Firefly luciferase is a bifunctional enzyme: ATP-dependent monooxygenase and a long chain fatty acyl-CoA synthetase

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Received 11 February 2003; revised 10 March 2003; accepted 12 March 2003

First published online 21 March 2003

Edited by Judit Ovádi

Abstract **Firefly luciferase can catalyze the formation of fatty acyl-CoA via fatty acyl-adenylate from fatty acid in the presence of ATP, Mg^{2+} and coenzyme A (CoA). A long chain fatty acyl-CoA (C_{16}–C_{20}), produced by luciferase from a North American firefly (*Photinus pyralis*) and a Japanese firefly (*Luciola cruciata*), was isolated and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. Of a number of substrates tested, linolenic acid ($C_{18:3}$) and arachidonic acid ($C_{20:4}$) appear to be suitable for acyl-CoA synthesis. This evidence suggests that firefly luciferase within peroxisomes of the cells in the photogenic organ may be a bifunctional enzyme, catalyzing not only the bioluminescence reaction but also the fatty acyl-CoA synthetic reaction.**
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Key words: Bioluminescence; Luciferin; CoA ligase; Adenylation; Intermediate; β-Oxidation

1. Introduction

Firefly luciferase (EC 1.13.12.7) is a well-characterized enzyme that is responsible for the bioluminescence reaction. It catalyzes the oxidation of firefly luciferin with molecular oxygen in the presence of ATP and Mg^{2+} to emit yellow-green light [1–4]. The initial reaction catalyzed by firefly luciferase is the formation of luciferyl adenylate with the release of inorganic pyrophosphate. The luciferase-bound luciferyl adenylate reacts rapidly with molecular oxygen to give light, CO_2, AMP and oxyluciferin (Fig. 1A). When excess luciferin and ATP are added to the reaction mixture of luciferase, a flash of light is observed and then a rapid inhibition of luminescence occurs. The addition of coenzyme A (CoA) to the reaction mixtures prevents this rapid inhibition [5]. In 1967, McElroy et al. [6] suggested that a luciferase from North American firefly, *Photinus pyralis*, is remarkably similar in catalytic mechanism to amino acyl-tRNA synthetase and fatty acyl-CoA synthetase. After two decades, the primary structure of firefly luciferase was determined by cDNA cloning [7] and significant sequence homology of *P. pyralis* luciferase to plant *p*-coumarate:CoA ligase (EC 6.2.1.12) [8] and rat long chain acyl-CoA synthetase (EC 6.2.1.3) [9] was reported. Further, conserved sequence motifs characteristic of adenylate formation were proposed in firefly luciferase [10], based on comparison with several adenylate-forming enzymes including *p*-coumarate:CoA ligase, fungal acetyl-CoA synthetase and rat long chain acyl-CoA synthetase. At the present time, over 12 cDNAs encoding luciferase from various firefly species have been obtained [11] and the crystal structure of *P. pyralis* luciferase has been determined by X-ray analysis [12]. However, the structure–function relationship for the luminescence reaction has not been well resolved [13,14]. Recently, it was reported that firefly luciferase can synthesize the adenosine(5′)tetraphospho(5′)adenosine (AppppA) from dehydroluciferyl adenylate, ATP and Mg^{2+}, and the formation of AppppA was inhibited by addition of CoA [15]. Furthermore, Ueda and Suzuki [16] reported that the long chain fatty acids (C_{12}–C_{20}) inhibited the light production in competition with firefly luciferin in the luciferase reaction.

With this background information, we have predicted the ability of firefly luciferase to synthesize fatty acyl-CoA via fatty acyl-adenylate as an intermediate from fatty acid in the presence of ATP, Mg^{2+} and CoA (Fig. 1B), and have now shown long chain fatty acyl-CoA synthetic activity in firefly luciferase.

