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Rotation of the c Subunit Oligomer in EF_0EF_1 Mutant cD61N*

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ATP synthases (F_0F_1-ATPases) mechanically couple ion flow through the membrane-intrinsic portion, F_0, to ATP synthesis within the peripheral portion, F_1. The coupling most probably occurs through the rotation of a central rotor (subunits $c_{10}\epsilon\gamma$) relative to the stator (subunits $ab_2\delta(\alpha\beta)_3$). The translocation of protons is conceived to involve the rotation of the ring of c subunits (the c oligomer) containing the essential acidic residue cD61 against subunits ab_2. In line with this notion, the mutants cD61N and cD61G have been previously reported to lack proton translocation. However, it has been surprising that the membrane-bound mutated holoenzyme hydrolyzed ATP but without translocating protons. Using detergent-solubilized and immobilized EF_0F_1 and by application of the microvideographic assay for rotation, we found that the c oligomer, which carried a fluorescent actin filament, rotates in the presence of ATP in the mutant cD61N just as in the wild type enzyme. This observation excluded slippage among subunit γ, the central rotary shaft, and the c oligomer and suggested free rotation without proton pumping between the oligomer and subunit a in the membrane-bound enzyme.

ATP synthases of bacteria, chloroplasts, and mitochondria use ion-motive force for the synthesis of ATP from ADP and phosphate (1–4). When operating in reverse (F-ATPase), the enzyme hydrolyzes ATP and generates ion-motive force. ATP synthase in its simplest bacterial form consists of eight different subunits, five in the F_1 portion, $(\alpha\beta)_3\gamma\delta\epsilon$, and three in F_0, $\mathbf{ab_2c}_{10}$ (5). F_1 catalyzes substrate conversion, and F_0 is responsible for ion translocation. ATP (6–12) and membrane-bound F_1 (13) drives the rotation of $\gamma(\epsilon)$ relative to the $(\alpha\beta)_3$ barrel. The counterpart of these rotor elements in F_1 is the ring of **c** subunits (the **c** oligomer) in F_0 (14–16). Therefore, subunits $\mathbf{ab}_2\delta(\alpha\beta)_3$ form the "stator," and subunits $\mathbf{c}_{10}\gamma\epsilon$ form the "rotor" (17–21).

Direct evidence for the relative rotation of $\mathbf{c}_{10}\gamma\epsilon$ against $\mathbf{ab}_2\delta(\alpha\beta)_3$ under the conditions of ATP synthesis is still lacking, because it has not yet been feasible to energize the oriented immobilized enzyme within a native-like ion-tight membrane environment. Instead, the rotation of the **c** oligomer was investigated by attaching the reporter (a fluorescently labeled actin filament) to $\mathbf{c}_{10}$ of detergent-solubilized and immobilized F_0F_1 and checking for ATP hydrolysis-driven rotation (14–16). The presence of detergent was inevitable in these approaches (just as in the one presented here). All of the groups observed that the activity was now insensitive to DCCD[1] and nearly insensitive to venturicidin, in contrast to the behavior of the membrane-bound enzyme. Upon the removal of the detergent, coupling was restored (16). Apparently, the enzyme became functionally uncoupled in the presence of the detergent.

