Identification of triphosphoribosyl-dephospho-CoA as precursor of the citrate lyase prosthetic group

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Abstract The γ-subunit of citrate lyase (EC 4.1.3.6) contains the prosthetic group 2′-(5″-phosphoribosyl)-3′-dephospho-CoA and serves as an acyl carrier protein (ACP). We recently showed that in *Escherichia coli* the proteins CitG and CitX are essential for holo-ACP synthesis and provided evidence that CitG catalyzes the formation of a prosthetic group precursor from ATP and dephospho-CoA, which is subsequently attached via phosphodiester linkage to apo-ACP by CitX. Here we prove that CitG indeed catalyzes the conversion of ATP and dephospho-CoA to adenine and 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA, the predicted precursor of the prosthetic group. Furthermore, this precursor was transferred by CitX to apo-ACP, yielding holo-ACP. Thus, our proposed mechanism for holo-ACP synthesis could be verified.

Key words: Citrate lyase; 2′-(5″-Triphosphoribosyl)-3′-dephospho-CoA; Prosthetic group; Acyl carrier protein; CitG; CitX; *Escherichia coli*

1. Introduction

Citrate lyase catalyzed the Mg^{2+}-dependent cleavage of citrate to acetate and oxaloacetate. This reaction represents the initial step in all known bacterial citrate fermentation pathways [1–4]. Citrate lyase of the enterobacterial species *Klebsiella pneumoniae* consists of three different subunits α (55 kDa), β (32 kDa) and γ (11 kDa). The native enzyme is composed of six copies of each of the three subunits, forming a 550 kDa complex [5,6]. Whereas the α- and the β-subunits possess catalytic activity, the γ-subunit serves as an acyl carrier protein (ACP) [7]. It carries the prosthetic group 2′-(5″-phosphoribosyl)-3′-dephospho-CoA (Fig. 1) which is attached via phosphodiester linkage to serine-14 in the enzymes from *K. pneumoniae* [8–12] and *Escherichia coli* [13]. As a prerequisite for catalytic activity, the thiol group of the enzyme-bound CoA derivative has to be converted to the acetyl-thioester derivative by the action of citrate lyase ligase (EC 6.2.1.22) [14,15]. The α-subunit then catalyzes the exchange of the acetyl group with a citryl group, forming citryl-ACP and acetate. The β-subunit subsequently mediates the Mg^{2+}-dependent conversion of citryl-ACP into acetyl-ACP and oxaloacetate [16].

In *K. pneumoniae*, the genes encoding the γ- (CitD), β- (CitE) and α-subunit (CitF) of citrate lyase are part of the *citCDEFG* operon, with *citC* encoding citrate lyase ligase [17]. This operon is induced under anoxic conditions in the presence of citrate and Na^+ ions. Its expression is strictly dependent on the CitA/CitB two-component regulatory system [18–20]. In *E. coli*, a similar gene cluster was identified, *citCDEFXG* [21], which differs by the presence of an additional gene (*citX*) [22]. Like in *K. pneumoniae*, expression of the *E. coli citC* operon appears to be regulated by the CitA/CitB (DpiB/DpiA) two-component system [23].

We could recently demonstrate [13] that the formation of holo-citrate lyase is dependent on CitG (33 kDa) and CitX (20 kDa). Expression of the *K. pneumoniae citCDEFG* genes in *E. coli* resulted in the formation of an inactive citrate lyase, because only the apo-form of the ACP was formed [17]. Coexpression of the *E. coli citX* gene could complement this deficiency and led to the formation of an active holo-citrate lyase [13]. Depending on the presence or absence of CitX and CitG, three different-sized ACP forms could be detected in vivo. Holo-ACP was only synthesized if CitX and CitG were present, whereas in the absence of the two proteins or of CitX alone, apo-ACP was formed. If CitG was absent, but CitX present, an intermediate-sized ACP was found that was identified as adenylylated ACP with the AMP residue attached to serine-14. The deduced function of CitX as an apo-ACP nucleotidyl transferase (EC 2.7.7) could be verified in vitro, where the enzyme also accepts CTP, GTP and UTP as substrates [13].