2. Materials and methods

2.1. Materials

All chemicals were obtained from commercial sources: CoA trisodium salt, GTP, UTP, sodium acetate, linoleic acid and ferulic acid (Wako Pure Chemicals, Osaka, Japan); propionic anhydride (Tokyo Kasei Kogyo, Tokyo, Japan); oleoyl-CoA, arachidonic acid sodium salt, *p*-coumaric acid, caffeic acid, 3-hydroxypicolinic acid, CTP, TTP, and ITP (Sigma, St. Louis, MO, USA); oleic acid sodium salt (Aldrich, Milwaukee, WI, USA); firefly luciferin sodium salt (Nacalai Tesque, Kyoto, Japan); palmitic acid sodium salt (Kanto Chemical, Tokyo, Japan); linolenic acid (NOF, Tokyo, Japan); ATP and AMP (Oriental Yeast, Osaka, Japan); recombinant firefly luciferases from *P. pyralis* (Promega, Madison, WI, USA) and *Luciola cruciata* (Wako); recombinant aequorin (Chisso, Tokyo, Japan); acyl-CoA synthetase from *Pseudomonas* sp. (Funakoshi, Tokyo, Japan); [α-^{32}P]ATP (3000 Ci/mmol) and [1-^{14}C]oleic acid (56.0 mCi/mmol) from NEN Life Science Products (Boston, MA, USA) and Amersham Pharmacia Biotech (Piscataway, NY, USA), respectively.

2.2. Assay for acyl adenylation activity using [α-^{32}P]ATP by TLC analysis

The reaction mixture (20 μl) contained [α-^{32}P]ATP (0.33 μCi = 5.6 nM), CoA (250 μM), $MgCl_2$ (5 mM), substrate (10 μM) and recombinant *P. pyralis* firefly luciferase (0.25 μg = 0.2 μM) in 100 mM Tris–HCl (pH 7.8). The reaction was started by adding luciferase and in-

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Abbreviations: CoA, coenzyme A; HPLC, high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; TLC, thin-layer chromatography

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doi:10.1016/S0014-5793(03)00272-2

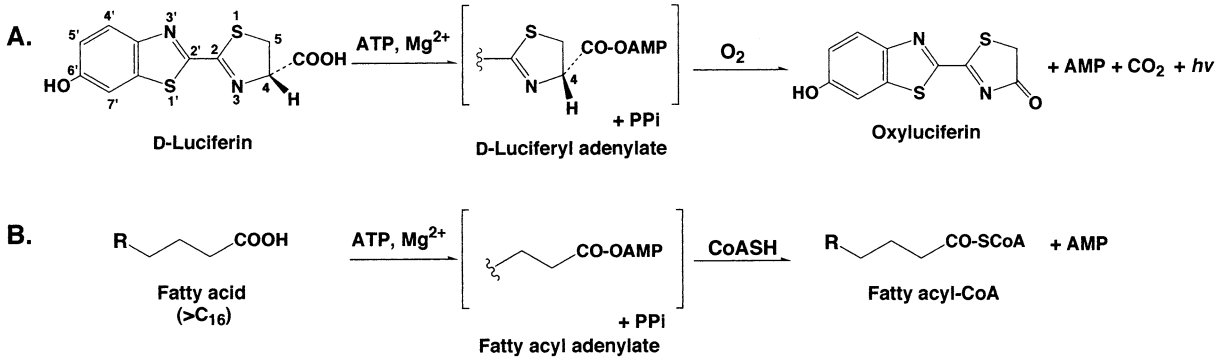

Fig. 1. Schematic representation of bioluminescence reaction (A) and fatty acyl-CoA synthetic reaction (B), catalyzed by firefly luciferase.

cubated at 30°C for 30 min. After adding 20 μl of ethanol to the reaction mixture, 2 μl of the reaction solution was spotted on thin-layer chromatography (TLC) plates (Silica gel 60 F_{254}, Merck). First development was performed in dioxane/50 mM acetic acid (4:1), followed by second development in dioxane/ammonium hydroxide/water (6:1:5) at the same level as the first development. The R_f values of [α-^{32}P]ATP and [α-^{32}P]AMP were identified to 0.17 and 0.51, respectively, comparing with the R_f values for authentic samples of ATP and AMP under a UV lamp at 254 nm. The radioactivity of [α-^{32}P]AMP was measured using an image analyzer (Fuji Film; a model BAS 2500) for 30 min exposure and was obtained by subtracting the background value. The intensity was calculated as a percentage of that produced with firefly luciferin as a substrate under the same reaction conditions and the data were analyzed by Student's *t*-test.