Our method to attach fluorescent actin filaments to the **c** oligomer via engineered Strep-tags not only was monospecific for the **c** oligomer of F_0, it turned out to be quite robust (15), for example, in that it allowed to wash away the detergent after immobilization without the complete loss of rotary activity of immobilized F_0F_1. After the washing of both untreated and DCCD-treated EF_0EF_1, the yield of rotating filaments with DCCD-treated EF_0EF_1 dropped to zero as expected in view of the inhibitory effect of DCCD, but it remained to some extent in controls. A partial loss of the rotary activity in the controls was probably caused by mechanical removal of immobilized enzymes/filaments from the surface. Hence, although this result was compatible with the notion that the observable rotating filaments were connected to fully DCCD-sensitive EF_0EF_1 (coupled enzyme), it did not fully exclude that the rotation of the **c** oligomer relative to subunit **a** was decoupled from proton control between subunits **a** and **c** by the presence of detergent (decoupled enzyme) in all samples whether they were DCCD-treated or not. To clarify this situation, we repeated the experiment with the EF_0EF_1 mutant **c**D61N. It lacks the acidic residue on subunit **c**, which is essential for proton translocation. The mutant is known to assemble normally but to be completely blocked in proton translocation both by F_0F_1 and by exposed F_0. Therefore, the mutant strain is unable to support the growth on non-fermentable carbon sources. However, despite ATP-driven proton pumping being completely blocked, the ATP hydrolytic activity of the membrane-bound enzyme remains unaffected (22, 23). In today's understanding of the rotary enzyme, this observation implies either that the rotation of the central shaft, subunit γ, became uncoupled from the rotation of the **c** oligomer or that the **c** oligomer remained mechanically coupled to and corotated with subunit γ but was "freewheeling" relative to the stator subunits $\mathbf{ab}_2\delta\,(\alpha\beta)_3$. Here we show the latter to be the case. EF_1EF_0(cD61N) is as effective in the filament rotation assay as the control. Although this finding fully agrees with the previously proposed protonic uncoupling of detergent-solubilized F_0F_1 (16, 20), it also suggests the free mobility of the **c** oligomer against the stator, mainly subunit **a**, not only in the detergent-solubilized but also in the membrane-embedded mutant enzyme.

MATERIALS AND METHODS

Chemicals and Enzymes—All of the restriction enzymes were purchased from New England Biolabs or MBI Fermentas (St. Leon-Rot,

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[1] The abbreviations used are: DCCD, dicyclohexyl-carbodiimide; Ni-NTA, nickel-nitrilotriacetic acid; TES, 2-{[2-hydroxy-1,1-bis (hydroxymethyl)ethyl]-amino}ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

Germany). Oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany). Streptactin-Sepharose was purchased from IBA (Göttingen, Germany). Nickel-nitrilotriacetic acid (Ni-NTA) horseradish peroxidase and Ni-NTA Superflow were from Qiagen (Hilden, Germany). Biotin-$PEAC_5$-maleimide was from Dojindo (via Gerbu Biotechnik, Gaiberg, Jena, Germany). The Lumi-Light Western blotting kit was obtained from Roche Molecular Biochemicals. Venturicidin A was obtained from Dr. B. Liebermann (Department of Pharmacology, University of Jena, Jena, Germany), but the supply exhausted in the meantime. Other reagents were of the highest grade commercially available.

Molecular Genetics—The complete cysteine-less plasmid pSE1 (β-His tag, Strep-tag at the C terminus of **c**) (15) was used as starting material. Site-directed mutagenesis was performed by standard PCR using the oligonucleotide primers 5′-ATTCTGATTGCTGGTCTGTTGCCG-3′, 5′-ATCGGGATAGCATTCACCAGACCCATAACG-3′, 5′-GGATACGGCCAGTACACTTAACTTTCATG-3′, and 5′-GGGTCTGGTGAATGCTATCCCGATCGC-3′. The *Bam*HI/*Xho*I fragment of pSE1 was substituted with the corresponding fragment carrying the **c**D61N mutation by restriction and religation. Successful cloning was confirmed by nucleotide sequencing. The resulting plasmid was called pKG1. pKG1 carried a His_6 tag at the N termini of subunits β, a C-terminal Strep-tag at subunits **c**, a point mutation in subunit **c** (D61N), and all of the Cys residues were replaced by Ala (24).

