ELSEVIER

Biochimica et Biophysica Acta 1553 (2002) 296–301

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

pH dependent inactivation of solubilized F_1F_0 ATP synthase by dicyclohexylcarbodiimide: pK_a of detergent unmasked aspartyl-61 in *Escherichia coli* subunit *c*

Francis Valiyaveetil, Joe Hermolin, Robert H. Fillingame *

Department of Biomolecular Chemistry, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706, USA

Received 6 September 2001; received in revised form 3 December 2001; accepted 5 December 2001

Abstract

The pH dependence of the reaction of dicyclohexylcarbodiimide with the essential aspartyl-61 residue in subunit *c* of *Escherichia coli* ATP synthase was compared in membranes and in a detergent dispersed preparation of the enzyme. The rate of reaction was estimated by measuring the inactivation of ATPase activity. The reaction with the detergent dispersed form of the enzyme proved to be pH sensitive with the essential aspartyl group titrating with a pK_a = 8. However, when measured with *E. coli* membranes, the reaction proved to be pH insensitive. The results suggest that the reacting aspartyl-61 residues are shielded from the bulk aqueous solvent when in the membrane, but then become aqueous-accessible following detergent solubilization. © 2002 Published by Elsevier Science B.V.

Keywords: ATP synthase; Subunit *c*; Essential carboxyl; Dicyclohexylcarbodiimide; pH dependence; ATPase inhibition; Proton transport

1. Introduction

ATP synthesis during oxidative and photophosphorylation is catalyzed by closely related F_1F_0 ATP synthases located in the inner membrane of mitochondria, the thylakoid membrane of chloroplasts and the cytoplasmic membrane of most eubacteria [1]. These enzymes utilize a transmembrane H^+ electrochemical gradient to drive ATP synthesis via a rotary catalytic mechanism [2–6]. The enzymes are composed of two structurally distinct sectors termed F_1 and F_0. The F_1 sector is bound at the membrane surface and contains the catalytic sites for ATP synthesis or hydrolysis. The F_0 sector traverses the membrane and catalyses proton translocation across the membrane. In *Escherichia coli*, the F_1 sector is composed of five types of subunits in an $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$ stoichiometry and the F_0 sector is composed of three subunits present in a stoichiometry now thought to be $a_1b_2c_{10}$ [7–10]. Proton transport is thought to drive rotation of an oligomeric ring of *c* subunits in the membrane, which in turn is coupled to rotation of the γ subunit within an immobilized $\alpha_3\beta_3$ hexamer of catalytic subunits bound at the surface of the membrane [2–6,11]. Both the *a* and the *c* subunits of the F_0 sector are believed to be involved in the transport of protons across the membrane. The *c* subunit spans the membrane as a hairpin of two α-helices and, in the case of *E. coli*, contains the essential aspartyl-61 residue at the center of the second

* Corresponding author. Fax: 608-262-5253.
E-mail address: rhfillin@facstaff.wisc.edu (R.H. Fillingame).

0005-2728/02/$ – see front matter © 2002 Published by Elsevier Science B.V.
PII: S0005-2728(01)00251-1