2.3. Requirements for oleoyl-CoA synthesis in firefly luciferase reaction

The reaction mixture (20 μl) contained [1-^{14}C]oleic acid (19.2 nCi = 17.3 μM), ATP (250 μM), CoA (250 μM), $MgCl_2$ (5 mM) and recombinant *P. pyralis* firefly luciferase (1.27 μg = 1 μM) in 100 mM Tris–HCl (pH 7.8). The reaction was started by adding luciferase and incubated at 30°C for 60 min. After adding 20 μl of ethanol to the reaction mixture, 2 μl of the reaction solution was spotted on TLC plate (Silica gel 60 F_{254}, Merck) and was developed in dioxane/ammonium hydroxide/water (3:0.5:2). Oleoyl-CoA (R_f = 0.50) was identified using the authentic sample of oleoyl-CoA under a UV lamp at 254 nm. The radioactivity of [1-^{14}C]oleoyl-CoA was measured in a BAS 2500 (Fuji) and the relative intensity was obtained by subtracting the background value.

2.4. Isolation of fatty acyl-CoA by reversed-phase HPLC

The reaction mixture (400 μl) contained substrate (17.3 μM; palmitic acid, oleic acid, linoleic acid, linolenic acid or arachidonic acid), NTP (250 μM; ATP, GTP, CTP, TTP, UTP or ITP), CoA (250 μM), $MgCl_2$ (5 mM) and recombinant firefly luciferase (25.4 μg = 1 μM) from *P. pyralis* or *L. cruciata* in 100 mM Tris–HCl (pH 7.8). The enzyme reaction was started by addition of luciferase and incubated at 30°C for 60 min. The reaction was terminated by adding 267 μl of acetonitrile and centrifuged at 10 000 rpm for 5 min. The resultant supernatant was subjected to reversed-phase high performance liquid chromatography (HPLC) using a Capcell Pak C18 (4.6×150 mm; Shiseido, Japan) and with a linear gradient of 40–70% acetonitrile in 25 mM KH_2PO_4 from 5 to 17 min, followed by 70% acetonitrile for 8 min at a flow rate of 0.8 ml/min. The fractions from the column were monitored using a multi-wavelength detector (195–650 nm; MD-2010 plus, Jasco). Elution times of palmitoyl-CoA, oleoyl-CoA, linoleoyl-CoA, linolenoyl-CoA and arachidonoyl-CoA were 18.4 min, 19.1 min, 17.1 min, 14.8 min and 16.2 min, respectively.

2.5. Identification of fatty acyl-CoA by MALDI-TOF-MS

The peak fraction isolated from HPLC analysis was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with an AutoFLEX (Bruker Daltonics) using 3-hydroxypicolinic acid as a matrix. The data were acquired in the negative reflector mode of operation.

2.6. Kinetic analyses

The reaction mixture (500 μl) contained linolenic acid (0.1–100 μM), ATP (250 μM), CoA (250 μM), $MgCl_2$ (5 mM) and recombinant *P. pyralis* firefly luciferase (6.32 μg = 0.2 μM) in 100 mM Tris–HCl (pH 7.8). The reaction was started by adding luciferase and incubated at 30°C for 10 min. The product, fatty acyl-CoA, was measured by reversed-phase HPLC as described in Section 2.4. Since a strong inhibition for fatty acyl-CoA synthetic activity of firefly luciferase was observed at high concentrations (over 20 μM) of various fatty acids, the K_m, V_{max} and K_{cat} values for linolenic acid were determined at concentrations from 10 to 20 μM of fatty acids by the method of Lineweaver–Burk plots.

2.7. Luminescence assay

The reaction mixture (100 μl) contained substrate (10 μM; fatty acid or firefly luciferin), ATP (250 μM), CoA (250 μM) and $MgCl_2$ (5 mM) in 100 mM Tris–HCl (pH 7.8). The reaction was started by the addition of enzyme (1.27 μg, recombinant *P. pyralis* firefly luciferase; 2 μg, acyl-CoA synthetase from *Pseudomonas* sp.) at 25°C. The initial intensity of the luminescence was determined with an Atto (Tokyo, Japan) model AB-2200 luminometer. The detection limit of the luminometer corresponded to the light intensity from 1 pg of recombinant aequorin (4.8×10^{15} photons/mg protein) as a light source.