Preparation of EF_0EF_1—E. coli strain DK8 (25) was transformed with pKG1, and cells were grown on minimal medium containing 10% (v/v) LB and 0.5% (w/v) glucose. Cells were collected at A_{600} = 0.8. The membranes were isolated and purified essentially according to Wise (26), and membrane proteins were extracted as described previously (15). After the addition of avidin, the octylglycoside extract of membranes from 25 g of collected cells containing 140 mg of total membrane protein was diluted with buffer A (20 mM TES (pH 7.5), 5 mM $MgCl_2$, 1 mM K-ADP, 15% (v/v) glycerol) to 1% octylglycoside (total volume, 100 ml) and then was applied batchwise to 5 ml of streptactin-Sepharose (settled volume, 5 mg streptactin/ml). Washing and elution were performed as described previously (15). Protein-containing fractions (2 mg of protein) were combined, and batchwise was adsorbed onto 1 ml of Ni-NTA Superflow. After washing, up to 100 μg of pure EF_0EF_1 eluted from the column. Protein determinations were carried out according to Sedmak and Grossberg (27) and SDS-gel electrophoresis with the Pharmacia Phast system (8–25% gradient gels). Staining was carried out with Coomassie Blue followed by silver (28). ATPase activity was measured with 0.1 μg of protein, 50 mM Tris/HCl (pH 8.0), 3 mM $MgCl_2$, 10 mM Na-ATP, 1% *N*-octyl-β-D-glucopyranoside.

Rotation Assay—Samples were filled into flow cells consisting of two coverslips (bottom, 26 × 76 mm^2; top, 21 × 26 mm^2; thickness, 0.15 mm (Menzel-Gläser/ProLabor, Georgsmarienhütte, Germany) separated by parafilm strips. Protein solutions were infused in the following order (2 × 25 μl/step, 4-min incubation): 1) 0.8 μM Ni-NTA-horseradish peroxidase conjugate in 20 mM Mops/KOH (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$ (buffer B); 2) 10 mg/ml bovine serum albumin in buffer B; 3) 5–10 nM EF_0EF_1 in 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 mg of bovine serum albumin/ml, 10% (v/v) glycerol, 1% (w/v) *N*-octyl-β-D-glucopyranoside (buffer C); 4) wash with buffer C; 5) 0.5 μM streptactin in buffer C; 6) wash with buffer C; 7) 200 nM biotinylated fluorescent-labeled F-actin (15) in buffer C (7-min incubation); 8) wash with buffer C; and 9) 20 mM glucose, 0.2 mg/ml glucose oxidase, 50 μg/ml catalase, 5 mM ATP in buffer C. The deliberate omission of either one single component of the chain Ni-NTA-horseradish peroxidase, EF_0EF_1, streptactin, and biotin-F-actin prevented the binding of fluorescent F-actin as evident from the absence of fluorescent filaments within in the flow cell. This ensured that the actin filaments were attached to subunit(s) **c** in the correct manner. Also, the rotating filaments only could be observed in the presence of ATP (15), whereas in its absence (or with ADP present), this number dropped to zero without affecting the number of immobilized filaments.

Video Microscopy—An inverted fluorescence microscope (IX70, lens PlanApo 100x/1.40 oil, fluorescence cube MWIG, Olympus, Hamburg, Germany) was equipped with a silicon-intensified tube camera (C2400-08, Hamamatsu, Herrsching, Germany) and connected to a VHS-PAL video recorder (25 frames/s). With this setup, the filaments of 5-μm length appeared as 3-cm long rods on a 14-inch monitor. A freshly chromatographed sample of EF_0EF_1 was loaded into the flow cell and labeled with fluorescent actin filaments. The rotation of single filaments was observed for up to 3 min. A single molecule rotation was followed up to 30 min after loading. Video data were captured (frame grabber FlashBus, Integral Technologies, Indianapolis, IN) and further processed by using the software ImagePro Plus 4.0 (Media Cybernetics, Silver Spring, MD) and Matlab 5.2 (The Math Works, Natick, MA).