transmembrane helix. Asp61, or the equivalent Asp/Glu in other species, is thought to undergo protonation–deprotonation as each *c* subunit of the oligomeric ring moves past a stationary subunit *a* [5,6]. Subunit *a* is believed to provide access channels to the proton-binding Asp61 residue, but the actual proton translocation pathway remains to be defined [12–16]. In the bacterium *Propionigenium modestum* an F_1F_0 ATP synthase of similar structure to that in *E. coli* competitively transports Na^+, Li^+ and H^+ as coupling ions [16]. Asp61 of *E. coli* subunit *c* specifically reacts with dicyclohexylcarbodiimide (DCCD) during treatment at slightly alkaline pH and the resultant modification was shown to inhibit proton translocation through the F_0 and coupled ATPase activity [17–19]. Modification of a single *c* subunit of the enzyme complex is sufficient for complete inhibition of enzymatic activity [20]. The reaction of DCCD is believed to take place with the protonated form of the carboxyl group [21,22]. The pH dependence of the DCCD modification reaction can therefore provide an indirect method for determining the pK_a of the carboxyl group. As examples, the pH dependence of the DCCD modification reaction has been used to determine the pK_a of the essential glutamate-65 of subunit *c* in a detergent solubilized preparation of Na^+ translocating F_1F_0 ATP synthase from *P. modestum* [23] and in the P-type H^+ ATPase from *Kluyveromyces lactis* [24]. Carbodiimides have also been used to determine the pK_a of carboxyl groups in other enzymes, e.g. thrombin [25,26]. In this report we have used the pH dependence of the reaction with DCCD to determine the pK_a of Asp61 of subunit *c* in a detergent dispersed preparation of *E. coli* F_1F_0. However, the reaction proved to be pH insensitive when carried out with *E. coli* membrane vesicles, indicating that the reactive carboxyl group is not generally exposed to the aqueous medium in native membranes.

2. Materials and methods

2.1. Cell growth and membrane preparation

Strain VF170 was constructed by transformation of strain MM180 (*pyr*E41, *ent*A403, *arg*H1, *rps*L109, *sup*E44, *srl*::Tn*10* [27]) with the plasmid pOM142 [28]. Plasmid pOM142 contains the eight structural genes of the *atp* (ATP synthase) operon in plasmid pBR322. Strain VF170 was grown on M63 minimal medium [29] containing 0.6% glucose, 0.2 mM uracil, 0.2 mM L-arginine, 0.02 mM dihydrobenzoic acid, 2 mg/l thiamine, supplemented with 10% LB medium (i.e. 1 g/l tryptone, 0.5 g/l yeast extract and 1 g/l NaCl) and 100 μg/ml ampicillin. Membranes were prepared in TMDG buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% (v/v) glycerol) by rupture of cells using a French press [27]. The F_1F_0 ATPase was extracted from membranes, suspended at 10–15 mg/ml in TMDG buffer plus 1 mM phenylmethylsulfonyl fluoride, using 0.5% Na-cholate and 0.5% Na-deoxycholate in the presence of 1 M KCl as described by Fillingame and Foster [30]. The solubilized detergent extract was stored at −80°C and used within a 2 week period, an interval during which no decrease in ATPase activity was observed.

2.2. Inhibition of the ATPase by DCCD

A 50 μl sample of membranes (20 mg/ml) or the detergent extracted enzyme (4–5 mg/ml) was diluted into 450 μl of CPTM incubation buffer (50 mM CHES, 50 mM PIPES, 50 mM Tris, 5 mM $MgSO_4$ adjusted to the desired pH with 5 N KOH). DCCD was added to the desired concentration using stock solutions prepared in ethanol with the volume of ethanol added being ≤1% of the total reaction volume. At the desired time, the reaction was terminated by dilution of aliquots of the CPTM incubation mixture into ATPase assay buffer. The extent of dilution varied from $1 \rightarrow 100$ to $1 \rightarrow 500$ depending upon the experiment and ATPase assay method used, but in each case was sufficient to stop the DCCD inhibition reaction. ATPase activity was assayed by one of two methods. In the first assay method, membrane or enzyme was diluted into 0.9 ml of ATPase assay buffer #1 (50 mM Tris–SO_4, pH 7.8, 1 mM $MgSO_4$) and 0.1 ml of a 10 mM ATP solution was added and incubated for 5–10 min at 30°C. The ATPase reaction was terminated by addition of 0.1 ml 10% SDS with rapid mixing. The amount of P_i released due to ATP hydrolysis was determined as described by LeBel et al. [31] with the following modifications. Briefly, to 0.1 ml of the reaction