3. Results and discussion

In the first step, to screen a suitable substrate for acyl-CoA formation by *P. pyralis* firefly luciferase, the acyl-adenylate-mediated reaction was monitored by the formation of [α-^{32}P]AMP from [α-^{32}P]ATP using TLC analysis. In the presence of [α-^{32}P]ATP, Mg^{2+} and CoA, the following substrates were examined: acetic acid (C_2), propionic acid (C_3), palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$), linolenic acid ($C_{18:3}$) and arachidonic acid ($C_{20:4}$), *p*-coumaric acid and its derivatives. The results suggest that long chain unsaturated fatty acids are the most suitable substrate for the adenylation reaction (Fig. 2). Arachidonic acid, in particular, has ca. 50% efficiency of production of AMP in comparison with that using firefly luciferin as a substrate under the same conditions. Other substrates of aromatic acids for plant *p*-coumarate:CoA ligase and short chain acids were not utilized efficiently by firefly luciferase. The identification of fatty acyl-CoA formation via fatty acyl-adenylate was performed using [1-^{14}C]oleic acid as a substrate and oleoyl-CoA as an authentic sample. Based on TLC analysis, [1-^{14}C]oleoyl-CoA was a product and ATP, Mg^{2+} and CoA were the essential cofactors for the reaction (Table 1). Other nucleotides including GTP, CTP, TTP, UTP and ITP did not stimulate the formation of oleoyl-CoA significantly (data not shown). Furthermore, the oleoyl-CoA product was also isolated by reversed-phase HPLC. A single major peak showing the identical retention time to the authentic oleoyl-CoA was separated by HPLC (Fig. 3A). The peak fraction isolated was analyzed

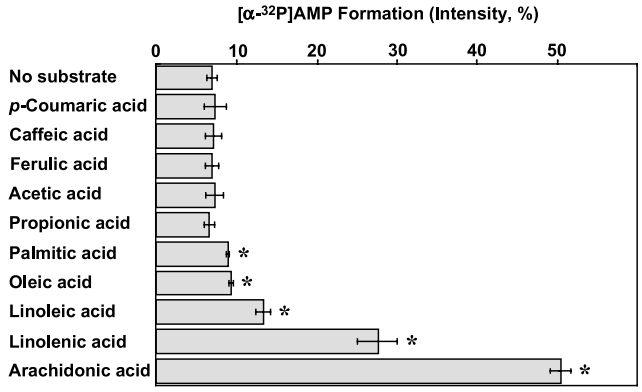

Fig. 2. Detection of acyl-adenylate formation by firefly luciferase. Acyl-adenylate formation was detected by the formation of [α-^{32}P]AMP from [α-^{32}P]ATP. The intensity of [α-^{32}P]AMP was calculated as a percentage of that produced with firefly luciferin as a substrate under the same reaction conditions. Horizontal bars in the shaded boxes represent the mean ± S.E.M. ($n = 3$). *$P < 0.05$ vs. 'No substrate'.

with MALDI-TOF-MS. The mass value obtained from the peak fraction was consistent with the predicted mass value of oleoyl-CoA (Fig. 3B) and the mass pattern of the product was also identical to that of authentic oleoyl-CoA. The CoA products formed by firefly luciferase with other fatty acids were also identified by the same procedure (Table 2). The conversion efficiency to fatty acyl-CoA from its fatty acid by *P. pyralis* luciferase was calculated from peak areas of HPLC analysis: 19.3% from palmitic acid, 52.8% from oleic acid, 63.3% from linoleic acid, over 98.0% from linolenic acid and over 98.0% from arachidonic acid under these reaction conditions. In the kinetic study on *P. pyralis* luciferase, the K_m and V_{max} values for linolenic acid were 13.6 μM and 0.127 μmol/min/mg protein, respectively. The K_{cat} value was calculated to 0.130/s. These values for linolenic acid were similar to the K_{cat} and K_m values for firefly luciferin ($K_{cat} = 0.125$/s, $K_m = 15$ μM) using recombinant *P. pyralis* luciferase [17].