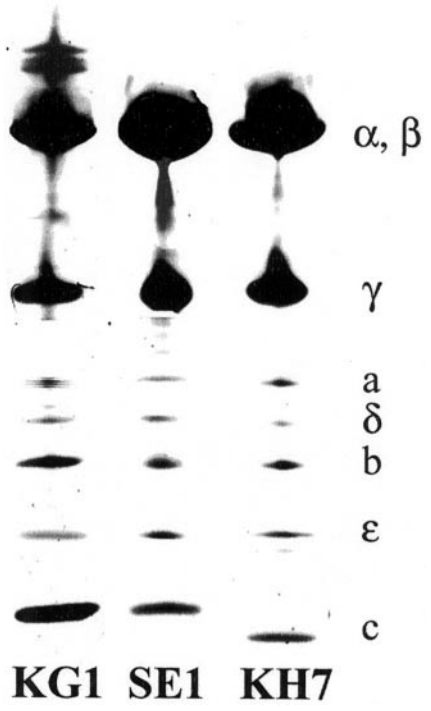

FIG. 1. **SDS-gel electrophoresis of 1 μg each of EF_0EF_1-KG1, EF_0EF1-SE1 (15), and a control (EF_0EF_1-KH7 (11)) after purification by streptactin-Sepharose and nickel-nitrilotriacetic acid affinity chromatography.** Pharmacia Phast gradient gel 8–25% silver/silicon tungstic acid stain (28) was used. The size difference between subunits **c** from EF_0EF_1-KG1, EF_0EF_1-SE1, and EF_0EF_1-KH7 is because of the C-terminal Strep-tag engineered into EF_0EF_1-KG1 and EF_0EF_1-SE1.

Other Methods—ATPase activity was measured at protein concentrations of 10 μg/ml in 50 mM Tris/HCl (pH 8.0), 3 mM $MgCl_2$, 10 mM Na-ATP, 1% octylglycoside. After incubation for 5 min at 37 °C, the reaction was stopped by the addition of trichloroacetic acid, and the released P_i was determined colorimetrically (29).

RESULTS AND DISCUSSION

EF_0EF_1 mutant SE1 (15) was used as starting material. In this mutant, all wild type cysteines are substituted by alanines (24), each β subunit carries an engineered His_6 tag at its N terminus, and each **c** subunit carries an engineered Strep-tag at its C terminus. The desired point mutation within subunit **c** (Asp61→ Asn) was introduced by PCR and confirmed by nucleotide sequencing. The resulting plasmid was called pKG1. Because the **c**D61N mutation causes uncoupling, EF_0EF_1-KG1 had to be prepared from cells grown on medium supplemented with LB and glucose. This yielded 30–100 μg of EF_0EF_1-KG1/8l culture volume. Typical activities after purification were 90 units/mg. Fig. 1 shows the results of an SDS-electrophoresis with purified EF_0EF_1-KG1, EF_0EF_1-SE1, and a control (EF_0EF_1-KH7 (11)).

As expected, ATPase activity from membranes isolated from DK8/pKG1 was not inhibited by DCCD in contrast to controls, which were reversibly (*i.e.* after the addition of 0.5% *N,N*-dimethyldodecylamine-*N*-oxide) inhibited by 70% after incubation with 50 μM DCCD for 1 day at room temperature. Also, venturicidin A (20 and 100 μM, 30-min incubation) did not inhibit the membrane-bound ATPase activity from EF_0EF_1-KG1, in contrast to wild-type-like controls (EF_0EF_1-SE1, EF_0EF_1-KH7). This finding is of limited value though in that the mutation might have compromised the venturicidin binding site (30). Fig. 2 summarizes the results of the filament rotation assay (8). *Panel A* shows typical time courses as ob-