To test whether AMP-ACP is an intermediate in the conversion of apo- into holo-ACP, an in vitro system was established that allowed the formation of holo-ACP with purified CitG and CitX as catalysts and apo-ACP, ATP and dephospho-CoA as substrates [13]. In this process, ATP could not be substituted by other nucleotides. Importantly, it was not possible to convert AMP-ACP into holo-ACP by incubation with CitG and dephospho-CoA. This result pointed to an alternative pathway of holo-ACP formation, where first a precursor of the prosthetic group is synthesized from ATP and dephospho-CoA by CitG. This precursor is subsequently transferred to apo-ACP by CitX. According to the structure of the prosthetic group and its precursors ATP and dephospho-CoA, 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA was deduced to be the intermediate formed by CitG. The proposed pathway for the conversion of apo- into holo-ACP is shown in Fig. 1.

In this work, we were able to verify this pathway by showing (i) that the products formed by CitG from ATP and dephospho-CoA are indeed adenine and 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA and (ii) that the latter compound is transferred by CitX to apo-ACP to yield holo-ACP.

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2. Materials and methods

2.1. Purification of proteins

Purification of *E. coli* apo-ACP_{His}, *E. coli* $CitG_{His}$, and *E. coli* $CitX_{Strep}$ was performed as described previously [13]. The purity of the proteins was checked by SDS–polyacrylamide gel electrophoresis (PAGE) [24] and subsequent staining of the gels with Coomassie brilliant blue. Protein concentrations were determined with the bicinchoninic acid protein assay [25] using ovalbumin as the standard.

2.2. CitG assay and reversed phase chromatography

The function of CitG was analyzed by incubating purified $CitG_{His}$ (30 μM) with ATP (30 μM) and dephospho-CoA (30 μM) at room temperature for 90 min. Subsequently the mixture was heated to 80°C for 7 min and heat-denatured CitG was removed by centrifugation. The compounds remaining in the supernatant were separated by reversed phase chromatography essentially as described previously [13]. A Hypersil ODS column (250 mm×4 mm, 5 μm particle size, Hewlett-Packard) was equilibrated with buffer A (0.2 M potassium phosphate pH 5.0) at a flow rate of 1 ml/min. Elution was performed with a linear gradient (0–60% within 18 min) of buffer B (0.2 M potassium phosphate pH 5.0 containing 20% (v/v) acetonitrile) with UV detection at 254 nm. The peak fractions were collected and stored at −20°C for further analysis.

2.3. Mass spectrometric analysis of the prosthetic group precursor

The fraction of the high performance liquid chromatography (HPLC) peak eluting at 13.5 min was evaporated to dryness and redissolved in half the volume water. 1 μl of this solution was mixed with the same volume of a saturated matrix solution (2,5-dihydroxybenzoic acid in an aqueous solution of 33% acetone) and allowed to dry at room temperature. Spectra (positive ion mode) were recorded with a Voyager Elite matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) in the delayed extraction and reflector mode using an accelerating voltage of 20 kV, a pulse delay time of 150 ns, a grid voltage of 75% and a guide wire voltage of 0.05%. Spectra were averaged from 32 scans. Calibration was external to the samples.

For the mass determination of apo-ACP_{His} and holo-ACP_{His}, a saturated matrix solution of sinapinic acid (3,5-dimethoxy-4-hydroxy-*trans*-cinnamic-acid) in 60% acetonitrile/0.1% trifluoroacetic acid was utilized. The samples were analyzed in delayed extraction linear mode using an accelerating voltage of 25 kV, a pulse delay time of 350 ns, a grid voltage of 92% and a guide wire voltage of 0.15%. Spectra were averaged from 50 scans. Calibration was external to the samples.

3. Results

3.1. In vitro synthesis of the prosthetic group precursor

In order to test our proposal that the first step in the synthesis of the citrate lyase prosthetic group is the CitG-catalyzed formation of a precursor from ATP and dephospho-CoA, purified *E. coli* $CitG_{His}$ (30 μM) was incubated for 90 min at room temperature with ATP (30 μM) and dephospho-CoA (30 μM). Subsequently the assay mixture was heated to 80°C for 7 min. After removal of the heat-denatured protein by centrifugation, the supernatant was subjected to reversed phase HPLC. The HPLC chromatogram presented in Fig. 2A shows the peaks observed for a reference sample that contained 30 μM of each ATP and dephospho-CoA and had also been incubated at 80°C. The peaks eluting at 5.6 min and 15.6 min represent ATP and dephospho-CoA, respectively. The third dominant peak eluting at 7.7 min results from a degradation product of dephospho-CoA. According to the pathway shown in Fig. 1, two products should be formed by CitG from ATP and dephospho-CoA, i.e. adenine and 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA. The elution profile obtained with the protein-free supernatant shown in