Table 1
Requirements of cofactors for fatty acyl-CoA synthesis by firefly luciferase

Reaction conditions	[1-^{14}C]Oleoyl-CoA relative intensity (%)[a]
Control[b]	764 (100)
– Luciferase	0 (0)
– ATP	11 (1)
– CoA	9 (1)
– Mg^{2+}	23 (3)

[a]Radioactivity was determined as relative intensity using an image analyzer.
[b]Control contains the complete components of luciferase, ATP, CoA and Mg^{2+}.

Thus, firefly luciferase acts as an enzyme that catalyzes the fatty acyl-CoA synthesis. To determine whether fatty acyl-CoA synthetic activity is a general property of firefly luciferase, a Japanese firefly *L. cruciata* luciferase (60.4% amino acid sequence identity to *P. pyralis* luciferase) was also examined and fatty acyl-CoA products were identified. As summarized in Table 2, these results showed that firefly luciferase has the catalytic ability to synthesize a long chain fatty acyl-CoA from various long chain fatty acids.

From the viewpoint of the luminescence reaction, firefly luciferase did not give any luminescence in the presence of fatty acid, ATP, Mg^{2+} and CoA. On the other hands, an acyl-CoA synthetase from *Pseudomonas* sp. (EC 6.2.1.3) having fatty acyl-CoA synthetic activity did not show luminescence activity in the presence of firefly luciferin, ATP, Mg^{2+} and CoA, and also did not produce luciferyl-CoA from luciferin (data not shown). The difference between the luminescence reaction and the fatty acyl-CoA synthetic reaction by firefly luciferase would be explained as follows: luciferase catalyzes the adenylation at the carboxyl group in both firefly luciferin and a long chain fatty acid with ATP. This adenylation step is presumably by an identical mechanism. The luminescence reaction will start by removing the proton at the C4 position in luciferyl adenylate, followed by addition of molecular oxygen and then give light emission (Fig. 1A) [2–4]. In

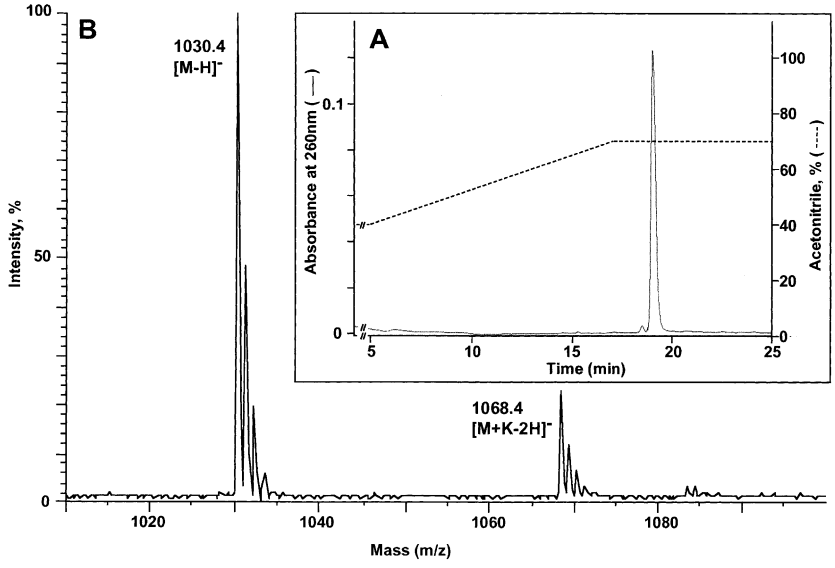

Fig. 3. Identification of oleoyl-CoA produced by firefly luciferase. A: Isolation of oleoyl-CoA by reversed-phase HPLC. The peak fraction at 19.1 min was subjected to MALDI-TOF-MS. B: MALDI-TOF-MS analysis of oleoyl-CoA produced by firefly luciferase. The signal at 1068.4 indicates the potassium adduct of oleoyl-CoA.