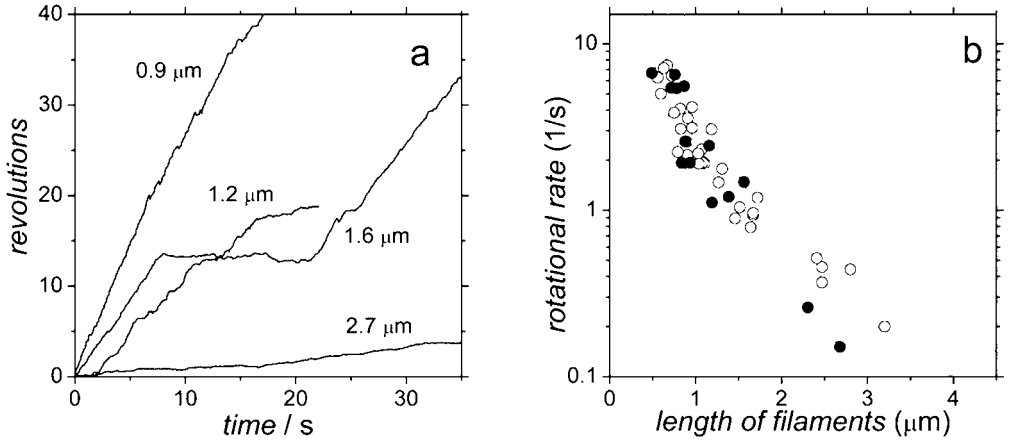

FIG. 2. *a*, typical time courses of the rotation of EF_0EF_1-KG1 subunit **c** oligomers. Images of rotating actin filaments in the presence of 5 mM Mg-ATP were recorded with a silicon-intensified tube camera (Hamamatsu C 2400–08) and analyzed with the ImagePro software. *b*, rotational rate in revolutions per second as a function of actin filament length. *Black dots*, EF_0EF_1-KG1; *open dots*, EF_0EF_1-SE1.

tained with EF_0EF_1-KG1. *Panel B* shows the dependence of the filament rotational rate from filament length. It is evident that EF_0EF_1-SE1 (15) and EF_0EF_1-KG1 were indistinguishable.

How do these results complement the proposal that detergent solubilized F_0F_1 is uncoupled from proton control (16), possibly by partial displacement of subunits **a** and **b** from their locations in the native enzyme (20)?

The exact structural consequences of the **c**D61N mutation are not known. They are expected to be small, because both the size and the polarity of Asp and Asn are very similar. Still the lack of an essential protonable group is sufficient to completely block proton conductance in both directions, passive under ATP synthesis and actively driven by ATP hydrolysis (23).

Assuming that ATP synthesis is driven by the rotation of subunits $\gamma\epsilon\mathbf{c_n}$, the failure to conduct protons is expected to prevent both rotation and ATP synthesis. However, ATP hydrolysis catalyzed by the membrane-bound enzyme is only diminished but not completely blocked (by 50% in the **c**D61N mutant and not at all in the **c**D61G mutant (23)). This finding in view of the structure of F_0F_1 either implies some sort of displacement of subunits $\gamma\epsilon$ from their **c** oligomer counterpart (with F_0F_1 still kept together by the stator subunits **a**, **b**, and δ) or continued corotation of $\gamma\epsilon\mathbf{c_n}$ without concomitant proton pumping. The latter is the case as we show here. Thus, "uncoupling" in EF_0EF_1-KG1 is brought about by ATP hydrolysis-driven freewheeling of the **c** oligomer.

The interaction of subunits $\gamma\epsilon$ and the **c** oligomer both in the wild-type enzyme and the mutant EF_0EF_1-KG1 withstands the strong mechanical strain between the ATP-hydrolyzing motor and either the drag force exerted on the actin filament or *in situ* the proton-motive force. In the **c**D61N mutant, the interactions between $\gamma\epsilon$ and the **c** oligomer are expected to be as strong as in the wild type enzyme, because the mutation is comparatively small and not likely to affect F_0-F_1 interactions at a distance of around 2.7 nm. Accordingly, we did not observe a more pronounced tendency of F_0 to dissociate from F_1 than with the wild type enzyme during preparation (data not shown).

To summarize, 1) the membrane-bound cD61N mutant hydrolyzes ATP without proton translocation; 2) the $\gamma\epsilon$-**c** oligomer interactions are strong enough to withstand considerable mechanical strain; and 3) solubilized wild type and mutant enzyme rotate $\gamma\epsilon\mathbf{c_n}$ upon ATP hydrolysis. These findings together indicate ATP hydrolysis-driven rotation of the **c** oligomer not only with solubilized but also with membrane-bound enzyme and irrespectively of the native or non-native location of subunits **a** and **b**. The expected sterical hindrances for the rotation of the **c** oligomer relative to subunits **a** and **b** would be smallest for the **c**D61G mutant and perhaps a little more pronounced for the **c**D61N mutant in accordance with the reported ATPase activities of the respective membrane-bound mutant enzymes (23).