mix, 0.3 ml of solution A (0.25% $CuSO_4$, 4.6% sodium acetate, 2 N acetic acid), 50 μl of solution B (5% ammonium molybdate) and 50 μl of solution C (2% *p*-methyl aminophenol sulfate, 5% sodium sulfite) were added, mixed and incubated at room temperature for 15 min. Color development was stopped by addition of 500 μl of stop solution (2% sodium arsenite, 2% sodium citrate, 2% acetic acid) and the absorbance at 850 nm determined. The assay was shown to be linear over the time interval and the range of protein concentrations used in the experiments. In a second type of assay, more typically used in other papers from this laboratory [27], 0.1 ml of 4 mM [γ-^{32}P]ATP was added to the enzyme or membrane in 0.9 ml ATPase assay buffer #2 (55.5 mM Tris–SO_4, pH 7.8, 0.222 mM $MgSO_4$) at 30°C and the reaction terminated after a 5 min incubation by addition of trichloroacetic acid [17]. Radioactive $^{32}P_i$ released in the assay was extracted as a molybdate complex into isobutanol-benzene and quantitated by scintillation counting [17]. Similar results were seen by either assay method.

2.3. Calculation of the kinetic parameters

To determine the pseudo-first order rate constant for the ATPase inactivation reaction, the time course of the inactivation reaction was plotted as fractional activity versus time and fit to Eq. 1:

$$y = a \exp^{(-k_{obs}t)} + c \quad (1)$$

where y corresponds to the fractional activity at time t. The fraction of total enzyme activity that can be inhibited by DCCD is given by a, c corresponds to the fraction of the enzyme activity that cannot be inhibited by DCCD and k_{obs} is the pseudo-first order rate constant. Second order rate constants for the DCCD inactivation reaction were calculated as follows: $k_2 = k_{obs}/[DCCD]$. For the determination of the pK_a of Asp61, the rate constants for the inactivation reaction, determined at different pH values, were fit to Eq. 2 [32]:

$$k_2 = k_h/(1 + [H^+]/K_a) + k_l/(1 + K_a/[H^+]) \quad (2)$$

where k_2 corresponds to the inactivation rate constant at proton concentration $[H^+]$, k_h and k_l are the inactivation rate constants at the highest and lowest $[H^+]$, and K_a is the dissociation constant of the ionizable group. The solver program of Excel Version 5.0 (Microsoft, Redmond, WA, USA) was used in fitting experimental data to Eqs. 1 and 2 and in generating the lines drawn in the figures shown.

3. Results

3.1. pH dependence of DCCD reaction with membrane versus detergent extracted enzyme

DCCD inhibits the ATPase activity of *E. coli* membranes in a time dependent manner. The rate of inactivation was little affected by the pH of the medium as indicated by the examples shown at pH 7.2 and pH 8.6 in Fig. 1A. From four similar experiments, the second order rate constant (k_2) for inactivation by DCCD was calculated to be