Table 2
Identification of fatty acyl-CoA produced by recombinant luciferase from *P. pyralis* and *L. cruciata* with MALDI-TOF-MS[a]

Product	Predicted $[M-H]^-$	Observed $[M-H]^-$	
		P. pyralis	*L. cruciata*
Palmitoyl-CoA	1004.3	1004.6	N.D.
Oleoyl-CoA	1030.4	1030.4	1030.4
Linoleoyl-CoA	1028.3	1028.4	1028.6
Linolenoyl-CoA	1026.3	1026.4	1026.6
Arachidonoyl-CoA	1052.3	1052.5	1052.6

N.D. = not determined.
[a]See Sections 2.4 and 2.5 for the details of reaction conditions and the procedures for determination of fatty acyl-CoA, respectively.

the case of fatty acyl-adenylate as an intermediate, fatty acyl-CoA is only produced in the presence of CoA in the same manner as other adenylate-mediated enzyme reactions (Fig. 1B) [9,10].

Considering the biological function of luciferase in the firefly, the enzyme is localized within peroxisomes in the cells of the photogenic organ [18,19], and the luciferase expressed in mammalian cells is distributed into peroxisomes. The signal sequence targeted into peroxisomes has also been identified at the carboxy-terminus of luciferase [20]. Since the peroxisome is the functional organelle for β-oxidation of long chain fatty acids [21], firefly luciferase could serve to synthesize a long chain acyl-CoA in the activation step leading to degradation of fatty acids. Our findings and the above evidence may suggest that firefly luciferase in peroxisomes keeps the catalytic functions in bioluminescence and fatty acid metabolism.

Acknowledgements: This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (A) from the Ministry of Education, Culture, Sports, Science and Technology.

References

[1] McElroy, W.D., Seliger, H.H. and White, E.H. (1969) Photochem. Photobiol. 10, 153–170.
[2] White, E.H., Rapaport, E., Seliger, H.H. and Hopkins, T.A. (1971) Bioorg. Chem. 1, 92–122.
[3] DeLuca, M. (1976) Adv. Enzymol. 44, 37–68.
[4] Wood, K.V. (1995) Photochem. Photobiol. 62, 662–673.
[5] Airth, R.L., Rhodes, W.C. and McElroy, W.D. (1958) Biochim. Biophys. Acta 27, 519–532.
[6] McElroy, W.D., DeLuca, M. and Travis, J. (1967) Science 157, 150–160.
[7] de Wet, J.R., Wood, K.V., DeLuca, M., Helinski, D.R. and Subramani, S. (1987) Mol. Cell. Biol. 7, 725–737.
[8] Schröder, J. (1989) Nucleic Acids Res. 17, 460.
[9] Suzuki, H., Kawarabayasi, Y., Kondo, J., Abe, T., Nishikawa, K., Kimura, S., Hashimoto, T. and Yamamoto, T. (1990) J. Biol. Chem. 265, 8681–8685.
[10] Toh, H. (1991) Protein Seq. Data Anal. 4, 111–117.
[11] Ye, L., Buck, L.M., Schaeffer, H.J. and Leach, F.R. (1997) Biochim. Biophys. Acta 1339, 39–52.
[12] Conti, E., Franks, N.P. and Brick, P. (1996) Structure 4, 287–298.
[13] Baldwin, T.O. (1996) Structure 4, 223–228.
[14] Branchini, B.R., Murtiashaw, M.H., Magyar, R.A., Portier, N.C., Ruggiero, M.C. and Stroh, J.G. (2002) J. Am. Chem. Soc. 124, 2112–2113.
[15] Fontes, R., Ortiz, B., de Diego, A., Sillero, A. and Sillero, M.A.G. (1998) FEBS Lett. 438, 190–194.
[16] Ueda, I. and Suzuki, A. (1998) Biophys. J. 75, 1052–1057.
[17] Branchini, B.R., Magyar, R.A., Murtiashaw, M.H. and Portier, N.C. (2001) Biochemistry 40, 2410–2418.
[18] Hanna, C.H., Hopkins, T.A. and Buck, J. (1976) J. Ultrastruct. Res. 57, 150–162.
[19] Keller, G.-A., Gould, S., DeLuca, M. and Subramani, S. (1987) Proc. Natl. Acad. Sci. USA 84, 3264–3268.
[20] Gould, S.J., Keller, G.-A., Hosken, N., Wilkinson, J. and Subramani, S. (1989) J. Cell Biol. 108, 1657–1664.
[21] Mannaerts, G.P. and Debeer, L.J. (1982) Ann. NY Acad. Sci. 386, 30–39.