How do these implications relate to the assumed rotary mechanism of F_0F_1? Proton transport through the F_0 portion of ATP synthase relies at least on two essential amino acid residues, Asp-61 on subunit **c** and Arg-210 on subunit **a** (*E. coli* numbering). A mechanism on how proton translocation might drive the rotation of the ring of **c** subunits (the **c** oligomer) relative to subunits **a** and **b** has been detailed previously (1, 3, 4, 17–20).

This model now would seem to be valid for all ATP synthases, because the proposed location of the acidic residue in subunit **c** of the sodium translocating ATP synthase close to the cytoplasmic side of the membrane (31) had to be abandoned as shown by recent cryoelectron microscopic data.[2]

The model features four assumptions. 1) The acidic residue **c**D61 is positioned at the center of the membrane. It is accessible for protons from both aqueous phases by two parallel but laterally off-set access channels. 2) There is a stochastic rotation of the **c** oligomer relative to subunit **a** driven by thermal impact (Langevin force). 3) It is limited by an electrostatic constraint, namely that the acidic residue on subunit **c** (Asp-61) is forcedly electroneutral (protonated) when facing the lipid core. 4) It is forcedly anionic (deprotonated) when opposing the permanently positively charged residue **a**R210, which is juxtaposed **c**D61 (for a detailed discussion *cf.* Refs. 31–33). The **c** oligomer thus rotationally fluctuates relatively to subunit **a** and progresses in one single direction by protonation of one Asp^- through one channel followed by the loss of another proton from a protonated Asp into the other channel located at the opposite side of the membrane. The model implicitly assumes that the interacting essential side chains are properly oriented without the requirement of large protein flexibility other than the thermal motion of the "rigid" **c** oligomer relative to subunit **a**.

This model both explains wild type features and the behavior of the **c**D61N mutant, *i.e.* the loss of passive and active proton translocation along with conservation of the ATPase activity of the membrane-bound enzyme, which corotates the *c* oligomer with or without proton pumping. However, the occurrence of the corotation in the mutant *in vivo* contradicts the fourth proposal above, because the postulated transient but essential

[2] W. Kühlbrandt and P. Dimroth, personal communication.

juxtaposition of a positive (**a**R210$^+$) and negative (**c**D61$^-$) charge is lacking in **c**D61N and **c**D61G.

In this context, the behavior of point-mutated strains containing **a**R210A is more difficult to understand. Both in accordance with expectations as predicted from the model and experiments, **a**R210A does not pump protons but allows for passive proton translocation (34). However, its membrane-bound ATP hydrolysis activity is largely inhibited. Because the mutation does not affect the F_1 part, the only explanation for this inhibition would be the blockage of the **c** oligomer rotation. These observations become better understandable by taking into account the proposed rotation of the helix with Asp-61 in subunit **c** and with Arg-210 in subunit **a** relative to the other helices in these subunits "swiveling" (35).[3] Proton translocation by F_0 would seem to involve both intersubunit as well as intrasubunit rotational movements.