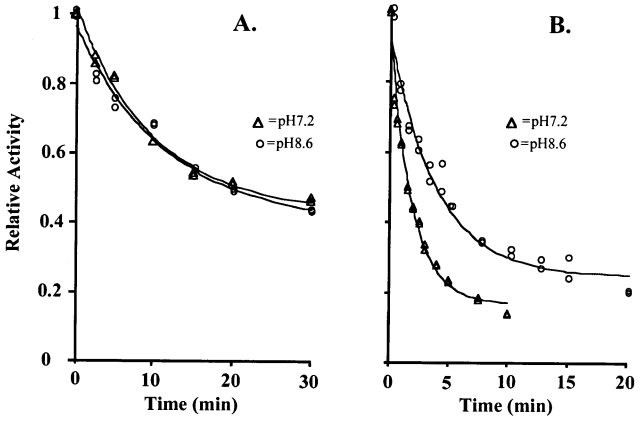

Fig. 1. Kinetics of inactivation of membrane bound or detergent extracted ATPase by DCCD. (A) Membranes were diluted to 2 mg/ml into CPTM buffer at 30°C at pH 7.2 (○) or pH 8.6 (△) and treated with 30 μM DCCD for the times indicated. Duplicate samples of treated membranes were then diluted 1→150 into ATPase assay buffer and the ATPase activity of each sample was determined in duplicate by a colorimetric assay for inorganic phosphate release. The lines are drawn as a fit to Eq. 1 with k_{obs} equal to 0.098 s^{-1} at pH 7.2 and 0.087 s^{-1} at pH 8.6. (B) The detergent extracted ATPase was diluted into 9 vols. of CPTM buffer at pH 7.12 (○) and pH 8.58 (△) and incubated with 30 μM DCCD at 30°C. Na-deoxycholate and Na-cholate were each present at a final concentration of 0.05% following this dilution into CPTM buffer. At various times thereafter, duplicate samples were diluted 1→100 into ATPase assay buffer and the ATPase activity of each sample determined in duplicate using the colorimetric assay (see Section 2). The lines are drawn as a fit to Eq. 1 with k_{obs} equal to 0.534 s^{-1} at pH 7.12 and 0.258 s^{-1} at pH 8.6.

$0.46 \pm .12 \times 10^4$ min^{-1} M^{-1} at pH 7.2 and $0.42 \pm .16 \times 10^4$ min^{-1} M^{-1} at pH 8.6.

We have routinely used a mixture of deoxycholate and cholate to extract *E. coli* F_1F_0 from the membrane for purification and reconstitution [30]. As shown in Fig. 1B, the detergent solubilized form of the enzyme is more rapidly inactivated by DCCD than the membrane bound form of the enzyme. Further, in contrast to the results seen with the membrane embedded enzyme, the rate of DCCD inactivation of the detergent solubilized enzyme varies markedly with pH (Fig. 1B). The pH dependence of DCCD inactivation of the detergent solubilized enzyme is characterized in greater detail below.

Using either membranes or detergent solubilized enzyme, a fraction of the ATPase is resilient to inhibition by DCCD. The fraction varies from 15 to 35% depending upon the conditions and dilutions done prior to the ATPase assay. Much of the DCCD-insensitive ATPase activity can be ascribed to disassociation of F_1 from the membrane embedded F_0 sector of the enzyme as the preparation is diluted for assay, as is documented in an earlier paper by Hermolin and Fillingame [20]. The fraction of DCCD-insensitive activity did increase with pH, i.e. from approx. 15–20% at pH 7.0 to 35% at pH 9.0. In the kinetic analysis below, this DCCD-insensitive fraction was subtracted during the curve fitting of the time course data to Eq. 1. The subtraction does lead to linear semilogarithmic plots of residual activity versus time ([20] and data not shown).

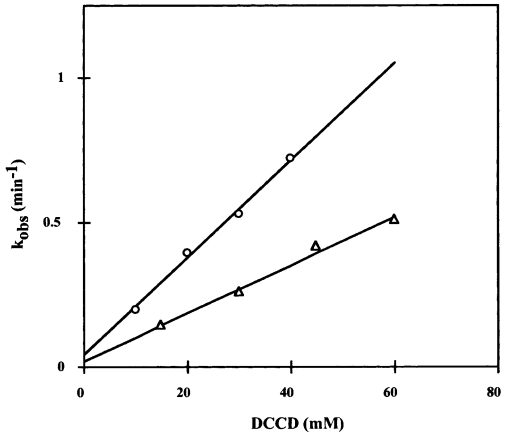

Fig. 2. Dependence of the rate of inactivation of detergent extracted ATPase on the concentration of DCCD. The pseudo-first order rate constants for the inhibition of the detergent extracted ATPase by DCCD at 30°C and at pH 7.12 (○) and pH 8.58 (△) were determined over a range of DCCD concentrations. Dilutions, incubations and ATPase assays were carried out as described in Fig. 1.