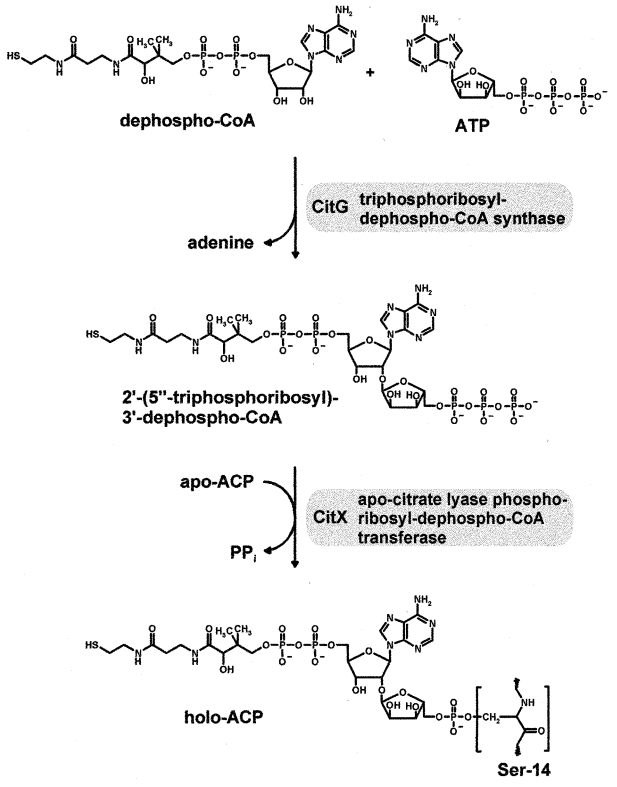

Fig. 1. Pathway for the synthesis of the 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group of citrate lyase in *E. coli* and *K. pneumoniae*.

Fig. 2B revealed residual amounts of ATP (5.5 min) and dephospho-CoA (15.7 min) and two dominant new peaks with retention times of 9.0 min and 13.5 min. If the protein-free supernatant was supplemented with 0.05 mM commercially available adenine, an increase of the peak eluting at 9 min was observed (Fig. 2C), showing that this peak represents adenine. Consequently, the compound eluting at 13.5 min was assumed to represent the prosthetic group precursor synthesized by CitG from ATP and dephospho-CoA. As described below, this assumption could be verified. In addition to the dominant new peaks at 9.0 min and 13.5 min, the protein-free supernatant of the CitG assay mixture contained a smaller new peak with a retention time of 5.9 min. This peak was shown to represent ADP.

3.2. Mass determination of the prosthetic group precursor

In order to determine the mass of the compound eluting at 13.5 min, the corresponding HPLC fraction was evaporated to dryness, redissolved in half the volume H_2O, and subjected to MALDI-TOF mass spectrometry. The obtained mass spectrum (Fig. 3) revealed seven major peaks with the following *m/z* values: 1060.6, 1098.6, 1136.5, 1174.5, 1212.5, 1250.5 and 1288.4. The calculated monoisotopic mass for the 1-fold protonated species $(M+H)^+$ of 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA, the precursor predicted by us, is 1060.6 Da, which corresponds exactly to the lowest *m/z* value measured. The six peaks with higher *m/z* values can be readily explained by the exchange of the protons of the phosphate groups by one to maximally six potassium ions originating from the buffer (see Fig. 1). This interpretation agrees with the calcu-

