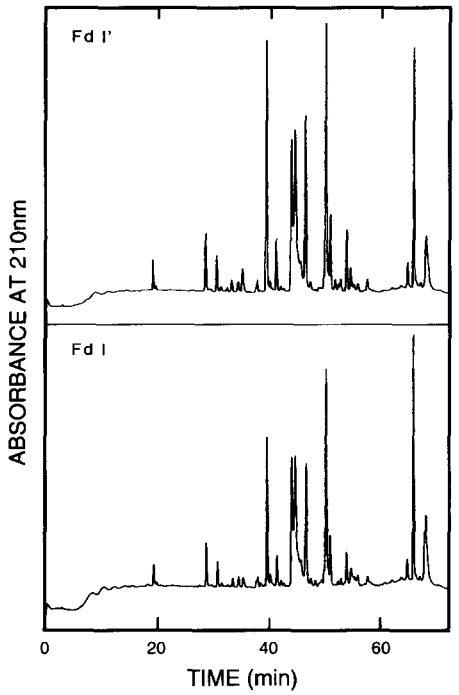

Fig. 5. HPLC elution profiles of peptides resulting from *S. aureus* protease digested ferredoxins. The column material was TSK ODS-120T. Eluent: (A) 0.06% trifluoroacetic acid in water, (B) 0.052% trifluoroacetic acid/80% CH_3CN. The gradient was from 2% B to 38% B in 60 min, followed by 38% B to 75% B in 30 min. The flow rate was 0.5 ml/min.

sulfate and sodium chloride. However, no such conversion could be evidenced.

Acknowledgements: The authors wish to express sincere gratitude to Dr. T. Taniguchi of Kochi Medical School for his analysis of the N-terminal amino acid sequences and also to Dr. Joseph A Gardner for reading the manuscript.

References

[1] Rouault, T.A., Hail, D.J., Downey, W.E., Philpott, C.C., Tang, C., Samaniego, F., Chin, J., Paul, I., Orloff, D., Harford, J.B. and Klausner, R.D. (1992) BioMetals 5, 131–140.
[2] Grabowski, R., Hofmeister, A.E. and Buekel, W. (1993) Trends Biochem. Sci., 18, 131–140.
[3] Hidalgo, E. and Demple, D. (1994) EMBO J. 13, 138–146.
[4] Imai, T., Matsumoto, T., Ohta, S., Ohmori, D., Suzuki, K., Tanaka, J., Tsukioka, M. and Tobari, J. (1983) Biochim. Biophys. Acta 743, 91–97.
[5] Nagayama, K., Ohmori, D., Imai, T. and Oshima, T. (1983) FEBS Lett. 158, 208–212.
[6] Imai, T., Saito, H., Tobari, J., Ohmori, D. and Suzuki, K. (1984) FEBS Lett. 165, 227–230.
[7] Howard, J.B., Lorsbach, T.W., Ghosh, D., Melis, K. and Stout, C.D. (1983) J. Biol. Chem. 258, 508–522.
[8] Hase, T., Wakabayashi, S., Matsubara, H., Ohmori, D. and Suzuki, K. (1978) FEBS Lett. 91, 315–319.
[9] Hase, T., Wakabayashi, S., Matsubara, H., Imai, T., Matsumoto, T. and Tobari, J. (1979) FEBS Lett. 103, 224–228.
[10] Trower, M.K., Marshall, J.E., Doleman, M.S., Emptage, M.K. and Sima Sariaslani, F. (1990) Biochim. Biophys. Acta 1037, 290–296.
[11] Kimura, T. and Tobari, J. (1963) Biochim. Biophys. Acta 73, 399–405.
[12] Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427.
[13] Weber, K. and Osbom, M. (1969) J. Biol. Chem. 244, 4406–4412 21.
[14] Massey, V. (1957) J. Biol. Chem. 229, 763–770.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Johnson, M.K., Czernuszewicz, R.S., Spiro, T.G., Fee, F.A. and Sweeney, W.V. (1983) J. Am. Chem. Soc., 105, 6671–6678.
[17] Ohmori, D., Yamakura, F., Suzuki, K., Imai, T. and Nagayama, K. (1986) in: Iron-Sulfur Protein Research (Matsubara, H., Katsube, Y. and Wada, K., Eds.) pp. 116–124, Japan Scientific Societies Press, Tokyo, Springer/Verlag, Berlin.
[18] Moura, I., Tavares, P. Moura, J.J.G., Ravi, N., Huynh, B.H., Liu, M.-Y. and LeGall, J. (1992) J. Biol. Chem. 267, 4489–4496.
[19] Buchanan, B.B. and Amon, D.I. (1971) Methods Enzymol. 23A, 413–439.
[20] Johnson, M.K., Spiro, T.G. and Mortenson, L.E. (1982) J. Biol. Chem. 257, 2447–2452.
[21] Moura, J.J. G., Moura, I., Kent, T.A., Lipscomb, J.D., Huynh, B.H., LeGall, J., Xavier, A.V. and E. Munck, (1982) J. Biol. Chem. 257, 6295–6267.
[22] Nagayama, K., Imai, T, Ohmori, D. and Oshima, T. (1984) FEBS Lett. 169, 79–84.
[23] Beinert, H., Emptage, M.H., Dreyer, J.L., Scott, R.A., Hahn, J.E., Hodgson, K.O. and Thomson, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 393–396.
[24] Guigliarelli, B., Bertrand, P., Mare, C., Paparassiliou, P., Hatehikian, E.C. and Gayda, J.P. (1986) Biochim. Biophys. Acta 810, 319–324.
[25] Morgan, T.V., Stephen, P.J., Burgess B.K. and Scott, C. D. (1984) FEBS Lett. 167, 137.