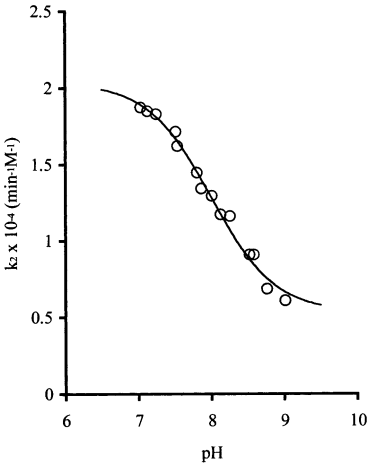

Fig. 3. pH dependence for inactivation of the detergent extracted ATPase by DCCD. The rate constants for the inhibition of the detergent extracted ATPase by 25 μM DCCD at 30°C were determined at various pH values between 7.0 and 9.0. The line is drawn from the experimental data using Eq. 2 with a fit to a pK_a value of 8.03 and a $k_{2(max)}$ and $k_{2(min)}$ of 2.0×10^4 min^{-1} M^{-1} and 0.50×10^4 min^{-1} M^{-1} respectively. Dilutions, incubations and ATPase assays were carried out as described in Fig. 1.

3.2. pH dependence of DCCD inhibition with detergent solubilized enzyme

The pseudo-first order rate constant for inhibition (k_{obs}) calculated according to Eq. 1 is expected to vary linearly with DCCD concentration. Examples of the linear dependence of k_{obs} with DCCD concentration are shown in Fig. 2. The rate constant for DCCD inactivation of the detergent solubilized enzyme proved to be strongly dependent upon the pH, as already indicated by the experiments shown in Figs. 1B and 2. The variation in the second order rate constant (k_2) over a range of pH values is shown in Fig. 3, with the rate of inactivation decreasing with increasing pH. The pH dependence of the k_2 for DCCD inactivation could be fit to an equation specifying a single group titrating with a pK_a of 8.03, as shown in Fig. 3. Values of 8.08 and 7.74 were obtained in two independent sets of experiments, the average pK_a being 7.95 ± 0.15. The pH dependence of the DCCD inactivation reaction fits with

the view that the protonated form of the Asp61 carboxyl group reacts with DCCD [21,22]. However, the fit of the titration curve might also indicate that the reaction with DCCD continues to a significant extent at high pH where the carboxyl group should be nearly completely ionized. Other possible interpretations are discussed below.

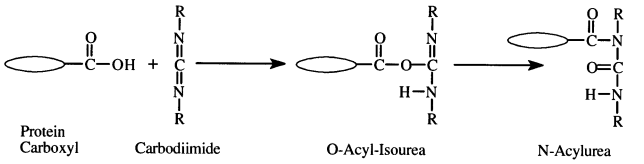

Fig. 4. Reaction of carbodiimide with protein carboxyl group occurs via *O*-acyl isourea intermediate and subsequent acyl migration to form the stable *N*-acyl urea.

4. Discussion

When *E. coli* membranes are treated with DCCD at slightly alkaline pH, the carbodiimide specifically modifies Asp61 of subunit *c* with resultant inhibition of ATPase activity [17]. As shown here, and as previously reported by Friedl et al. [33], the rate of DCCD reaction with the membrane integrated form of the *E. coli* enzyme is not affected by the pH of the suspending aqueous medium. The pH insensitivity of the reaction could be due to lack of ionization of Asp61 when buried in the hydrophobic environment of the membrane. On the other hand, DCCD reacts more rapidly with the detergent extracted F_1F_0 complex and the rate of reaction varies markedly with pH as is shown in Fig. 3. The simplest interpretation is that the detergent extraction leads to greater exposure of Asp61 residues of the oligomeric *c* ring to protonation from the aqueous medium, and perhaps greater exposure to DCCD as well. In the model of the oligomeric *c* ring proposed by Dmitriev et al. [34], the protonated Asp61 carboxylate is packed in a hydrophobic cavity between neighboring *c* subunits where it would be inaccessible to the aqueous medium and also shielded from the fatty acyl phase of the lipid bilayer. If DCCD reacts from the hydrophobic phase of the lipid bilayer, as is widely presumed [35,36], it would have to insert between the *c* subunits composing the ring to react. Detergent extraction of F_0 from the membrane may increase the flexibility of subunit *c* packing and account for the increased rate of reaction.