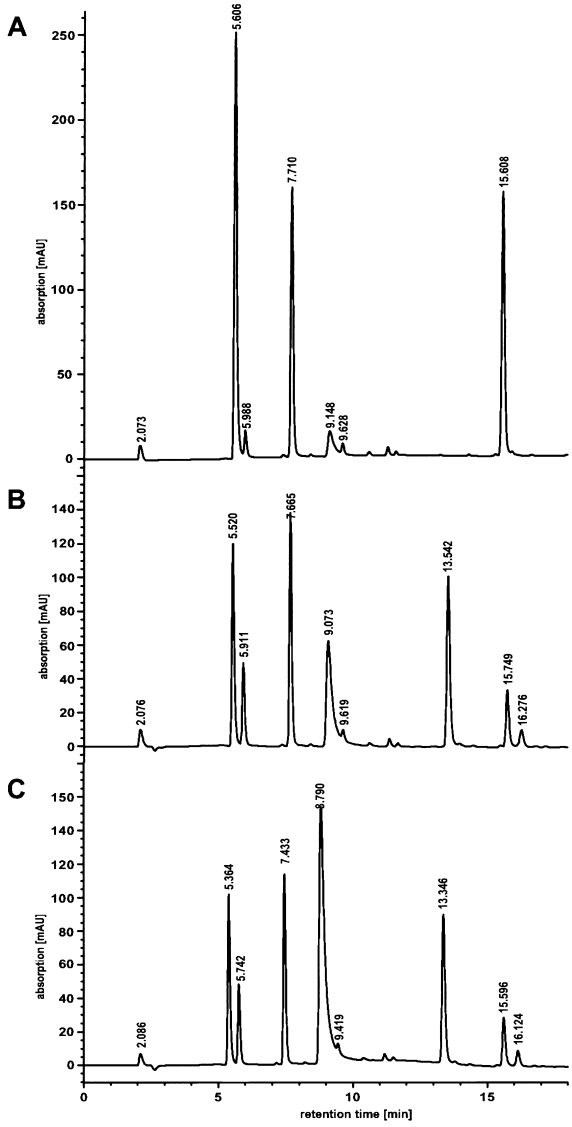

Fig. 2. HPLC analysis of substrates used and products formed in vitro by CitG. $CitG_{His}$ (30 μM) was incubated for 90 min at room temperature with ATP (30 μM) and dephospho-CoA (30 μM). After removal of the heat-denatured protein by centrifugation, the supernatant was subjected to reversed phase chromatography either without added adenine (B) or together with 0.05 mM adenine (C). As a reference, a sample containing 30 μM of both ATP and dephospho-CoA was analyzed (A). For detection, the UV absorption at 254 nm was measured.

lated mass difference of 38 Da between two consecutive mass peaks.

3.3. *In vitro conversion of apo-ACP and triphosphoribosyl-dephospho-CoA into holo-ACP by CitX*

Final confirmation of 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA as a precursor of the prosthetic group of citrate lyase ACP required that the compound is transferred by CitX to apo-ACP yielding holo-ACP. For this purpose, purified apo-ACP_{His} (31.5 μM) and $CitX_{Strep}$ (1.7 μM) were mixed either in the presence or the absence of the HPLC-purified prosthetic group precursor. At different time points after start of the reaction, samples were taken and analyzed by SDS–PAGE (Fig. 4). In the samples containing triphosphoribosyl-dephospho-CoA, a conversion of the apo-ACP_{His} (apparent molecular mass 12.5 kDa) to holo-ACP_{His} (apparent molecular mass 15.0 kDa) was observed as shown by the retarded migration. In the samples taken 15 min and 30 min after start of the reaction, about 50% of apo-ACP_{His} had been converted to holo-ACP_{His}. This incomplete conversion was due to the limiting amount of prosthetic group precursor, since a further addition of precursor at 30 min led to further conversion of the apo-form (Fig. 4, lane 8). In the samples where the potential precursor of the prosthetic group was omitted, no conversion of apo-ACP_{His} into a larger-sized from was detected.

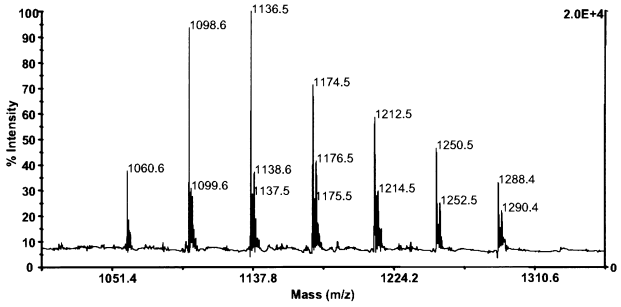

Fig. 3. MALDI-TOF mass spectrum obtained from the HPLC-purified precursor (retention time 13.5 min) of the citrate lyase prosthetic group.