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REFERENCES

1. Boyer, P. D. (1997) *Annu. Rev. Biochem.* **66,** 717–749
2. Junge, W., Lill, H., and Engelbrecht, S. (1997) *Trends Biochem. Sci.* **22,** 420–423
3. Fillingame, R. H. (2000) *Nat. Struct. Biol.* **7,** 1002–1004
4. Noji, H., and Yoshida, M. (2001) *J. Biol. Chem.* **276,** 1665–1668
5. Jiang, W., Hermolin, J., and Fillingame, R. H. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4966–4971
6. Duncan, T. M., Bulygin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10964–10968
7. Sabbert, D., Engelbrecht, S., and Junge, W. (1996) *Nature* **381,** 623–626
8. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997) *Nature* **386,** 299–302
9. Katoyamada, Y., Noji, H., Yasuda, R., Kinosita, K., and Yoshida, M. (1998) *J. Biol. Chem.* **273,** 19375–19377
10. Omote, H., Sambonmatsu, N., Saito, K., Sambongi, Y., Iwamoto- Kihara, A., Yanagida, T., Wada, Y., and Futai, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 7780–7784
11. Noji, H., Häsler, K., Junge, W., Kinosita, K., Jr., Yoshida, M., and Engelbrecht, S. (1999) *Biochem. Biophys. Res. Commun.* **260,** 597–599
12. Hisabori, T., Kondoh, A., and Yoshida, M. (1999) *FEBS Lett.* **463,** 35–38
13. Zhou, Y. T., Duncan, T. M., Bulygin, V. V., Hutcheon, M. L., and Cross, R. L. (1996) *Biochim. Biophys. Acta* **1275,** 96–100
14. Sambongi, Y., Iko, Y., Tanabe, M., Omote, H., Iwamoto-Kihara, A., Ueda, I., Yanagida, T., Wada, Y., and Futai, M. (1999) *Science* **286,** 1722–1724
15. Pänke, O., Gumbiowski, K., Junge, W., and Engelbrecht, S. (2000) *FEBS Lett.* **472,** 34–38
16. Tsunoda, S. P., Aggeler, R., Noji, H., Kinosita, K., Yoshida, M., and Capaldi, R. A. (2000) *FEBS Lett.* **470,** 244–248
17. Engelbrecht, S., and Junge, W. (1997) *FEBS Lett.* **414,** 485–491
18. Jones, P. C., Hermolin, J., Jiang, W., and Fillingame, R. H. (2000) *J. Biol. Chem.* **275,** 31340–31346
19. Hutcheon, M. L., Duncan, T. M., Ngai, H., and Cross, R. L. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8519–8524
20. Tsunoda, S. P., Aggeler, R., Yoshida, M., and Capaldi, R. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 898–902
21. Junge, W., Pänke, O., Cherepanov, D., Gumbiowski, K., Müller, M., and Engelbrecht, S. (2001) *FEBS Lett.* **504,** 152–160
22. Hoppe, J., Schairer, H. U., Friedl, P., and Sebald, W. (1982) *FEBS Lett.* **145,** 21–29
23. Fillingame, R. H., Peters L. K, White, L. K., Mosher, M. E., and Paule, C. R. (1984) *J. Bacteriol.* **158,** 1078–1083
24. Kuo, P. H., Ketchum, C. J., and Nakamoto, R. K. (1998) *FEBS Lett.* **426,** 217–220
25. Klionsky, D. J., Brusilow, W. S. A., and Simoni, R. D. (1984) *J. Bacteriol.* **160,** 1055–1060
26. Wise, J. G. (1990) *J. Biol. Chem.* **265,** 10403–10409
27. Sedmak, J. J., and Grossberg, S. E. (1994) *Anal. Biochem.* **79,** 544–552
28. Krause, I., and Elbertzhagen, H. (1987) in *Silver Stain for Dried PAA Gels Lasting 5 Minutes* (Radola, B. J., ed) Technische Universitat, Elektrophoreseforum, TU München, Germany
29. LeBel, D., Poirier, G. G., and Beaudoin, A. R. (1978) *Anal. Biochem.* **85,** 86–89
30. Galanis, M., Mattoon, J. R., and Nagley, P. (1989) *FEBS Lett.* **249,** 333–336
31. Dimroth, P., Wang, H., Grabe, M., and Oster, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4924–4928
32. Junge, W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4735–4737
33. Dimroth, P. (2000) *Biochim. Biophys. Acta* **1458,** 374–386
34. Valiyaveetil, F. I., and Fillingame, R. H. (1997) *J. Biol. Chem.* **272,** 32635–32641
35. Fillingame, R. H., Jiang, W., Dmitriev, O. Y., and Jones, P. C. (2000) *Biochim. Biophys. Acta* **1458,** 387–403

[3] R. H. Fillingame, personal communication.