The exact mechanism by which carbodiimides react with carboxyl groups in proteins is uncertain but it is widely assumed that the reaction occurs with the protonated form of the carboxyl group [21,22]. The reaction is known to take place in two steps with the initial step being reaction of the carbodiimide with the carboxyl group to form a protonated *O*-acyl isourea which then undergoes a rearrangement reaction to form a stable *N*-acyl isourea (Fig. 4) [21,22,37]. The reaction with aqueous accessible carboxyl groups is known to be pH dependent and the pK_a for the process is usually assumed to correspond to the pK_a of the reacting carboxylate residue. A requirement that the carboxyl group be protonated for the initial step to take place was initially proposed [21,22], although subsequently others have suggested that the reaction can occur by general acid catalysis where proton transfer to one of the carbodiimide nitrogens is required during *O*-acyl isourea formation [37,38]. The reaction could be a concerted one where the proton is donated to the carbodiimide nitrogen by the reacting carboxyl group [25]. A concerted mechanism would explain why different carboxyl residues in proteins show different pK_a values in reactions with the same carbodiimide.

The titration curve shown in Fig. 3 suggests a $pK_a = 8$ for the reacting Asp61 residues in the detergent extracted F_1F_0 complex. The titration curve is notable in that the reaction continues at a significant rate at alkaline pH, i.e. at approx. 25% of $k_{2(max)}$. The apparent plateau at alkaline pH may indicate that only a fraction of the solubilized enzyme is perturbed to expose the carboxylates under these conditions of detergent treatment, i.e. 75% of the potentially reactive enzyme in this case. Because of significant disassociation of F_1 from F_0 at pH >9, it was not possible to extend the experimental measurements beyond the range shown. The extrapolated $k_{2(min)}$ at alkaline pH corresponds to the k_2 seen with the membrane embedded form of the enzyme, where the DCCD-reactive carboxyl group appears to be shielded from the aqueous medium.

In the case of the Na^+-translocating F_1F_0 ATP synthase of *P. modestum*, the Glu65 carboxylate of subunit *c* (equivalent to *E. coli* Asp61) was concluded to reside near the surface of the membrane,

based in part on the pH dependence of its reaction with DCCD and protection from reaction by the binding of Na^+ [39]. Na^+ was also shown to activate the detergent extracted form of the enzyme in a co-operative manner (Hill coefficient = 2.6) suggesting aqueous accessibility of at least three binding sites [23]. These studies were done with a purified form of the enzyme in the presence of Triton X-100, as were subsequent Na^+ occlusion studies [40]. These experiments with the detergent solubilized *P. modestum* enzyme have been cited in support of a 'one channel' model for ion transport through F_0 wherein most of the Glu65 residues of the oligomeric *c* ring are freely accessible to the aqueous medium at the cytoplasmic face of the membrane [39]. In retrospect, it seems possible that the aqueous accessibility of Glu65 is influenced by the presence of Triton, and based upon the studies reported here, it would be of interest to compare features to the membrane embedded form of the *P. modestum* enzyme.

Acknowledgements

This work was supported by United States Public Health Research Grant GM23105.