Apo-ACP_{His} and the product formed in vitro by CitX from apo-ACP_{His} and 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA were analyzed by MALDI-TOF mass spectrometry. The obtained chromatograms are shown in Fig. 5. The apo-ACP_{His} yielded a mass of 11 755.1 Da, which deviates 0.002% from the calculated average mass $(M+H)^+$ of 11 755.3 Da. The putative holo-ACP_{His} sample yielded a mass of 12 637.8 Da. Substraction gives a mass difference of 882.7 Da, which exactly corresponds to the predicted mass of the prosthetic group. This experiment confirmed that 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA indeed represents the precursor of the

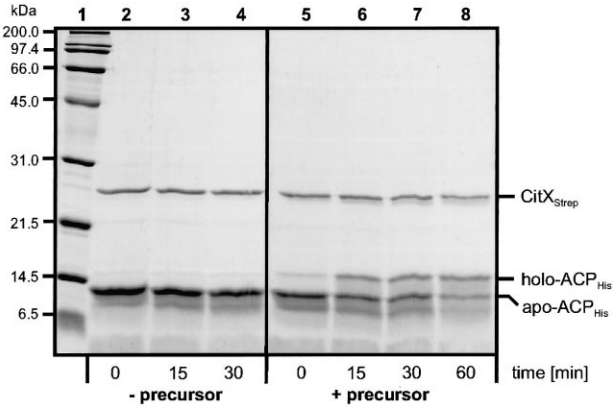

Fig. 4. In vitro conversion of apo-ACP into holo-ACP by CitX with the HPLC-purified prosthetic group precursor. Purified apo-ACP_{His} and $CitX_{Strep}$ were incubated in the absence or presence of 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA. After 30 min at room temperature, the latter assay mixture was supplemented again with 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA. Aliquots were taken at the indicated times and subjected to SDS–PAGE. The gel was stained with Coomassie brilliant blue. Lane 1 contains a protein standard.

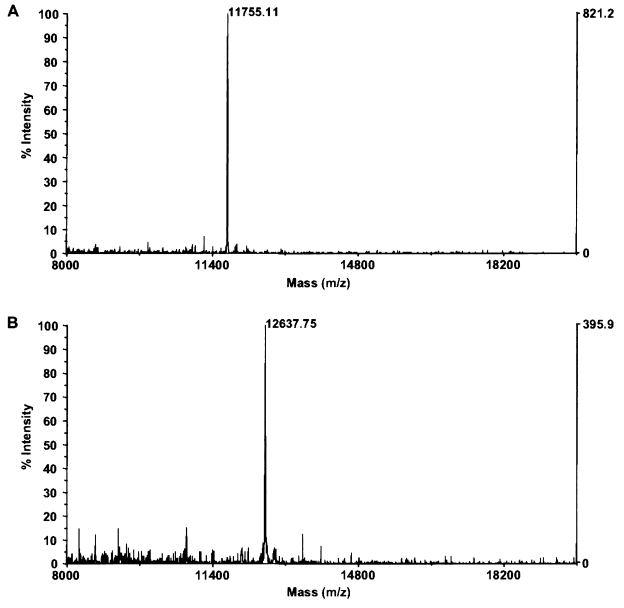

Fig. 5. MALDI-TOF mass spectra of apo-ACP_{His} (A) and the in vitro synthesized holo-ACP_{His} (B).

citrate lyase prosthetic group. Furthermore, it demonstrated that CitX alone is sufficient to catalyze the transfer of the prosthetic group precursor to apo-ACP resulting in the formation of holo-ACP.

4. Discussion

In this work, studies are presented that finally verified our recent proposal on the reactions involved in the biosynthesis of the citrate lyase prosthetic group 2′-(5″-phosphoribosyl)-3′-dephospho-CoA (Fig. 1). In the first step, CitG catalyzes an unusual α-1,2-glycosidic linkage between ATP and dephospho-CoA, leading to the formation of the prosthetic group precursor 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA and adenine, both of which were identified in our in vitro system. Thus, CitG functions as a 2′-(5″-triphosphoribosyl)-3′-dephospho-CoA synthase. In the second step, the prosthetic group precursor is transferred by CitX to apo-ACP, resulting in the formation of holo-ACP and pyrophosphate (data not shown). Thus, CitX functions as apo-citrate lyase phosphoribosyl-dephospho-CoA transferase. The attachment of the prosthetic group occurs via a phosphodiester linkage to serine-14 of the γ-subunit in the citrate lyase enzymes of *K. pneumoniae* and *E. coli*.

By performing two separate in vitro experiments in order to investigate the two reaction steps of the synthesis of the citrate lyase prosthetic group, it was shown that CitG and CitX are able to act independently of each other. However, the fact that in some bacteria CitX and CitG are fused [13] indicates that under physiological conditions the two enzymes closely interact. We observed that the same amount of the prosthetic group precursor was produced by CitG in vitro if the substrates ATP and dephospho-CoA were present either in the same concentration as CitG or in 5-fold excess (data not shown). This indicates that the prosthetic group precursor is not released from CitG but remains attached to it until the group is captured by CitX and then transferred to apo-ACP.