References

[1] A.E. Senior, Physiol. Rev. 68 (1988) 177–231.
[2] H. Noji, R. Yasuda, M. Yoshida, K. Kinosita Jr., Nature 386 (1997) 299–302.
[3] Y. Sambongi, Y. Iko, M. Tanabe, H. Omote, A. Iwasmoto-Kihara, I. Ueda, T. Yanagida, Y. Wada, M. Futai, Science 286 (1999) 1722–1724.
[4] O. Pänke, K. Gumbiowski, W. Junge, S. Engelbrecht, FEBS Lett. 472 (2000) 34–38.
[5] W. Junge, Proc. Natl. Acad. Sci. USA 96 (1999) 4735–4737.
[6] P. Dimroth, H. Wang, M. Grabe, G. Oster, Proc. Natl. Acad. Sci. USA 96 (1999) 4924–4929.
[7] D.L. Foster, R.H. Fillingame, J. Biol. Chem. 257 (1982) 2009–2015.
[8] D. Stock, A.G.W. Leslie, J.E. Walker, Science 286 (1999) 1700–1705.
[9] W. Jiang, J. Hermolin, R.H. Fillingame, Proc. Natl. Acad. Sci. USA 98 (2001) 4966–4971.
[10] R.H. Fillingame, W. Jiang, O.Y. Dmitriev, J. Bioenerg. Biomembr. 32 (2000) 433–439.
[11] S.P. Tsunoda, R. Aggeler, M. Yoshida, R.A. Capaldi, Proc. Natl. Acad. Sci. USA 98 (2001) 898–902.
[12] R.H. Fillingame, in: T.A. Krulwich (Ed.), The Bacteria, Vol. 12, Academic Press, New York, 1990, pp. 345–391.
[13] G. Deckers-Hebstreit, K. Altendorf, Annu. Rev. Micobiol. 50 (1996) 791–824.
[14] R.K. Nakamoto, C.J. Ketchum, M. Al-Shawi, Annu. Rev. Biophys. Biomol. Struct. 28 (1999) 205–234.
[15] B.D. Cain, J. Bioenerg. Biomembr. 32 (2000) 365–371.
[16] P. Dimroth, U. Matthey, G. Kaim, Biochim. Biophys. Acta 1459 (2000) 506–513.
[17] R.H. Fillingame, J. Bacteriol. 124 (1975) 870–883.
[18] J. Hoppe, W. Sebald, Proc. Natl. Acad. Sci. USA 77 (1980) 785–789.
[19] R.H. Fillingame, Annu. Rev. Biochem. 49 (1980) 1079–1113.
[20] J. Hermolin, R.H. Fillingame, J. Biol. Chem. 264 (1989) 3896–3903.
[21] H.G. Khorana, Chem. Rev. 53 (1953) 145–166.
[22] D.G. Hoare, D.E. Koshland Jr., J. Biol. Chem. 242 (1967) 2447–2453.
[23] C. Kluge, P. Dimroth, Biochemistry 32 (1993) 10378–10386.
[24] I. Velazquez, F. Martinez, J.P. Pardo, Arch. Biochem. Biophys. 346 (1997) 294–302.
[25] V.W.F. Chan, A.M. Jorgensen, C.L. Borders, Biochem. Biophys. Res. Commun. 151 (1988) 709–716.
[26] C.L. Borders, V.W.F. Chan, L.A. Miner, Y.M. Weerasuriya, FEBS Lett. 255 (1989) 365–368.
[27] M.E. Mosher, L.K. White, J. Hermolin, R.H. Fillingame, J. Biol. Chem. 260 (1985) 4807–4814.
[28] Y. Zhang, M.O. Oldenberg, R.H. Fillingame, J. Biol. Chem. 269 (1994) 10221–10224.
[29] J.H. Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992.
[30] R.H. Fillingame, D.L. Foster, Methods Enzymol. 126 (1986) 545–557.
[31] D. LeBel, G.G. Poirier, A.R. Beaudoin, Anal. Biochem. 85 (1978) 86–89.
[32] W.W. Cleland, Methods Enzymol. 63 (1979) 103–136.
[33] P. Friedl, B.I. Schmid, H.U. Schairer, Eur. J. Biochem. 73 (1977) 461–468.
[34] O.Y. Dmitriev, P.C. Jones, R.H. Fillingame, Proc. Natl. Acad. Sci. USA 96 (1999) 7785–7790.
[35] S. Engelbrecht, W. Junge, FEBS Lett. 414 (1997) 485–491.
[36] M.L. Hutcheon, T.M. Duncan, H. Ngai, R.L. Cross, Proc. Natl. Acad. Sci. USA 98 (2001) 8519–8524.
[37] A. Williams, I.T. Ibrahim, Chem. Rev. 81 (1981) 589–636.
[38] I.T. Ibrahim, A. Williams, J. C. S. Chem. Commun. (1980) 25–27.
[39] P. Dimroth, G. Kaim, U. Matthey, Biochim. Biophys. Acta 1365 (1998) 87–92.
[40] G. Kaim, U. Matthey, P. Dimroth, EMBO J. 17 (1998) 688–695.