The presence of a 2′-(5″-phosphoribosyl)-3′-dephospho-CoA prosthetic group is not restricted to citrate lyase but also occurs in the ACPs of citramalate lyase (EC 4.1.3.22) from *Clostridium tetanomorphum* [26], as well as in malonate decarboxylase (EC 4.1.1) from *Malonomonas rubra* [27], *K. pneumoniae* [28], and *Acinetobacter calcoaceticus* [29]. Recent studies with *K. pneumoniae* malonate decarboxylase have shown that the biosynthesis pathway of the prosthetic group involves the same reactions as shown in Fig. 1, but uses genetically distinct enzymes (Hoenke, Wild and Dimroth, submitted).

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References

[1] Bott, M. (1997) Arch. Microbiol. 167, 78–88.
[2] Dimroth, P. (1988) in: The Roots of Modern Biochemistry (Kleinkauf, H., Döhren, H.v. and Jaenicke, L., Eds.), pp. 191–204, Walter de Gruyter and Co., Berlin.
[3] Antranikian, G. and Giffhorn, F. (1987) FEMS Microbiol. Rev. 46, 175–198.
[4] Subramanian, S. and Sivaraman, C. (1984) J. Biosci. 6, 379–401.
[5] Dimroth, P. and Eggerer, H. (1975) Eur. J. Biochem. 53, 227–235.
[6] Singh, M., Srere, P.A., Klapper, D.G. and Capra, J.D. (1976) J. Biol. Chem. 251, 2911–2915.
[7] Dimroth, P., Dittmar, W., Walther, G. and Eggerer, H. (1973) Eur. J. Biochem. 37, 305–315.
[8] Dimroth, P. (1976) Eur. J. Biochem. 64, 269–281.
[9] Robinson, J.B., Singh, M. and Srere, P.A. (1976) Proc. Natl. Acad. Sci. USA 73, 1872–1876.
[10] Singh, M., Robinson, J.B. and Srere, P.A. (1977) J. Biol. Chem. 252, 6061–6068.
[11] Beyreuther, K., Böhmer, H. and Dimroth, P. (1978) Eur. J. Biochem. 87, 101–110.
[12] Oppenheimer, N.J., Singh, M., Sweeley, C.C., Sung, S.-J. and Srere, P.A. (1979) J. Biol. Chem. 254, 1000–1002.
[13] Schneider, K., Dimroth, P. and Bolt, M. (2000) Biochemistry 39, 9438–9450.
[14] Buckel, W., Buschmeier, V. and Eggerer, H. (1971) Hoppe-Seylers Z. Physiol. Chem. 352, 1195–1205.
[15] Schmellenkamp, H. and Eggerer, H. (1974) Proc. Natl. Acad. Sci. USA 71, 1987–1991.
[16] Dimroth, P. and Eggerer, H. (1975) Proc. Natl. Acad. Sci. USA 72, 3458–3462.
[17] Bott, M. and Dimroth, P. (1994) Mol. Microbiol. 14, 347–356.
[18] Bott, M., Meyer, M. and Dimroth, P. (1995) Mol. Microbiol. 18, 533–546.
[19] Meyer, M., Dimroth, P. and Bott, M. (1997) J. Mol. Biol. 269, 719–731.
[20] Kaspar, S., Perozzo, R., Reinelt, S., Meyer, M., Pfister, K., Scapozza, E.L. and Bott, M. (1999) Mol. Microbiol. 33, 858–872.
[21] Blattner, F.R. et al. (1997) Science 277, 1453–1474.
[22] Pos, K.M., Dimroth, P. and Bott, M. (1998) J. Bacteriol. 180, 4160–4165.
[23] Ingmer, H., Miller, C.A. and Cohen, S.N. (1998) Mol. Microbiol. 29, 49–59.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Smith, P.K. et al. (1985) Anal. Biochem. 150, 76–85.
[26] Dimroth, P. and Loyal, R. (1977) FEBS Lett. 76, 280–283.
[27] Berg, M., Hilbi, H. and Dimroth, P. (1996) Biochemistry 35, 4689–4696.
[28] Schmid, M., Berg, M., Hilbi, H. and Dimroth, P. (1996) Eur. J. Biochem. 237, 221–228.
[29] Koo, J.H. and Kim, Y.S. (1999) Eur. J. Biochem. 266, 683–690.